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Original article

Effects of soluble extracts from *Leishmania infantum* promastigotes, *Toxoplasma gondii* tachyzoites on TGF-β mediated pathways in activated CD4$^+$ T lymphocytes

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Abstract

Interference with transforming growth factor-β-mediated pathways helps several parasites to survive for long periods in immunocompetent hosts. Macrophages and dendritic cells infected by *Toxoplasma*, *Leishmania* and *Plasmodium* spp. produce large amounts of transforming growth factor-β and induce the differentiation of antigen-specific T-regulatory cells. Mechanisms not mediated by antigen-presentation could also account for the expansion of T-regulatory cells in parasitic diseases and they also might be mediated through transforming growth factor-β-receptor activated pathways. We explored the properties of soluble extracts from *Leishmania infantum* promastigotes, *Toxoplasma gondii* tachyzoites, *Trichinella spiralis* muscle *larvae* to expand the pool of T-regulatory cells in a population of polyclonally activated T cells in the absence of accessory cells, and compared their effects to those induced by *Plasmodium falciparum* extracts. Similarly to *P. falciparum, L. infantum* extracts activate the latent soluble form of transforming growth factor-β and that bound to the membrane of activated T lymphocytes. The interaction of the active cytokine with transforming growth factor-β receptor induces *Foxp3* expression by activated lymphocytes, favoring their conversion through the T-regulatory phenotype. Both *Toxoplasma gondii* and *L. infantum* extracts are able to induce transforming growth factor-β production by activated T cells in the absence of accessory cells.

Keywords: Transforming growth factor; T-regulatory cells; *Leishmania infantum*; *Toxoplasma gondii*

1. Introduction

Immune responses to parasitic infections are rarely able to achieve total pathogen clearance or the development of sterilizing and long-lasting immunity; this deficiency often ensures advantages for both host and parasite: modulation of anti-pathogen immune responses may limit damage to host tissues and at the same time allow for low-level parasite persistence which, in turn, may be required for maintenance of long-term resistance to reinfection [1]. Several parasites modulate anti-parasitic immune responses by inducing the production of the suppressive cytokines IL-10 and transforming growth factor (TGF)-β by accessory cells. IL-10 suppresses the functions of antigen-presenting cells (APCs) as well as those of B and T lymphocytes [2]. TGF-β has proven essential for regulating T cell-mediated inflammation and maintaining immune homeostasis and tolerance. In fact an important mechanism through which TGF-β suppresses effector T cells is the promotion of regulatory T cells (Tregs) maintenance and differentiation [3,4].

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TGF-β was shown to be involved in the expansion of both native and induced Foxp3$^+$ Tregs in malaria [5–7], toxoplasmosis [8], and leishmaniasis [9]. Both TCR-dependent and independent mechanisms have been demonstrated to account for Treg expansion during parasitic infections [10,11]. In malaria infection accessory cells engulfed with parasites as well as activated T lymphocytes produce high amounts of TGF-β and IL-10 which might easily induce the conversion of bystander effector T cells to the regulatory phenotype [11]. Furthermore several parasites directly interfere with TGF-β mediated pathway to subvert protective immune responses: a homolog of human TGF-β is produced by most filarial parasites [12,13] while enzymatic molecules activating latent TGF-β are produced by *Plasmodium falciparum*, *Leishmania chagasi*, and by the intestinal helminth parasite *Heligmosomoides polygyrus* [14–16]. Activation of latent TGF-β on the surface of activated T cells is a requirement for triggering signals from TGF-β receptor which regulate the expression of *Foxp3* gene, the master regulator of T regulatory cell function [17,18]. Either *P. falciparum* extracts or *H. polygyrus* excretory-secretory proteins were shown to be able to activate TGF-β receptor on the surface of activated lymphocytes [15,19]. The effects of live parasites or their soluble extracts on the modulation of immune responses are well defined and considered as possible tools for modulating the responses in autoimmune diseases [20].

In this study we evaluated the parasitic extracts from *Leishmania infantum* promastigotes, *Toxoplasma gondii* tachyzoites and from the nematode *Trichinella spiralis* to test their interaction with activated T lymphocytes in terms of T-regulatory phenotype differentiation in absence of antigen presenting cells. The effects of *L. infantum*, *T. gondii* and *T. spiralis* extracts were compared with that of *P. falciparum* extracts. In particular, the mechanisms of interference with TGF-β-mediated pathways and the production of suppressive cytokines by activated T lymphocytes treated with parasitic extracts were studied.

2. Materials and methods

2.1. Antibodies and reagents

Anti-CD25-PE and anti-CD4-FITC antibodies were obtained from BD Biosciences – Pharmingen (San Jose, CA, USA); allophycocyanin-anti-Foxp3 (FJK-16s) staining kit was from eBiosciences (San Diego, CA, USA). Antibodies to TLR 2, 4 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). CD4$^+$ T cell and CD25$^+$ cell separation kit were obtained by Miltenyi Biotec (Bergisch, Gladbach, Germany). Dynabeads CD3/CD28 T cell expander was purchased by Invitrogen (Paisley, UK).

Anti-phospho- and total-SMAD 2/3 monoclonal antibodies were obtained by Cell Signaling (Cambridge, UK). RPMI 1640, DMEM 4.5 g/l glucose, antibiotics (penicillin/streptomycin), L-glutamine, heat inactivated fetal bovine serum were purchased from Celbio (Pero, Italy) and used for T cell culture. Protease inhibitor cocktail was purchased from Sigma Aldrich. Native human thrombospondin protein (TSP), purified from platelets, was purchased from Calbiochem (Merck, Millipore, Billerica, MA).

2.2. Donors

Buffy coats from 8 healthy donors never exposed to either parasitic infection were supplied by Transfusional Center of Azienda Ospedaliera Careggi (Firenze, Italy).

2.3. Parasite extracts preparation

T. gondii tachyzoites (RH strain) were grown in human foreskin fibroblast (HFF) monolayers in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Nu-Serum (Gibco), and antibiotics at 37 °C in a humid 5% CO_2 atmosphere. Tachyzoites were recovered by passage through a 27 gauge syringe needle to release parasites from infected HFF and subsequently filtered through 3 μm pore-diameter filters to remove host-cell debris.

L. infantum promastigotes (IPT1 strain) were cultured in Evans' modified Tobie's medium supplemented with 10% fetal calf serum (HyClone) and gentamicin at 22 °C.

T. spiralis muscle *larvae* from the reference strain ISS3 were collected from experimental infected Swiss CD1 mice fresh muscles, by artificial HCl-peptic digestion as reported [21]. After isolation the muscle *larvae* were washed in PBS and frozen in liquid nitrogen. *Trichinella* reference strains are maintained in Swiss CD1 mice at the International *Trichinella* Reference Center (http://www.iss.it/site/trichinella/, Istituto Superiore di Sanità, Rome, Italy).A laboratory strain (3D7) of *P. falciparum* was cultured in group O+ human RBCs suspended in RPMI medium 1640 (Gibco) containing 10% heat-inactivated O+ human serum. Schizont-stage parasites were purified by sedimentation through 60% Percoll.

Crude extracts of *T. gondii*, *L. infantum*, *T. spiralis* and *P. falciparum* were obtained by subjecting parasites to three freeze–thaw cycles (−156 °C – 37 °C) and by repeated centrifugations of the lysate at 13,000 rpm to obtain soluble fractions; protein concentration was determined by Bio-Rad assay in 0.2 μ Millex filtered supernatants. *P. falciparum* extracts were used at a concentration of 10 μg/ml in all the experiments. Heat-inactivated parasitic extracts (HIPE) were obtained by treating each crude parasitic extract at 80 °C for 30 min and used as negative control.

2.4. Protease inhibitors treatment

Crude extracts of *T. gondii*, *L. infantum* and *T. spiralis* were treated with protease inhibitors cocktail containing aprotinin, trypsin, cysteine proteases, leupeptin; cathepsin B was purchased by Sigma Aldrich and used according to manufacturer's recommendations.

2.5. Preparation, isolation and culture of cells

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats of 8 healthy donors by gradient

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centrifugation using Ficoll–Paque (GE Healthcare Italia, Milan, Italy), according to the manufacturer's recommendations and subjected to two cycles of plastic adherence to eliminate monocytes.

$CD4^+$ cells were isolated from non adherent fraction of PBMCs by using a $CD4^+$ T cell separation kit (Miltenyi) according to the manufacturer's recommendations and cultured in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (Celbio) with anti-CD3/CD28 antibodies coupled to beads (1 bead/5–10 effector cells). $CD25^+$ cells were affinity purified from the $CD4^+$ enriched fraction by using anti-CD25 mAb conjugated beads (Miltenyi) according to the manufacturer's recommendations. The purity of populations was checked by cytofluorimetric analysis with specific antibodies and was always >90% (median = 95 ± 2.38). However the percentage of $CD14^+$, $CD11c^+$ and $CD19^+$ was always <0.1%.

We used $CD4^+$ cells from the same donors ($n = 5$) for experiments reported in Figs. 1, 2 and 5.

PCR experiments:

2.6. Flow cytometry

To evaluate Foxp3 expression, $CD4^+$ cells were stained with anti-CD4-FITC and anti-CD25-PE antibodies followed by intracellular staining with anti-Foxp3-allophycocyanin according to the manufacturer's protocol (eBiosciences). To evaluate TLR expression $CD4^+$ cells were stained with anti-CD4-allophycocyanin and anti-TLR 2 or TLR 4 antibodies (Santa Cruz, CA, USA) followed by FITC secondary Ig. Cells were analyzed by FACSCalibur (BD Biosciences) by using CellQuest Pro software (BD Biosciences). The area of positivity was determined by using an isotype-matched control mAb. Ten thousand events for each sample were acquired.

2.7. RT2-PCR

RNA was extracted by using SV Total RNA Isolation system from Promega (Madison,WI) according to the manufacturer's recommendation. 200 ng of total RNA were reverse transcribed by using Stratascript kit (Stratagene, Santa Clara, CA, USA) and amplified by using primers for Foxp3 and Actin as house keeping (HK) genes, obtained from Superarray-Qiagen (Hilden, Germany). Amplifications process was performed with PowerSybr Green PCR Master MIX (Applied Biosystems, Foster City, CA) according to manufacturer's recommendations on an ABI PRISM 7900. Data were normalized to the mean value of HK genes and the relative amount of mRNA was calculated by using the $2^{-\Delta CT}$ method [22].

2.8. Immunoarray

TGF-β and IL-10 concentration in culture supernatants were determined by using IL10 Milliplex kit (Millipore, Bedford, MA, USA) and multispecies TGFβ1 kit (Invitrogen) and BIOPLEX apparatus in polystyrene immunoplates (Corning, Corning, USA). For TGF-β measurement samples were acid-treated according to the manufacturer's recommendations.

2.9. Latent TGF-β activation assay

Recombinant HuLat TGF-β (20 ng/ml) was incubated in duplicate in eppendorf tubes in the presence or absence of different concentrations of parasite extracts for 2 h at 37 °C or of the same concentrations of heat-inactivated extracts. Control tubes were loaded with Recombinant HuLat TGF-β (20 ng/ml) and 200 ng/ml of human thrombospondin (TSP) (Calbiochem) or with *P. falciparum extracts* (10 μg/ml).

At the end of the incubation, one aliquot of each sample was acid-treated with HCl 1 N for 10 min at room temperature and neutralized with 1 M NaOH/HEPES. Samples were then plated in triplicate in ELISA plates of R&D-DUO set TGFβ-kit. Bioactive TGF-β was measured by using mouse anti–human TGF-β1 specific for the active molecule as the coating antibody and chicken biotinylated anti–human TGF-β1 as the detecting antibody; recombinant human TGF-β1 was used as standard (all reagents from R&D Systems). Selected experiments were performed as above by using parasite extracts before and after treatment with protease inhibitor cocktail (SIGMA ALDRICH), see 2.4.

2.10. Western blot analysis

$CD25^+$ cells were obtained by magnetic adsorption from $CD4^+$ cells activated with anti-CD3/CD28 antibodies. Cells were cultured in serum free medium at 37 °C for 2 h with or without 10 μg/ml of *P. falciparum*, 10 μg/ml of *L. infantum*. Heat-inactivated extracts of *L. infantum* and of *P. falciparum* were used as negative control. $CD25^+$ cells were lysed in RIPA buffer containing phosphatase inhibitor cocktail 1 and 2 protease inhibitor (Sigma Aldrich, Milano, Italy), 2 mM PMSF and 1% Triton X-100, run on SDS-PAGE, blotted onto nitrocellulose filters, Hybond-ECL membranes (Amersham, Little Chalfont, UK), and stained with mouse monoclonal antibody against phospho-SMAD2 (Ser465/467)(138D4) (1:1000 dilution; Cell Signaling, Danvers, MA, USA). β-Actin was detected simultaneously as a loading control by using anti-β-actin (Santa-Cruz Biotech, Santa Cruz, CA), 1:3000 dilution. The membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (Santa Cruz) and the reaction was visualized by ECL detection system as recommended by the manufacturer (Amersham). Membranes were stripped for 30 min at 56 °C with StripAblot Stripping Buffer (Euroclone, Pero, MI, Italy) and stained with 1:1000 anti-total-SMAD2 (Cell Signaling, Cambridge, UK).

2.11. TLR assay

HEK-293-hTLR2, HEK-293-hTLR4/CD14/MD2 were plated in 96 well (2×10^5/well) with medium alone, *P. falciparum* extracts (10, 100 μg/ml), *L. infantum* extracts (10,

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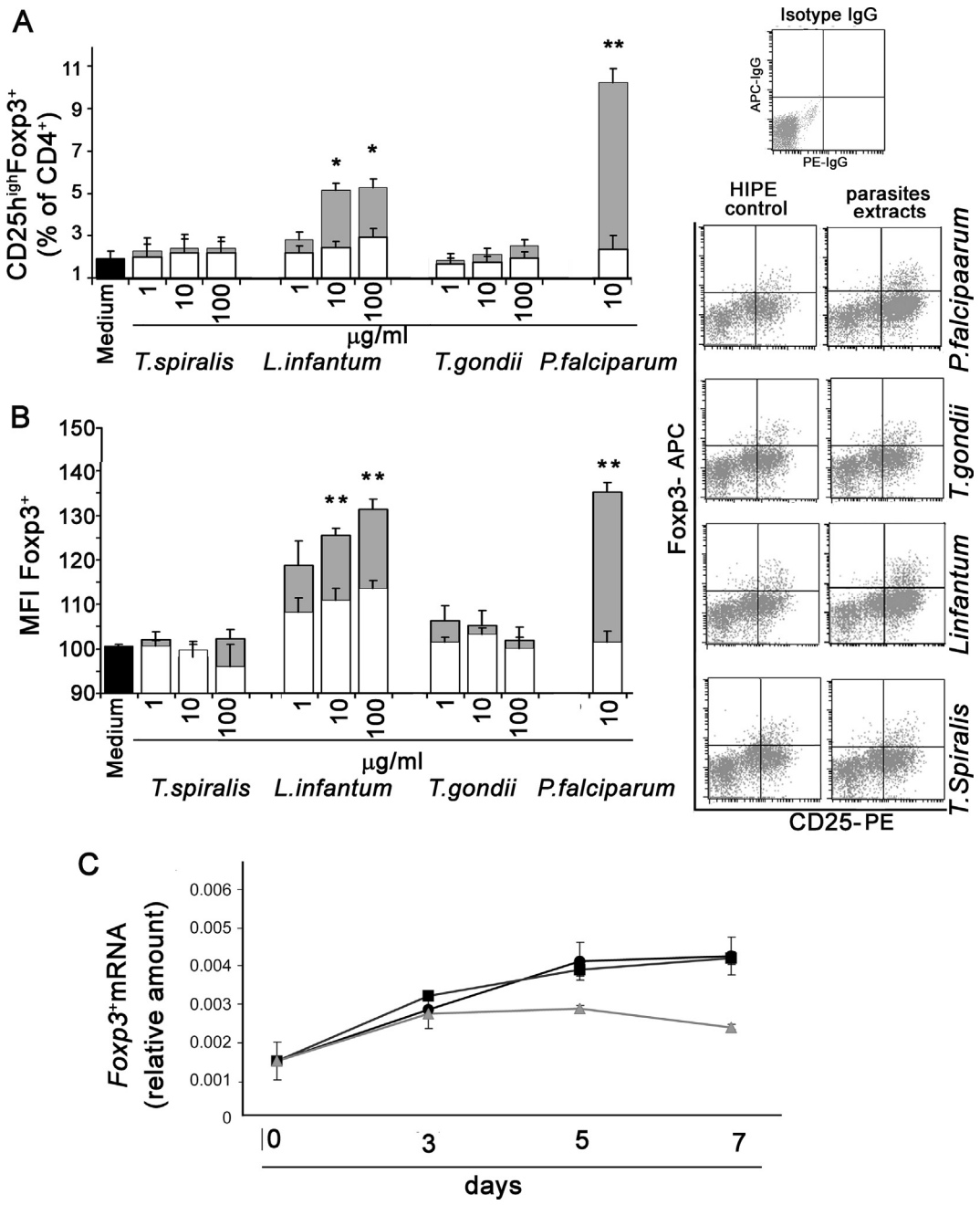

Fig. 1. **Effect of parasite extracts on the expansion of cells with a T regulatory phenotype.** $CD3^+$ $CD4^+$ T cells were isolated from PBMC of 5 normal healthy donors and cultured at 10^6/ml for 5 days with anti-CD3/CD28-microbeads (at 1 bead/5−10 cells) and in the presence of different concentrations (1, 10, 100 µg/ml) of extracts from *L. infantum* promastigote, *T. gondii* tachyzoites and *T. spiralis* muscle *larvae* (grey columns) or with the same concentrations of heat-inactivated parasite extracts (HIPE − white column). Cells were also cultured with medium alone (black columns) or with *P. falciparum* extracts (10 µg/ml) before (grey column) and after heat-treatment (white columns) as control. After 5 days anti-CD3/CD28 activated cells were stained with FITC anti-CD4, APC-anti-Foxp3, anti-CD25 or isotype control and analyzed by cytofluorimetry. Facsplot shown were carried out at day 5. **Panel A** shows results as mean percentage of $CD25^{high}Foxp3^+$ cells ± SE in cultures of $CD4^+$ T cells activated with anti-CD3/CD28 antibodies. **Panel B** shows results as mean of Median Fluorescence Intensity (MFI) of Foxp3 ± SE. Statistical analysis was performed by Student's *t*-test. * = $p \leq 0.05$; ** = $p \leq 0.005$. **Panel C**: Time course analysis of *Foxp3* gene expression: $CD4^+$ cells from 3 different healthy donors were activated with anti-CD3/CD28 antibodies and cultured in the presence or absence of *P. falciparum* extracts (10 µg/ml), *L. infantum* extracts (10 µg/ml). Cells were lysed at the times indicated and *Foxp3* gene expression assessed by RT-PCR with specific primers. The data are represented as relative amount of mRNA (means ± SE of technical replicates) of one representative experiment out of three performed.

100 µg/ml) and *T. gondii* extracts (10, 100 µg/ml) for 8 h. HKLM (3×10^7 cells/ml), LPS (20 ng/ml), were used as positive controls for TLR2, TLR4 triggering respectively. At the end of the culture, conditioned media were harvested and IL-8 production, as a major cytokine induced by TLR4 [23] was assessed by Immunoplex array (Millipore) using Bioplex apparatus.

TLR4-based IL-8 reporter cell line as a tool for screening of the LPS-mediated immunomodulators to control TLR4 signaling pathway (P3305).

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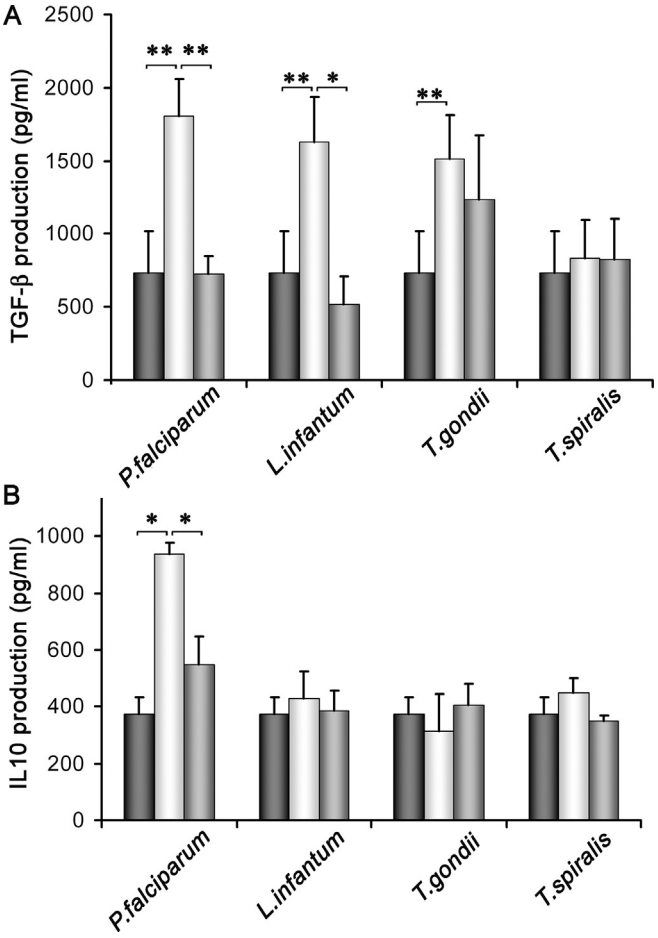

Fig. 2. **Effect of *parasitic extracts* on TGF-β and IL-10 production by CD4$^+$ cells.** Culture supernatants of anti-CD3/CD28-activated CD4$^+$ cells used for the cytofluorimetric analysis (Fig. 1) were recovered after 5 days. Cytokine concentration was determined by specific immunoarray. White columns represent data of cytokine measurement in cells activated with anti-CD3/CD28-microbeads (at 1 bead/5–10 cells) and stimulated with 10 μg/ml of extracts from *L. infantum* promastigote, *T. gondii* tachyzoites, *T. spiralis* muscle *larvae* and from *P. falciparum*. Grey column represent data of cytokine measurement of the same cells stimulated with the relative heat-inactivated extracts (HIPE) at 10 μg/ml. **Panel A** shows results as mean of TGF-β production ± SE. **Panel B** shows results as mean of IL-10 production ± SE. The histogram shows data from 5 experiments (mean ± SE). Statistical analysis was performed by Student's *t*-test. * = $p \leq 0.05$; ** = $p \leq 0.005$.

3. Results

3.1. Effects of parasite extracts on Treg induction in resting or activated CD4$^+$ cells

To investigate whether parasitic molecules could directly interact with T lymphocytes inducing their differentiation to Foxp3$^+$ T regulatory cells, we cultured purified CD4$^+$ cells from 5 different healthy donors, either resting or activated by anti-CD3/CD28 antibodies, with different concentrations of soluble extracts from *L. infantum* promastigotes, *T. gondii* tachyzoites and *T. spiralis* muscle *larvae* in the presence or absence of anti-CD3/CD28 coupled beads [24]. Heat-inactivated parasitic extracts (HIPE) or medium alone were used as "negative" controls while *P. falciparum* extracts at 10 μg/ml was used as positive control [15]. We chose to quantify the expansion of CD25^{high}Foxp3$^+$Treg populations in cells activated by anti-CD3/CD28 antibodies after 5 days of culture, because at this time the effects of TCR-activation on the expression of *Foxp3* gene by CD4$^+$ T lymphocytes are not evident ([25,26], our data in Fig. 1C).

Culture supernatants were also recovered at this time for cytokine measurement. Fig. 1, panel A shows the percentage of activated cells recorded by cytofluorimetric analysis after 5 days of culture. At this time, the percentage of Tregs with a T regulatory phenotype (CD25^{high}Foxp3$^+$) was markedly induced in activated CD4$^+$ T cells by *L. infantum* extracts at concentration ≥10 μg/ml and by *P. falciparum* extracts, in agreement with our previous report [15]. *T. gondii* or *T. spiralis* soluble extracts did not induce significant changes in the percentage of Tregs among activated T cells. Resting CD4$^+$ T lymphocytes cultured in the presence or absence of parasitic extracts for the same time (5 days) also did not show any change in the percentage of Treg cells. (Supplementary Information Fig. S_1). Foxp3 fluorescence intensity was also increased in cultures with *L. infantum* and *P. falciparum* extracts compared to control cultures (Fig. 1, panel C), suggesting that parasite molecules could activate pathways involved in *Foxp3* gene expression.

To confirm the results obtained by cytofluorimetric analysis, we analyzed the kinetic expression of *Foxp3* gene in anti-CD3/CD28 activated CD4$^+$ T cells isolated from 3 additional healthy donors cultured as above reported. According to previous report [10], Fig. 1, panel D shows that, in cells activated by anti-CD3/CD28 antibodies, the expression of *Foxp3* gene is significantly higher after 72 h of culture compared to day 0. However, in the absence of any additional stimuli, it declines to basal levels following 5–7 days. In contrast, in cells cultured in the presence of *L. infantum* or *P. falciparum* extracts the expression of *Foxp3* gene is significantly higher from that recorded at day 0 as well as from that expressed by cells cultured in the absence of parasitic extracts.

3.2. Effect of parasitic extracts on the production of suppressive cytokines by activated T lymphocytes

Antigen-activated T lymphocytes usually produce high amounts of effector cytokines. However, in selected conditions and under the coordinate action of antigen presenting cells or of their cytokines they also produce suppressive cytokines as TGF-β and IL-10.

Indeed in some parasitic infections, a high production of TGF-β and IL-10 by activated T cells has been reported even in absence of accessory cells [11,15]. Thus we studied the effect of parasitic extracts on the production of TGF-β and IL-10 by activated T lymphocytes. Supernatants of the CD4$^+$ T lymphocytes activated with anti-CD3/CD28 antibodies and cultured in the presence or absence of the parasitic extracts before and after heat-treatment (see Fig. 1a), were harvested after 5 days of culture and the concentration of TGF-β and IL10 evaluated by specific Immunoarray. Fig. 2 shows the results obtained with 10 μg/ml of each extract: with the

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exception of *T. spiralis* all parasite extracts significantly induced TGF-β production compared to unstimulated cultures. Heat-treatment almost completely abolished the TGF-β production induced by *P. falciparum* and *L. infantum* extracts. In contrast no significant differences in TGF-β concentrations were recorded in cultures stimulated with *T. gondii* extracts before and after heat-treatment. The production of IL-10 was significantly increased only in cultures of activated T cells with *P. falciparum* extracts.

3.3. Effect of parasitic extracts on latent TGF-β activation

It is known that several parasites directly interfere with TGF-β- mediated pathway to subvert protective immune responses: either the production of a human TGF-β homolog or the ability to activate latent TGF-β has been reported in helminths, nematodes and protozoa [12–16,19].

Thus we studied the ability of *L. infantum*, *T. gondii*, or *T. spiralis* extracts to activate latent TGF-β converting it in soluble recombinant form in comparison with the activity of *P. falciparum* extracts. Recombinant latent human TGF-β (rLat TGF-β), comprising TGF-β plus LAP (R&D Systems), was added to different concentrations of each parasitic extracts at a final concentration of 20 ng/ml and incubated for 2 h at 37 °C. Human thrombospondin, TSP (Calbiochem), at a concentration of 200 ng/ml, was used as a positive control. Bioactive TGF-β was measured through ELISA assays using specific antibodies to the active molecule. Total amount of immunereactive TGF-β was determined following acid-treatment of selected samples. In order to investigate the chemical nature of the molecule/s involved in latent TGF-β activation we denatured the protein content of *L. infantum* extracts through heating at 80 °C or we treated them with a protease inhibitor cocktail containing inhibitors of cysteine and serine proteases and of cathepsin B (SIGMA). Fig. 3 shows that similarly to *P. falciparum* extracts, also *L. infantum* extracts were able to activate the soluble form of latent TGF-β. Surprisingly this activity was maximal at 10 μg/ml and did not further increase with higher concentrations of extracts likely suggesting the occurrence of steric hindrance or the presence of inhibitor molecules in high concentrations of parasitic extracts. Both heat-treatment and treatment with protease inhibitor cocktail completely abolished the activation of latent TGF-β suggesting that enzymatic proteins with protease activity are responsible for such activity. Neither *T. gondii* nor *T. spiralis* extracts were able to activate latent TGF-β (data not sown).

3.4. Effect of parasitic extracts on the activation of membrane-bound latent TGF-β

The ability of *L. infantum* extracts to activate latent TGF-β suggests that parasitic molecules also activate the TGF-β bound to the membranes of activated T cells and trigger the TGF-β receptor signaling leading to *Foxp3* gene expression. Since membrane-bound TGF-β is expressed by $CD25^+$ cells following TCR activation [27], in a different set of

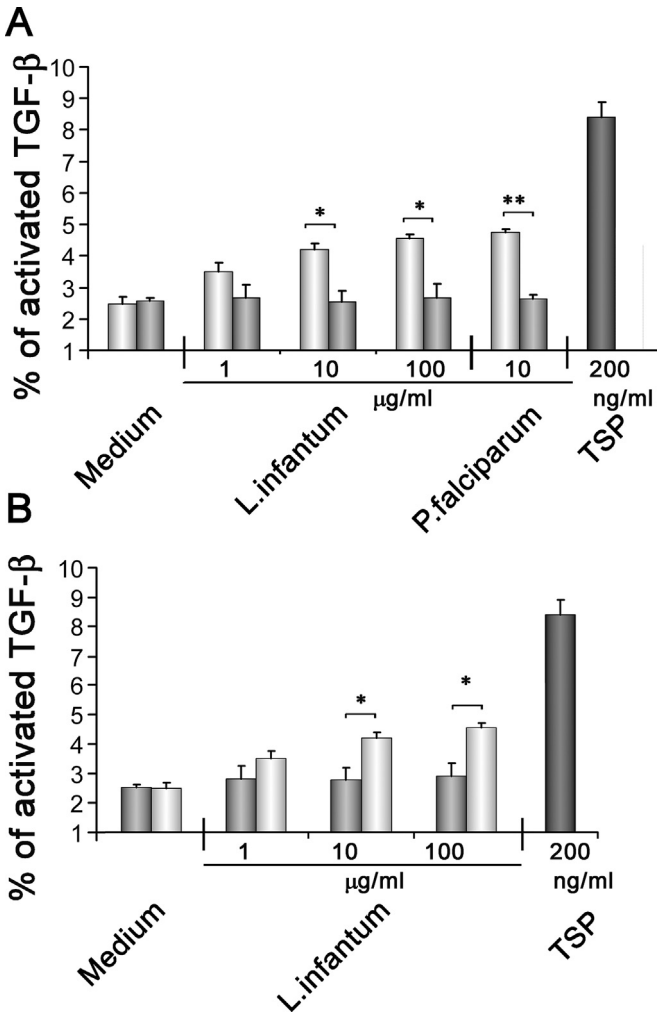

Fig. 3. **Effect of parasitic extracts on latent TGF-β activation.** Panel A: Recombinant latent human TGF-β (rLatTGF-β), comprising TGF-β plus LAP (R&D Systems), was added at a final concentration of 20 ng/ml to different concentrations of *L. infantum* extracts (white column) or to 10 μg/ml of *P. falciparum* extracts (white column) in eppendorf tubes (in duplicate). Heat-inactivated parasitic extracts (HIPE) or Heat-treated medium (grey columns) were used as control. TSP, from human platelets (Calbiochem) (black column) was used as positive control at a concentration of 200 ng/ml. Samples were incubated for 2 h at 37 °C. At the end of incubation one aliquot of each sample was treated with HCl 1 N, neutralized. All samples were plated in triplicate and bioactive TGF-β was measured through ELISA assays using specific antibodies according to R&D Systems recommendations. Data are shown as activated/total TGF-β ratio × 100. Results from three different experiments (mean ± SE) are shown. Statistical analysis was performed by Student's *t*-test: * = $p \leq 0.05$; ** = $p \leq 0.005$. **Panel B**: rLatTGF-β, was added at a final concentration of 20 ng/ml to different concentrations of *L. infantum* extracts (white column) or *L. infantum* extracts treated with protease inhibitor cocktail (grey column) in eppendorf tubes (in duplicate). TSP, from human platelets (Calbiochem) (black column) was used as positive control at a concentration of 200 ng/ml. One aliquot of each sample was treated with HCl 1 N, neutralized and plated in triplicate. Bioactive TGF-β was determined as in panel A. Data are shown as activated/total TGF-β ratio × 100. Results from three different experiments ((mean ± SE) are shown. Statistical analysis was performed by Student's *t*-test: * = $p \leq 0.05$; ** = $p \leq 0.005$.

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experiments we isolated these cells from cultures of $CD4^+$ cells activated with anti-CD3/CD28 coupled beads for three days. Activated $CD25^+$ cells were thus cultured for 2 h with an active concentration of *L. infantum* extracts (10 μg/ml) in serum free medium. Heat-inactivated *L. infantum* extracts or *P. falciparum* extracts were used as negative and positive control respectively. Phosphorylated SMAD 2/3 proteins are major mediators of TGF-β receptor signaling and considered as the most important factors governing *Foxp3* transcription [28]. Thus, phosphorylation of SMAD 2/3 proteins by Western Blot analysis was used as a measure of TGF-β receptor activation. Fig. 4 shows that stimulation of activated $CD25^+$ cells with *L. infantum* or *P. falciparum* extracts, in the absence of any support of external TGF-β, induces phosphorylation of SMAD 2/3 proteins. In contrast, the same parasitic extracts do not activate SMAD 2/3 phosphorylation in activated $CD25^-$ cells which express TGF-β receptor but do not express membrane-bound latent TGF- β [29,30].

3.5. TLR expression by activated $CD4^+$ cells

Several Pattern Recognition Receptors recognize parasitic molecules [31]. TLR2 and TLR4 which recognize parasitic glycosylphosphatidylinositol anchors and glycoinositolphospholipids respectively are also expressed by activated T lymphocytes and are involved in T lymphocyte functions such as survival, IL2 production, TH1/TH17 shift and sensitivity to T regulatory [32,33]. We first investigated the possibility that culture in the presence of parasitic extracts could affect the expression of TLR2 and TLR4 as reported in different experimental systems [15]. As shown in Fig. 5, we found that the percentage of activated T cells expressing TLR4 was significantly increased in cultures with high concentrations of *T. gondii* extracts compared to unstimulated cultures. Activated $CD4^+$ cells cultured with *P. falciparum* extracts also increased their TLR4 expression. However in both cases no significant differences were recorded between cultures stimulated with parasitic extracts before and after heat-treatment. These data suggest that, beside TCR-stimulation, the molecular pathways leading to TLR4 expression T lymphocytes could be amplified by heat-stable molecules present in these parasitic extracts through unknown molecular pathways. In contrast no increase of TLR2 expression was observed (data not shown). Since all of parasitic extracts used in the experiments reported above moderately activate the TLR4 pathway at concentrations ≥50 μg/ml (Supplementary Informations, Fig. S_1), we suggest that this pathway might be involved either in Treg differentiation, either in TGF-β production.

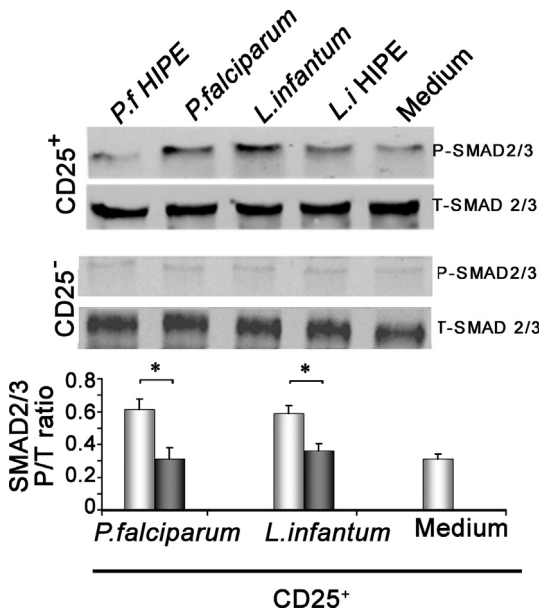

Fig. 4. **Effect of parasitic extracts on the activation of membrane-bound latent TGF-β.** $CD3^+$ $CD4^+$ T cells isolated from PBMC of 3 normal healthy donors were cultured at 10^6/ml with anti-CD3/CD28-microbeads (at 1 bead/5–10 cells) for 5 days. Activated $CD25^+$ T and $CD25^-$ T cells were isolated by Miltenyi isolation kit according to the manufacturer's recommendations, cultured for 2 h in serum free medium with 10 μg/ml of *L. infantum* extracts, *P. falciparum* extracts and HIPE as control. The phosphorylation of SMAD 2/3 proteins was measured through Western Blot analysis with specific antibodies and normalized on total SMAD 2/3 protein. The histogram represents the data of the densitometric analysis of SMAD 2/3 phosphorylation in $CD25^+$ T cells only. Results are expressed as the ratio between Phosphorilated-SMAD 2/3 (P) and total protein (T) of 3 different experiments (mean ± SE). Statistical analysis was performed by Student's *t*-test. $p \leq 0.05$ was considered as significant (*).

4. Discussion

The main role played by Tregs in balancing protective immunity and pathogenesis in parasitic diseases gave rise to considerable efforts in understanding the mechanisms governing their expansion in the course of infection. In this study we isolated purified primary human CD4(+) T cells and stimulated them with anti-CD2/CD3/CD28 antibody-coated beads as an in vitro surrogate system for antigen presenting cell-T cell interaction; in this model, virtually deprived of any antigen-presenting or accessory cells, we investigated the ability of *L. infantum*, promastigotes, *T. gondii* tachyzoites, *T. spiralis* muscle *larvae* to directly interact with TCR-activated lymphocytes and to induce their differentiation to T regulatory cells. Data obtained from cytofluorimetric analysis show that, among the parasites tested, only *L. infantum* extracts are able to expand the Treg population. This activity is dose-dependent and it is completely abolished by heat-treatment of extracts at 80 °C. Previously we showed that, in the same experimental model, *P. falciparum* extracts expand a Treg population with $CD25^{high}Foxp3^+$ phenotype endowed with potent suppressive activity: the parasitic molecules responsible for Treg expansion activate the latent TGF-β complex either in soluble or membrane-bound form [10,16]; active TGF-β, through the interaction with TGF-β receptor expressed on the membrane of effector T cells, force their conversion to the T regulatory phenotype [10]. In this study we show that, similarly to *P. falciparum* extracts, also *L. infantum* extracts activate the TGF-β receptor pathway as assessed by the phosphorylation of SMAD 2/3, transcription factors involved in the regulation of Foxp3 gene expression [34]. The activation of TGF-β receptor by parasitic extracts is evident in $CD25^+$ T cells but not in

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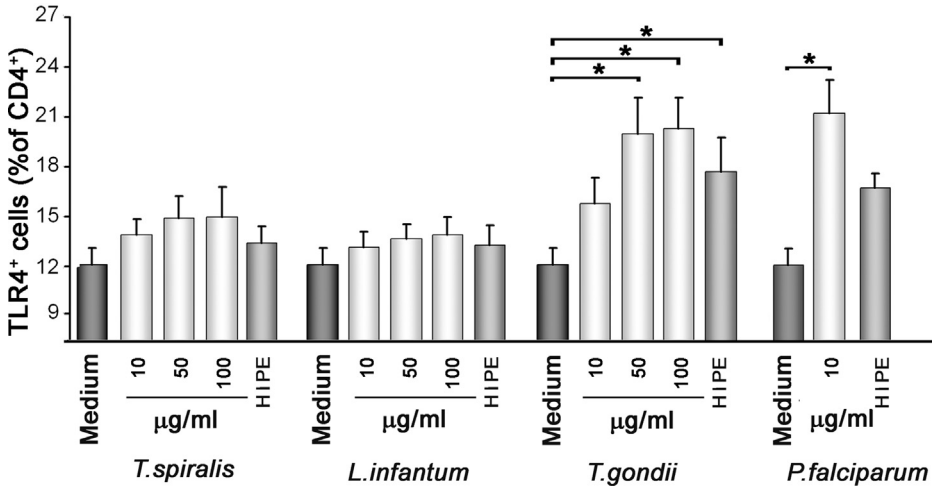

Fig. 5. **Effects of parasitic extracts on TLR expression by activated CD4$^+$ cells.** CD3$^+$ CD4$^+$ T cells from PBMC of 5 donors were cultured at 10^6/ml for 5 days with anti-CD3/CD28-microbeads (at 1 bead/cells) and in the presence of different concentrations (10, 50, 100 µg/ml) of extracts from *L. infantum* promastigote, *T. gondii* tachyzoites and *T. spiralis* muscle *larvae* (grey columns) or with the same concentrations of heat-inactivated parasite extracts (HIPE – white column). Cells were also cultured with *P. falciparum* extracts (10 µg/ml) as positive control (grey column) or with 10 µg of heat-inactivated *P. falciparum* (white columns) or culture medium (black columns) as negative control. After 5 days anti-CD3/CD28- activated cells were stained with APC anti-CD4, aTLR4 followed by FITC secondary Ig or isotype control and analyzed by cytofluorimetry. Results are shown as mean of % of TLR4$^+$CD4$^+$ cells ±SE. Statistical analysis was performed by Student's *t*-test: * = $p \leq 0.05$.

CD25$^-$ T cells. Although both the cellular subsets produce TGF-β and express TGF-β receptors, only CD25$^+$ T cells produce GARP, a transmembrane protein which presents the latent form of TGF-β1 on the cell surface [35].

The activation of membrane-bound TGF-β is one of the most important mechanisms that induce the "infectious tolerance", a process whereby one regulatory lymphoid population confers suppressive capacity on another. *L. infantum* and *P. falciparum* extracts might induce "infectious tolerance" by directly activating the TGFβ-receptor on T cells without the interference of dendritic cells. This mechanism might have a role in maintaining immune homeostasis, limiting the inflammatory reaction and the specific immunological response during the course of parasitic infection in vivo. Indeed the property of *L. infantum* extracts to activate the soluble form of latent TGF-β complex might favor the persistence of parasites within infected macrophages, through the induction of TGF-β-mediated anti-inflammatory mechanisms.

We attempted to identify the nature of the molecules involved in the latent TGF-beta activation. Our data show that either treatment with a cocktail containing inhibitors of cysteine and serine proteases and of cathepsin B and heat-treatment at 80 °C completely inhibited latent TGF-β activation by *L. infantum* extracts suggesting the role of protein molecules with proteases activity. Our results are in agreement with those reported by Gantt et al. showing the property to activate latent TGF-β of live *L. chagasi* promastigotes [14] likely because *L. infantum* and *L. chagasi* (the most prevalent species in South America) belong to the same species [36].

Since we used *L. infantum* promastigote extracts we hypothesize that the function of these protein(s) might be relevant during the maturation to amastigote stage but it is highly likely that proteins with such activity are produced also in the amastigote stage. Indeed proteins able to activate latent TGF-β are secreted in the supernatant of macrophages infected with *L. chagasi* [14]. Furthermore Somanna et al. [37] cloned the cathepsin B homolog from *L. chagasi* and showed that it is expressed in both the amastigote and promastigote form of the parasite. Taking into account such activity, we suggest that part of the immunomodulation induced by soluble protein extracts of *Leishmania* spp., used as antigens in vaccine formulations in both veterinary and human fields [38] might also be attributable to the activation of latent TGF-β either in soluble or membrane-bound form.

Apart from the cellular-mediated dialog conferring suppressive capacity to a lymphoid population, the infectious tolerance might be mediated directly by the secretion of suppressive cytokines as TGF-β and IL-10 [2–4,34,39]. TGF-β plays a key role in maintaining the balance between immunoprotection mediated by T cells and immunopathology either in experimental models of malaria and toxoplasma infection and in human diseases [5,29,30]. Macrophages and dendritic cells mostly account for TGF-β production following parasite uptake; nevertheless, the production of these cytokines by activated CD4$^+$ T cells following a direct stimulation with parasites or parasite products has also been reported [10,11,15]. Our data show that the synthesis of IL10 by CD4$^+$ T lymphocytes is significantly induced only by *P. falciparum* extracts according to our previous report [10]; indeed the production of TGF-β is induced also by *T. gondii* and by *L. infantum* extracts.

The pathways involved in TGF-β production by CD4$^+$ T cells following parasite or parasitic molecules stimulation are largely unknown. *Schistosoma mansoni* soluble egg antigen (SEA) directly act on CD4$^+$ T cells via TLR2 to induce the secretion of bioactive TGF-β [40]. *P. falciparum* extracts activate TGF-β receptor activation through membrane bound TGF-β molecules and TGF-β dependent signals positively regulate TGF-β expression [41]. Similar mechanisms are suggested for

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the production of TGF-β observed with *L. infantum* extracts which also activate TGF-β receptor signaling. The involvement of this pathway in TGF-β production is further supported by data showing that heating at 80 °C or treatment with protease inhibitors of *L. infantum* extracts strongly inhibit their ability to induce TGF-β production. Apart from TGF-β-induced TGF-β production also the interaction of parasitic molecules with known and/or unknown Pattern Recognition Receptors might play a role in the induction of cytokine synthesis by *L. infantum* and/or *P. falciparum* extracts.

In this study we evaluated the expression of only two PRR involved in recognition of parasitic molecules, namely the TLR2 and the TLR4 [31,42,43]. The expression of TLRs by T lymphocytes usually is induced and/or amplified by TCR-activation; neverthless we could observe significant differences in the expression of TLR4 by activated T lymphocytes following stimulus with *T. gondii* and with *P. falciparum* extracts. Interestingly the TLR4-inducing activity of the two parasitic extracts was not abolished by heat-treatment suggesting it was mediated by heat-stable molecules interacting with unknown receptors in T cells. In this study we did not further investigate the mechanisms of TLR4 expression or the role of TLR4 in the functional properties of $CD4^+$ T cells. Previously we reported, in the context of specific antigenic stimulation, that *P. falciparum* extracts increased the sensitivity of Tregs to TLR4 ligands [15]. Pathways mediated by TLR4 activation were shown to be involved in selected T reg functions as survival and *Foxp3* gene expression. Despite the moderate activation of TLR4 pathway *of P. falciparum*, *T. gondii* and *L. infantum* extracts, we were not able to correlate TLR4 with the production of TGF-β (data not shown) in our experimental systems. Further studies are needed to understand the role of TLR4 in $CD4^+$ T cell functional properties during parasitic diseases.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.micinf.2014.08.002.

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