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Interleukin 10 and dendritic cells are the main suppression mediators of regulatory T cells in human neurocysticercosis

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Summary

Neurocysticercosis is caused by the establishment of *Taenia solium* cysticerci in the central nervous system. It is considered that, during co-evolution, the parasite developed strategies to modulate the host's immune response. The action mechanisms of regulatory T cells in controlling the immune response in neurocysticercosis are studied in this work. Higher blood levels of regulatory T cells with $CD4^{+}CD45RO^{+}$forkhead box protein 3 $(FoxP3)^{high}$ and $CD4^{+}CD25^{high}FoxP3^{+}CD95^{high}$ phenotype and of non-regulatory $CD4^{+}CD45RO^{+}FoxP3^{med}$ T cells were found in neurocysticercosis patients with respect to controls. Interestingly, regulatory T cells express higher levels of cytotoxic T lymphocyte antigen 4 (CTLA-4), lymphocyte-activation gene 3 (LAG-3), programmed death 1 (PD-1) and glucocorticoid-induced tumour necrosis factor receptor (GITR), suggesting a cell-to-cell contact mechanism with dendritic cells. Furthermore, higher IL-10 and regulatory T cell type 1 (Tr1) levels were found in neurocysticercosis patients' peripheral blood, suggesting that the action mechanism of regulatory T cells involves the release of immunomodulatory cytokines. No evidence was found of the regulatory T cell role in inhibiting the proliferative response. Suppressive regulatory T cells from neurocysticercosis patients correlated negatively with late activated lymphocytes ($CD4^{+}CD38^{+}$). Our results suggest that, during neurocysticercosis, regulatory T cells could control the immune response, probably by a cell-to-cell contact with dendritic cells and interleukin (IL)-10 release by Tr1, to create an immunomodulatory environment that may favour the development of *T. solium* cysticerci and their permanence in the central nervous system.

Keywords: cysticerci, dendritic cells, neurocysticercosis, regulatory T cells, *Taenia solium*

Introduction

Neurocysticercosis (NC) is an endemic disease in most countries of Latin America, Asia and Africa. NC is caused by the larval stage of *Taenia solium* (cysticercus), which establishes in the central nervous system (CNS) after reaching it in the form of a hexacanth embryo (oncosphere). The incidence of NC has increased progressively in developed countries of North America and Europe due to immigration [1,2].

NC affects immunocompetent hosts, and it is noteworthy that the parasite is able to establish and develop in different areas of the CNS, circumventing the host immune response. Cellular immunity is suppressed in the most severe forms of the disease [3,4]. This suppression can be attributed to the action of peripheral and central regulatory T cells (T_{regs}), which were found increased in these severe NC patients. Indeed, *T. solium* cysticerci have been reported as able to promote T_{reg} generation both *in vivo* and *in vitro* [5,6]. It is feasible that the parasite could drive the immunity elicited by the host to promote a favourable environment for its own survival [6]. This strategy has been demonstrated in other parasitic diseases, such as cerebral malaria and cutaneous leishmaniasis [7,8].

It is well known that T_{regs} can modulate the specific immunity through different suppressive mechanisms [9]. T_{regs} may produce interleukin (IL)-10, IL-35 and transforming growth factor (TGF)-β, which can act together to

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inhibit the production of proinflammatory cytokines and the proliferative immune response [10–12]. Moreover, IL-35 and TGF-β may also promote *de-novo* generation of T_{regs} [13,14]. Another known mechanism for T_{reg} action involves higher expression levels of the IL-2 receptor α chain (CD25), resulting in a deprivation of IL-2 for effector T cells and the inhibition of T cell proliferation. In recent years, further modulatory functions of T_{regs} have been reported. T_{regs} express the ectoenzymes CD39 and CD73, which produce adenosine; in turn, this nucleoside suppresses the function of effector T cells by activating the adenosine receptor 2A ($A_{2A}R$) [9]. T_{regs} also express cytotoxic T lymphocyte antigen 4 (CTLA-4) (CD152) and lymphocyte-activation gene 3 (LAG-3) (CD223), which can interact with CD80/CD86 and major histocompatibility complex II (MHC-II) (CD74) [9,15] suppressing the activation and maturation of dendritic cells (DC) and inducing a tolerogenic phenotype [9,16,17] which, in turn, induces *de-novo* T_{reg} generation [18]. T_{regs} also express programmed death 1 (PD-1) (CD279), which can prevent CD28-mediated activation and down-regulate the expression of proinflammatory cytokines [19,20]. Glucocorticoid-induced tumour necrosis factor receptor (TNFR) family-related gene (GITR) (CD357) is another molecule expressed by T_{regs} that may inhibit T cell activation [21].

This study is aimed to explore the mechanisms that mediate the function of T_{regs} during NC. This information will improve our understanding of the host–parasite relationship and the factors that participate in the control of the immune response during this parasitic disease.

Materials and methods

NC patients and control subjects

A total of 20 NC patients (13 male, mean age 54·15 ± 9·97 years and seven female, mean age 36·29 ± 4·54 years) who attended at Instituto Nacional de Neurología y Neurocirugía and Centro Médico Nacional Siglo XXI in Mexico City were included into the study. Diagnosis was based on clinical manifestations (seizures, focal deficit and intracranial hypertension) and on neuroradiological studies (showing viable cysticerci). Blood samples from NC patients were collected before any cysticidal treatment was administered. Cerebrospinal fluid (CSF) samples were obtained by lumbar puncture from 11 patients. Blood samples from 16 healthy volunteers (six male, mean age 36·33 ± 11·33 and 10 female, mean age 42·60 ± 10·67) were also included as controls.

A number of central (CSF) and peripheral (blood) immunological parameters were evaluated.

Nineteen NC patients showed multiple vesicular cysticerci, while one patient had only a single parasite. Eighteen patients exhibited extraparenchymal cysts, 16 in the subarachnoid space of the base and two in the ventricles. Two patients showed parenchymal cysticerci.

Ethical considerations

The present study fulfilled all regulations for research with human subjects as required by Mexican law and international regulations. It also complied with all ethical aspects considered in the General Rules of Health for Clinical Investigation. Ethics Committee at Instituto Nacional de Neurología y Neurocirugía, México, approved the protocol. Written informed consent was obtained from all participants. Patients were informed that the samples obtained would be used for this work.

Cell phenotype

The phenotype of cell populations in peripheral blood mononuclear cells (PBMCs) and CSF was determined. The following antibodies were used to characterize the phenotype of regulatory T cells: mouse anti-human CD4 allophycocyanin (APC) cyanin 7 (Cy7) [isotype immunoglobulin (Ig)G1k], mouse anti-human CD25 APC (isotype IgG1k), rat anti-human forkhead box protein 3 (FoxP3) phycoerythrin (PE) (isotype IgG2ak), mouse anti-human CD45RO peridinin chlorophyll (PerCP) Cy5·5 (isotype IgG2aK) and mouse anti-human CD127 fluorescein isothiocyanate (FITC) (isotype IgG1K). To measure the expression of molecules participating in the mechanism associated with cell–cell contact, the following antibodies were used in PBMCs: mouse anti-human CD4 APC Cy7 (isotype IgG1k), mouse anti-human CD25 APC (isotype IgG1k), mouse anti-human CTLA-4 (CD152) PE (isotype IgG2ak) and mouse anti-human LAG-3 (CD223) PerCP Cy5·5 (goat isotype IgG).

The presence of PD-1 (CD279) was assessed using the mouse anti-human PD-1 PerCP Cy5 (isotype IgG1K) antibody on $CD4+CD25^{high}$ peripheral T_{regs}. The presence of other regulatory molecules on $CD4+CD25^{high}$ T_{regs} was assessed using the mouse anti-human GITR FITC (isotype IgG1K) or mouse anti-human CD95 FITC (isotype IgG1K) antibodies. The PBMC regulatory T cell type 1 (Tr1) phenotype was assessed by using the mouse anti-human CD4 APC Cy7 (isotype IgG1k), mouse anti-human CD25 APC (isotype IgG1k) and rat anti-human IL-10 PE (isotype IgG1k) antibodies. Anti-human FoxP3, anti-human CTLA-4 and anti-human IL-10 antibodies were used for intracellular staining with the eBioscience kit (eBioscience, Santa Clara, CA, USA). For intracellular staining with anti-human IL-10 antibodies, cells were pretreated with 2 μl brefeldin A (5 mg/ml) for 4 h at 37°C/5% CO_2.

The dendritic cells (DCs) phenotype from PBMCs was characterized using the following antibodies: mouse anti-human CD11c PerCp-eFluor 710 (isotype IgG1k), mouse anti-human human leucocyte antigen D-related (HLA-DR) (CD74) FITC (isotype IgG2ak), mouse anti-human CD86 APC (isotype IgG1k) and mouse anti-human CD40 PE (isotype IgG1k). The phenotype of tolerogenic DCs from PBMCs was determined by using the following antibodies: mouse anti-human CD11c PerCp-eFluor 710 (isotype

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IgG1k), mouse anti-human signalling lymphocytic activation molecule 1 (SLAMF1) (CD150), PE (isotype IgG1k), mouse anti-human CD205 FITC (isotype IgG2bk) and mouse anti-human ILT3 (CD85K) APC (isotype IgG1k).

To assess lymphocyte activation, PBMCs were stained by using the following antibodies: mouse anti-human CD3 FITC (isotype IgG1k), mouse anti-human CD4 PerCP Cy5·5 (isotype IgG1k), mouse anti-human CD69 PE (isotype IgG1k) and mouse anti-human CD38 PE (isotype IgG1k).

Most antibodies and reagents used for cell staining were purchased from Affymetrix/ eBioscience. All antibodies were titrated for optimal detection of positive populations prior to use, following the manufacturer's recommended concentrations.

Measurements for cytometry analysis of T_{regs} included: (1) classic phenotype ($CD4^{+}CD25^{high}FoxP3^{+}$) [22]; (2) suppressive phenotype ($CD4^{+}CD25^{high}FoxP3^{+}CD127^{low}$): in this context, a low or negative expression of CD127 was associated with suppressive T_{regs} according to previous reports [23]; (3) activated phenotype ($CD4^{+}CD45RO^{+}FoxP3^{high}$); (4) resting phenotype ($CD4^{+}$CD45RO-$FoxP3^{low}$); and (5) non-T_{reg} phenotype ($CD4^{+}CD45RO^{+}FoxP3^{medium}$) [24,25]. For cytometry analysis of the classic T_{reg} phenotype, cells were gated according to CD4 expression and forward-scatter properties. Gated cells were then analysed for double $CD25^{high}$ and $FoxP3^{+}$ expression.

To analyse the T_{reg} suppressive phenotype, total lymphocytes were first gated according to forward-/side-scatter properties; $CD4^{+}$ and $CD25^{high}$-positive cells were selected from these gated cells. A third gate was established according to FoxP3 and CD127 expression. All events read as $FoxP3^{+}/CD127^{low}$ were considered to be suppressive T_{regs}. For other T_{reg} phenotypes, $CD4^{+}$ lymphocytes were gated according CD45RO and FoxP3 expression. $CD45RO^{+}/FoxP3^{high}$ cells were regarded as active T_{regs}; $CD45RO^{+}/FoxP3^{medium}$ cells were considered as non-T_{regs} and cells CD45RO-/$FoxP3^{low}$ were regarded as resting T_{regs}.

T_{reg} cells were also characterized from $CD4^{+}$ gated cells according to the double expression of $CD25^{high}$, along with one of the following: CTLA-4, LAG-3, PD-1, IL-10, GITR or CD95.

To analyse the phenotype of DCs, cells were first gated according to forward-/side-scatter properties. Gated cells were then analysed for double expression of CD11c and one of the following: HLA-DR, CD86 or CD40 for active DCs, and SLAMF1, CD205 or immunologlobulin-like transcript 3 (ILT3) for tolerogenic DCs.

For the analysis of CD4 activated populations, cells were gated according to CD3 expression and forward-scatter properties. Total $CD3^{+}$ cells were analysed for double expression of CD4 and CD69 or CD4 and CD38.

Most antibodies and reagents used for cell staining were purchased from Affymetrix/ eBioscience.

Fluorescence activated cell sorter (FACS)Calibur and FACSAria cytometers were used for data acquisition. The Cytometer Cell Quest software (Becton Dickinson, Franklin Lakes, NJ, USA) was used for data analysis.

Cytokines

IL-2, IL-4, IL-6, IL-10, IL-35 and TGF-β levels in plasma and CSF were measured with commercial sandwich enzyme-linked immunosorbent assay (ELISA) kits. TGF-β was measured with the human/mouse TGF-β1 ELISA Ready-Set-Go kit, which includes a heat-and acid-treatment to measure all TGF-β1 isoforms. Detection limits were: 2 pg/ml for IL-12, IL-4, IL-6, IL-10 and IL-35; 4 pg/ml for IL-5; and 60 pg/ml for TGF-β. All analyses were run in duplicate.

Most ELISA kits were purchased from Affymetrix/eBioscience. Human IL-35 kit was purchased from Biolegend (San Diego, CA, USA).

Lymphocyte proliferation

PBMCs were recovered from 10 ml of venous blood from 20 patients and 16 controls by Ficoll-Paque (Amersham Life Sciences, Little Chalfont, UK). PBMCs, 10×10^{6} cell/ml, were labelled with carboxyfluorescein diacetate succinimidyl ester (CFSE) 5 μM in phosphate-buffered saline (PBS) supplemented with 10% fetal bovine serum and incubated for 5 min at 37°C. Cells were washed three times with cool RPMI-1640 (Gibco BRL, Grand Island, NY, USA), 10% fetal calf serum (FCS) (Gibco BRL). After washing, 1×10^{5} cells were incubated with 200 μl of RPMI-1640 plus 1% antibiotic (penicillin and streptomycin) (Gibco BRL) supplemented with 10% human AB type serum and 50 μM of 2-mercaptoethanol (Sigma, St Louis, MO, USA). Cells were stimulated with either phytohaemagglutinin A (PHA) 1 : 50 (Gibco BRL), total extract of *T. solium* cysticercal soluble antigens (20 μg/well) or antigens from vesicular fluid (20 μg/well). After 4 days of culture, cells were harvested and the total proliferative response was analysed with CFSE marker. For analysing proliferative T_{regs}, lymphocyte cells were gated according to forward-/side-scatter properties. Proliferative cells were gated according to CFSE and CD4 markers. A new gate was set in the proliferative zone of $CD4^{+}$ according to the CD25 and FoxP3 markers. T_{regs} were considered as $CD4^{+}CD25^{high}FoxP3^{+}$. Data were acquired using a FACScalibur cytometer and analysed with the Cytometer Cell Quest software (Becton Dickinson).

Cysticercal antigens

Briefly, *T. solium* cysticerci were obtained from muscles of naturally infected pigs and cultivated for 3 days in RPMI-1640 plus 1% antibiotic (penicillin and streptomycin) and 10% FCS (both purchased from Gibco BRL) for 3 days before use, to eliminate host molecules. At this time of culture, no anti-cysticerci immunoglobulins were detected by ELISA (data not shown) in cysticercal extracts, an indication that harvested cystecerci were mostly free of the host's

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immunological molecules. After culture, cysticerci were frozen, macerated and centrifuged for 30 min at 3500 ***g***. The supernatant containing soluble proteins was collected as total extract. The vesicular fluid was collected by puncturing each cysticercus.

Statistical analysis

Data were processed in SPSS version 21 (IBM Corporation). Variables are reported as mean ± standard deviation (s.d.). Differences between groups were calculated with Student's *t*-test or Mann–Whitney *U*-test, as appropriate. A *P*-value less than 0·05 was regarded as significant.

Results

Blood levels of regulatory T cells are increased in NC patients

The percentage of $CD4^+$ cells in peripheral blood did not differ significantly between healthy controls and NC patients. As shown in Table 1, significant differences between patients and controls were found in the analysed T_{reg} phenotypes. Higher levels of activated T_{regs} $CD4^+CD45RO^+FoxP3^{high}$ ($P = 0.001$) and $CD4^+CD25^{high}FoxP3^+CD95^{high}$ ($P = 0{\cdot}002$) and non-T_{reg} cells $CD4^+CD45RO^+FoxP3^{med}$ ($P = 0.001$) were found in the peripheral blood of NC patients with respect to healthy controls. No differences were observed in the levels of resting T_{reg} $CD4^+CD45RO\text{-}FoxP3^{low}$ phenotype, either in suppressive T_{reg} $CD4^+CD25^{high}FoxP3^+CD127^{low}$ or in the classical T_{reg} $CD4^+CD25^{high}$ $FoxP3^+$ phenotypes.

T_{regs} are found in the cerebrospinal fluid of NC patients

Cells were found in the CFS of 11 patients in concentrations ranging from $6{\cdot}8 \times 10^4$ to $2{\cdot}78 \times 10^6$ cells/ml. Different T_{reg} phenotypes were detected: classical $CD4^+CD25^{high}FoxP3^+$ ($7{\cdot}39 \pm 4{\cdot}94$ cells/ml); activated $CD4^+CD45RO^+FoxP3^{high}$ ($5{\cdot}10 \pm 6{\cdot}28$ cells/ml); suppressive $CD4^+CD25^{high}FoxP3^+CD127^{low}$ ($35{\cdot}10 \pm 36{\cdot}50$ cells/ml); resting $CD4^+CD45RO\text{-}FoxP3^{low}$ ($1{\cdot}76 \pm 0{\cdot}59$ cells/ml) and non-T_{reg} $CD4^+CD45RO\text{-}FoxP3^{med}$ ($13{\cdot}81 \pm 1{\cdot}762$ cells/ml). A *k*-related test was performed comparing the level of the aforementioned cell populations ($P = 0{\cdot}038$). Positive correlations were found between the central and peripheral levels of suppressive T_{regs} ($r = 0{\cdot}982$, $P < 0{\cdot}001$) and between the central levels of activated T_{regs} with the peripheral levels of suppressive T_{regs} ($r = 0{\cdot}982$, $P = 0{\cdot}003$).

Table 1. Levels of regulatory T cell phenotypes in neurocysticercosis (NC) patients and controls.

Phenotype	NC patients (%)	Controls (%)	P
$CD4^+CD25^{high}FoxP3^+$	4·80 ± 2·83	3·01 ± 2·68	0·064
$CD4^+CD45RO^+FoxP3^{high}$	2·19 ± 0·96	0·98 ± 0·87	0·001*
$CD4^+CD45RO^+FoxP3^{med}$	8·83 ± 2·75	5·24 ± 2·50	0·001*
$CD4^+CD45RO^-FoxP3^{low}$	4·34 ± 1·62	3·56 ± 2·45	0·308
$CD4^+CD25^{high}FoxP3^+CD127^{low}$	15·37 ± 14·68	17·96 ± 14·9	0·606
$CD4^+CD25^{high}FoxP3^+CD95^{high}$	1·58 ± 1·19	0·40 ± 0·47	0·002*
$CD4^+$	35·98 ± 16·58	36·14 ± 11·17	0·974

*Significantly different between NC patients and controls with 95% confidence. FoxP3 = forkhead box protein 3.

T_{regs} express molecules known to be involved in cell-to-cell contact with DCs

As shown in Fig. 1, NC patients express higher levels of CTLA-4, LAG-3, GITR and PD-1 in T_{regs} than healthy controls ($P < 0{\cdot}05$). A positive correlation was found between CTLA-4^+ T_{regs} and LAG-3^+ T_{regs} ($r = 0{\cdot}892$, $P < 0{\cdot}001$).

As DCs present the ligands for the suppressive molecules CTLA-4, LAG-3, GITR and PD-1, their phenotype was studied. As shown in Table 2, increased levels of HLA-DR and the suppressive molecules SLAMF1 and CD205 were found in NC patients with respect to controls.

Levels of regulatory cytokines in NC patients

The three main regulatory cytokines were found in CSF in widely varying concentrations among patients. IL-10 ranged from 23·64 to 319·14 pg/ml, TGF-β from 245·24 to 7305·65 pg/ml and IL-35 from 1·10 to 26·80 pg/ml. Central TGF-β levels correlated positively with the percentage of central activated T_{regs} ($r = 0{\cdot}989$, $P = 0{\cdot}011$) and central suppressive T_{regs} ($r = 0{\cdot}976$, $P = 0{\cdot}004$).

Additionally, linear regression analyses showed that both CSF TGF-β and IL-35 levels are related to peripheral activated T_{reg} phenotype ($P = 0{\cdot}048$ and $P = 0{\cdot}011$, respectively).

In the periphery, only IL-10 levels were found increased ($P = 0{\cdot}003$) (Fig. 2). Interestingly, Tr1 cells ($CD4^+CD25^{high}IL\text{-}10^+$), the main producers of IL-10, were also found increased in NC patients ($P = 0{\cdot}028$) with respect to controls ($1{\cdot}04 \pm 1{\cdot}32$ and $0{\cdot}26 \pm 0{\cdot}33$, respectively). A positive correlation was found between Tr1 and CTLA-4^+ T_{regs} ($r = 0{\cdot}868$, $P < 0{\cdot}001$), as well as LAG-3^+ T_{regs} ($r = 0{\cdot}705$, $P = 0{\cdot}003$) and PD-1^+ T_{regs} ($r = 0{\cdot}528$, $P = 0{\cdot}043$).

Peripheral IL-35 level correlated positively with the three central phenotypes of T_{regs}: suppressive ($r = 0{\cdot}969$, $P = 0{\cdot}001$), classic ($r = 0{\cdot}914$, $P = 0{\cdot}002$) and activated ($r = 0{\cdot}989$, $P = 0{\cdot}001$).

T_{reg} levels are not increased in *in-vitro* proliferating lymphocytes

Both specific and non-specific proliferative immune responses were evaluated (Table 3). The specific proliferative response induced by total and vesicular cysticercal extracts was increased significantly in NC patients compared to controls ($P < 0{\cdot}05$). The non-specific proliferative response to PHA was similar in

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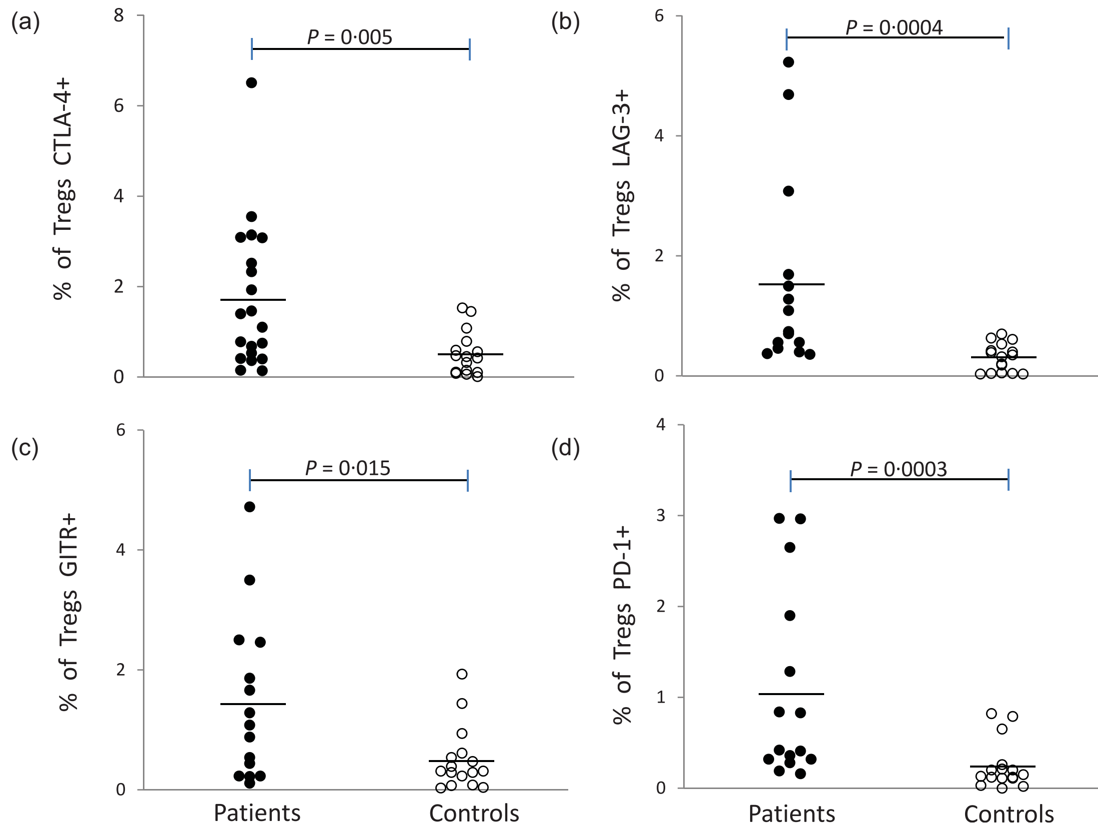

Fig. 1. Regulatory T cell (T_{reg}) expression levels of cytotoxic T lymphocyte antigen 4 (CTLA-4), lymphocyte-activation gene 3 (LAG-3), glucocorticoid-induced tumour necrosis factor receptor (GITR) and programmed cell death 1 (PD-1) in neurocysticercosis (NC) patients and controls. Individual data are shown for T_{regs}: (a) CTLA-4$^+$, (b) LAG-3$^+$, (c) GITR$^+$ and (d) PD-1$^+$. Total CD4$^+$ cells were gated according to the double expression of CD25^{high} and CTLA-4, LAG-3, PD-1 or GITR. *Significantly different between NC patients and controls with 95% confidence.

both groups. The percentage of T_{regs} was not modified in the proliferating cells, disregarding the proliferation observed.

Effector immune response during NC

To explore the effector immune response exerted by CD4 T cells, the levels of early (CD69) and late (CD38) activation in CD4 cells were determined. Both CD4$^+$CD69$^+$ and CD4$^+$CD38$^+$ levels were similar in patients and controls ($4·39 \pm 3·84$ *versus* $5·83 \pm 10·51$, respectively, for CD4$^+$CD69$^+$ and $17·89 \pm 13·20$ *versus* $17·96 \pm 11·13$, respectively, for CD4$^+$CD38$^+$). A statistically significant negative correlation between peripheral suppressive T_{regs} and CD4$^+$CD38$^+$ cells was found in NC patients ($r = -0·527$, $P = 0·025$). In addition, the levels of IL-2, IL-4, IL-5 and IL-6 cytokines, most of them inflammatory, were measured in CSF and plasma (Fig. 3). IL-5 and IL-6 were found in CSF. Interestingly, lower IL-5 and IL-6 peripheral levels were found in NC patients with respect to controls ($P < 0·05$). A positive correlation was also found between peripheral IL-2 levels and both central classic T_{regs} ($r = 0·783$, $P = 0·022$) and activated T_{regs} ($r = 0·989$, $P = 0·001$).

Table 2. Levels of regulatory and activating molecules on dendritic cells in neurocysticercosis (NC) patients and controls.

Phenotype	NC patients (%)	Controls (%)	P
SLAMF1$^+$CD11c$^+$	$7·77 \pm 5·71$	$3·43 \pm 1·25$	0·015*
CD205$^+$CD11c$^+$	$22·63 \pm 15·83$	$10·25 \pm 9·68$	0·029*
ILT3$^+$CD11c$^+$	$38·45 \pm 27·87$	$35·08 \pm 20·28$	0·715
HLA-DR$^+$CD11c$^+$	$71·16 \pm 19·80$	$57·18 \pm 16·51$	0·048*
CD86$^+$CD11c$^+$	$43·86 \pm 24·83$	$39·16 \pm 19·39$	0·573
CD40$^+$CD11c$^+$	$9·07 \pm 8·41$	$6·52 \pm 8·38$	0·430

*Significantly different between NC patients and controls with 95% confidence. HLA-DR = human leucocyte antigen D-related; SLAMF1 = signalling lymphocytic activation molecule 1; ILT3 = immunologlobulin-like transcript 3.

Discussion

T_{reg} recruitment from the periphery to the central nervous system in human NC has been reported previously [5]. To deepen our understanding of this phenomenon, the levels

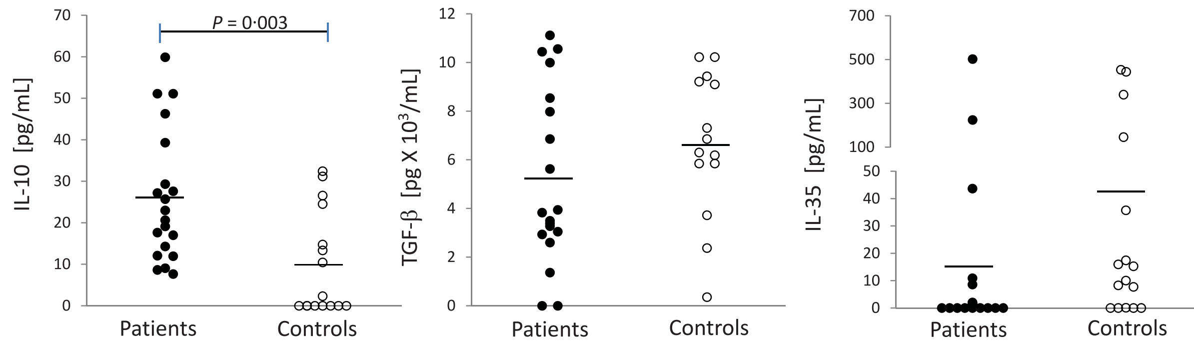

Fig. 2. Peripheral levels of regulatory cytokines. Plasma was tested by enzyme-linked immunosorbent assay (ELISA) for interleukin (IL)-10, transforming growth factor (TGF)-β and IL-35 in patients and controls. *Significantly different between neurocysticercosis (NC) patients and controls. $P < 0{\cdot}005$.

of several T_{reg} phenotypes, both in the CNS and in peripheral blood, are reported here. In this context, the case of T_{regs} with low CD127 expression, which has been reported as a suppressive phenotype, is interesting [23]. Previous studies have shown that the differential expression of CD45RO and FoxP3 could distinguish activated and functional T_{regs} [24,25]. According to our data, NC patients show an increase in the levels of both activated T_{regs} and non-T_{regs} , but not of resting T_{regs}. These findings could be associated with the plasticity exhibited by T_{regs} [26]. Depending on the environment, T_{reg} cells could lose or re-acquire the suppressive function [27]. Sakaguchi *et al.* have proposed that the changes in the expression of FoxP3 and in the functionality of T_{regs} could be mediated by epigenetic changes [28]. Certain conditions promote stable epigenetic changes, allowing the maintenance of $FoxP3^+$ expression and the suppressive function in the long term [27,28]. Thus, it is possible that non-T_{reg} cells could be in transition to T_{regs} during NC.

The observed increase in the level of phenotypically activated T_{regs} in NC patients allows us to propose that T_{regs} have an active and functional phenotype during NC. More importantly, the levels of two phenotypes associated with a suppressive function ($CD4^+CD45RO^+FoxP3^{high}$ and $CD4^+CD25^{high}FoxP3^+CD127^{low}$) are correlated between the periphery and the site where the parasite establishes (CSF), a finding that may suggest that T_{regs} are exercising a suppressive action in CSF.

A mechanism through which T_{regs} exert their function is direct cell–cell contact [9]. In this situation, T_{regs} interact with DCs through PD-1, CTLA-4, LAG-3 and GITR, inducing a tolerogenic DC phenotype [20,29]. These molecules were found highly expressed in T_{reg} cells from NC patients with respect to controls. A positive significant correlation between CTLA-4^+ T_{regs} and LAG-3^+ T_{regs} suggests that T_{regs} could express both molecules during NC. It is known that CTLA-4 has 10 times more affinity to its ligand than CD80/CD86, and LAG-3 interacts with HLA-DR in DCs, preventing antigen presentation [9].

The significant increase in the expression of HLA-DR, SLAMF1 and CD205 on DCs suggest a tolerogenic phenotype, as reported previously *in vitro* [6]. Additionally, increased plasmatic levels of Tr1 and IL-10 were found in NC patients. Thus, it is feasible that T_{regs} promote a tolerogenic DC phenotype which, in turn, induces Tr1 cells, as reported previously [30]. The positive correlations between T_{reg} CTLA-4, LAG-3 and PD-1 with Tr1 in NC patients support this supposition. It is probable that the release of IL-10 by Tr1 cells could promote an immunoregulatory environment during NC.

In addition, other cytokines such as TGF-β and IL-35, which could be produced by T_{regs}, may also participate in controlling the immune response. Our data demonstrate the presence of TGF-β, IL-35 and IL-10 in CSF from NC patients. In this context, a positive correlation was found between central TGF-β levels and central suppressive

Table 3. Proliferative levels of PBMCs after specific and non-specific stimulus.

	Total proliferation (%)			T_{reg} proliferation (%)		
Stimulus	Patients	Controls	*P*	Patients	Controls	*P*
PHA	47·95 ± 32·42	44·41 ± 20·71	0·710	6·45 ± 16·07	14·62 ± 16·44	0·218
Cysticercal vesicular extract	24·10 ± 31·28	4·79 ± 5·96	0·024*	0·11 ± 0·17	0·34 ± 0·76	0·336
Cysticercal total extract	22·98 ± 30·53	5·49 ± 3·80	0·046*	0·36 ± 0·55	1·05 ± 1·74	0·236

*Significantly different between neurocysticercosis (NC) patients and controls with 95% confidence. PHA = phytohaemagglutinin; T_{reg} = regulatory T cell.

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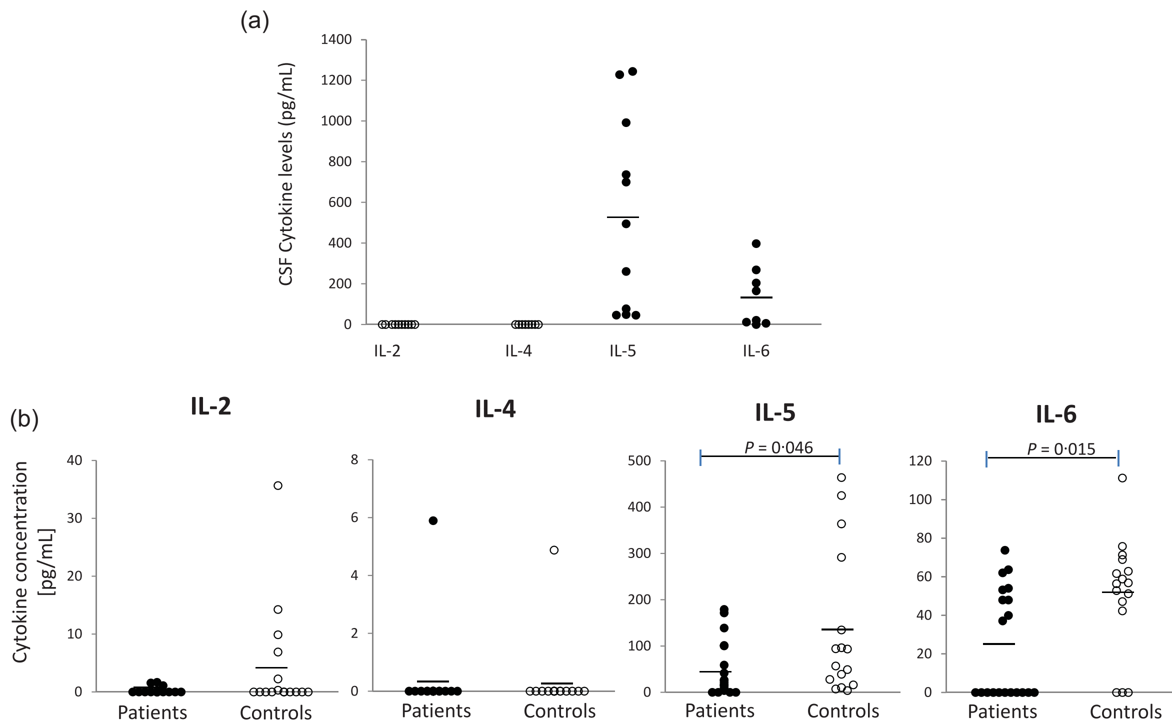

Fig. 3. Inflammatory cytokines in the central and peripheral compartments. Cerebrospinal (CSF) and plasma were tested by enzyme-linked immunosorbent assay (ELISA) for interleukin (IL)-2, IL-4, IL-5 and IL-6. (a) Central (CSF, a *k*-related test was performed, $P < 0{\cdot}001$), and (b) peripheral levels in patients and controls. *Significantly different between neurocysticercosis (NC) patients and controls. $P < 0{\cdot}005$.

and activated T_{reg} phenotypes ($CD4^+CD25^{high}FoxP3^+CD127^{low}$ and $CD4^+CD45RO^+FoxP3^{high}$). Moreover, we found a significant relation between central TGF-β and IL-35 levels and peripheral activated T_{regs} ($CD4^+CD45RO^+FoxP3^{high}$), suggesting an important interaction between the central response and that of periphery, as TGF-β and IL-35 promotes T_{regs} [13,31]. Interestingly, peripheral IL-35 levels correlate positively with suppressive, classic and activated T_{reg} phenotypes at the central compartment, suggesting the importance of these peripheral cytokines in the central T_{reg} response as IL-35 promotes T_{regs} [31]. Finally, our data show that central levels of IL-10, TGF-β and IL-35 in NC patients could be relevant for the parasite establishment by promoting a regulatory-driven, permissive environment.

Conversely, considering that severe NC patients show a depressed peripheral cellular immune response [3] and that previous findings suggest that cysticerci may promote T_{reg} cells *in vitro* [6], we challenged the hypothesis that T_{regs} would increase its proliferation, exceeding that of effector cells during parasite stimulation. The number of T_{regs} was determined at the end of the assay, but no difference was found between patients and controls. Thus, our hypothesis was discarded and T_{regs} do not seem to be involved in controlling the specific proliferative immune response.

With regard to the inflammatory response, lower levels of IL-5 and IL-6 were found in NC patients, which suggest that T_{regs} could be inhibiting effector T cells [22,23]. Additionally, a negative correlation was found between suppressive T_{regs} ($CD4^+CD25^{high}FoxP3^+CD127^{low}$) and T $CD4^+CD38^+$cells, which supports this idea.

Interestingly, IL-2 correlates positively with classic and activated central T_{regs}. These data are consistent with the fact that IL-2 is a growth factor for T_{regs} [32]; it also suggests that IL-2 could be used by T_{regs} during NC.

T_{regs} play a preponderant role in controlling the immune response in other parasitic infections. In fact, depleting these cells decreases the number of parasites in Chagas' disease [33]. A number of studies describe some mechanisms for T_{reg} suppressive actions and their effect in parasitic diseases. For example, in the infections by *Plasmodium vivax*, *Wuchereria bancrofti* and *Mansonella perstans*, host T_{regs} express CTLA-4, LAG-3 and GITR, suggesting a possible interaction between T_{regs} and DCs. In addition, the presence of T_{regs} is related to higher parasite loads [34,35]. Conversely, parasites such as *Trypanosoma cruzi*, *Plasmodium falciparum*, *P. vivax* and *Heligmosomoides polygyrus* exploit the production of imunomodulatory cytokines such as IL-10 and TGF-β by T_{regs} to promote parasite growth [34,36–38]. This study is the first to extensively characterize the different Treg populations and their mechanisms of action during human NC. Our data suggest that T_{regs} may act by

promoting a modulatory environment that favours the survival of the parasite.

In conclusion, our data suggest that T_{regs} suppress the immune response against the parasite during neurocysticercosis, probably by interacting with DCs through CTLA-4, LAG-3, PD-1 and GITR. This interaction could be promoting a tolerogenic DC phenotype which, in turn, induces Tr1 cells. Tr1 cells could then release IL-10 and create an immunomodulatory environment that would favour the development of *T. solium* cysticerci and its permanence in the host's central nervous system. Further studies will be conducted to confirm that T_{regs} actually suppress the immune response through direct contact with DCs.

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Disclosure

The authors declare no conflict of interest regarding the publication of this paper.

Author contributions

A. A.-S., D. D. Á.-L., D. C.-H., M. H. and F. G.-V. performed the experiments; A. F. and J. V.-P. provided samples from human patients; A. A.-S. and G. C. performed the statistical analysis; A. A.-S., G. F., E. S. and L. A.-P. designed the study protocol. A. A.-S., G. C., G. F., E. S. and L. A.-P. wrote the paper.

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