UNIVERSIDAD AUTÓNOMA DE SAN LUIS POTOSI
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Análisis del número y la función de células T reguladoras en niños con parasitosis intestinal

TESIS QUE PRESENTA
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Quantitative and functional analyses of T regulatory cells in children with chronic parasite infection

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Key words: regulatory T cells, helminthes, lymphocytes, immune activation

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ABSTRACT

Introduction. Diseases caused by helminths and protozoa are highly prevalent in the third world, mainly in children. It has been reported that chronic parasite infections induce a persistent activation of the immune system that results in defective responsiveness of T cells with a decrease in immunocompetence. Since regulatory T cells can exert a key effect on immunocompetence, we performed a quantitative and functional analysis of different subsets of regulatory T cells in children with chronic parasitic infections.

Patients and Methods: Ninety three native children (6-12 years old) from a tropical community of the municipality of Cd. Valles,San Luis Potosi, México, were divided in two groups, with or with out parasite infection (n=52, and 41, respectively) according to the coproparasitoscopic examination. Different regulatory T cell subsets, and activated T cells were detected in peripheral blood mononuclear cells (PBMC) by immunostaining with specific monoclonal antibodies and flow cytometry analysis, whereas the suppressive function of CD4+CD25+ lymphocytes was assessed by a carboxyfluorescein (CFSE) dilution cell proliferation assay.

Results: Levels of CD3+HLA-DR+ T cells were similar in children with and without parasitic infection. However, a significantly enhanced number of CD3+CD69+ lymphocytes was detected in children with parasitic infection. Although the levels of CD4+CD25^{high} and CD4+Foxp3+ cells tended to be higher in parasitized children. no significant differences were detected when compared to those of control group. In contrast, the number of CD8+CD28- T suppressor cells (Ts) was significantly increased in parasitized children with chronic immune activation (with high levels of either CD3+HLA-DR+ or CD3+CD69+ cells) compared to control group. In addition, enhanced levels of CTLA-4+ lymphocytes was detected in cells from helminth-parasitized children ($p<0.05$). Functional assays showed that parasitized children with chronic immune activation had an apparently diminished proliferative

response of T cells to CD3/CD28 stimulation compared to controls. However, no apparent differences were detected in the suppressive function of natural Treg cells in the two groups studied

Conclusion: Parasitized children with chronic immune activation show increased levels of Ts cells (CD8+CD28-), and apparently diminished responsiveness of T cells. However, they show normal numbers of Treg lymphocytes (CD4+CD25^{high} and CD4+Foxp3+), with a normal suppressive function of these cells These data suggest that Ts lymphocytes, but no natural Treg cells seem to have an important role in the diminished immunocompetence observed in parasitized children with chronic immune activation.

INTRODUCTION

Infections by intestinal parasites is a highly prevalent condition in tropical countries of the third world, mainly in children from families with low income (1). Thus, an important proportion of children in these countries are infected by *Entamoeba histolytica, Ascaris lumbricoides*, *Giardia lamblia*, *Trichuris trichiura*, *Hymenolepis nana*, *Necator americanus*, *Enterobius vermicularis*, *Strongyloides stercolaris* and *Taenia sp*. Deficient sanitary conditions, as a part of the low socioeconomic class as well as lack of access to clean water, deficient sewage elimination systems and tropical weather have been described as risk factors for parasite infections (2).

The immune response against intestinal infection by parasites is mainly mediated by Th2 cells (9, 10), with increased synthesis of IgE and IgG4, and eosinophilia (9, 10, 37). It has been shown that this type of response is able to kill extracellular parasites in vitro. However, since IgG4 may block the mechanisms mediated by IgE it is still unknown whether or not this type of response is beneficial for the host in vivo (10).

Regulatory T cells (Treg) suppress immune responses, mainly the proliferation and cytokine production of CD4+ and CD8+ effector T cells (18, 21, 24). This effect has a key role in the maintenance of immune tolerance and prevents the development of autoimmune diseases (15, 16, 17). In addition, Treg cells have an important role in the modulation of immune response against microbial pathogens, and they are clearly involved in the balance between tissue inflammation and the development of effector mechanism that kill the pathogen (12,13).

Several subsets of CD4+ and CD8+ T cells with regulatory activity have been described (12, 24, 25, 30, 38, 43). Natural Treg cells represent 5 to 10% of peripheral CD4+ T cells and constitutively express the cytotoxic T lymphocyte antigen-4 (CTLA-4), the glucocorticoid-induced TNF-receptor related gene (GITR), CD25 (α-chain of IL-2 receptor) (22) and the transcription factor Foxp3. The latter

molecule is a member of the forkhead family of transcription factors that bind to DNA and has been demonstrated that has a key role in the differentiation and suppressive function of these cells (14, 32), which synthesize transforming growth factor-β (TGF-β) and IL-10. These cells are anergic when are stimulated in vitro through CD3, but proliferate upon addition of exogenous IL-2 (39). Natural Treg cells show a remarkable suppressive effect both in vitro and in vivo on the activation and proliferation of naive CD4+ (18, 11) and CD8+ T cells (20, 21) in an antigen-non-specific manner, via a mechanism that requires cell-cell contact and that is apparently independent of TGF-β and IL-10 (11, 39).

It has been shown that the removal of CD4+CD25+ cells enhances the immune response against protozoan infections such as *Leishmania major*. Interestingly, this maneuver leads to a complete eradication of parasites from the infection site (8). On the other hand, it has been observed that chronic infections by helminths are associated to persistent immune activation, with an unbalanced immune profile (5, 6). The peripheral T cells obtained from these infected individuals show a low response to parasite antigens with deficient proliferation, and synthesis of Th1 cytokines (3, 4). In addition, these patients show high levels of CD4+CTLA-4+ and CD4+CD25+ as well as activated (CD3+HLA-DR+) T cells, and enhanced synthesis of TGF-β (29). These phenomena decreased 6 to 12 months after deworming procedure (7).

CD8+CD28- suppressor T cells (Ts) are class I MHC-restricted, and also they are able to render dendritic antigen presenting cells (APC) tolerogenic, down-regulating thus the immune response. These tolerogenic cells show a diminished expression of costimulatory molecules as CD80, and CD86, and an increased synthesis of the inhibitory receptors ILT3 and ILT4 (23). Ts cells seem to have an important role in oral tolerance as well as in the regulation of inflammation in gut (26-28).

In this work, we have explored the status of regulatory T cells in children with chronic parasitic infections. We found enhanced levels of CD8+CD28- suppressor cells in patients with parasitic infections, with no significant differences in the number or function of natural Treg cells compared to control group. These data suggest that T suppressor, but apparently not natural Treg cells, could contribute to the diminished immunocompetence seen in children with chronic parasitic infection.

MATERIAL AND METHODS

Individuals. Ninety three children from a Tenek indian community (La Subida) of the municipality of Ciudad Valles, San Luis Potosi, México were studied They were 52 females and 41 males, with a mean age of 8.5 yr (range 6-12), and were divided in two groups, with and without parasite infection, according to the results of the coproparasitoscopic examination. No significant differences were found in age, height and weight between parasitized and non-parasitized children (Table 1). A written informed consent was obtained from the parents of all children before entering the study.

Cells. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque density gradient centrifugation (Sigma Chemical Co., St Louis, MO), washed two times with phosphate-buffered saline (PBS) and resuspended $1x10^6$ cells/mL in RPMI 1640 culture medium (Hyclone, Laboratories, Inc, Logan, UT), supplemented with 10% fetal bovine serum (GIBCO BRL), 2 mM L-glutamine (Sigma), 50 U/mL penicillin and 50 μg/mL streptomycin (Sigma). In some experiments, cells were stimulated with 10 μg/mL of the T3b anti-CD3 mAb and 10 μg/mL of anti-CD28 mAb (BD PharMingen, San Jose, CA) for 72 hs at 37°C and 5% CO_2.

Flow cytometry analysis. Cells were immunostained with the indicated monoclonal antibodies (mAbs), e.g., anti-CD4-phycoeritrin (PE), and an anti-CD25-fluorescein isothiocyanate (FITC), or anti-CD28-PE, and anti-CD8-FITC (Becton-Dickinson, San Jose, CA), washed, fixed with 1% p-formaldehyde, and analyzed using the Cell Quest software and a FACSCalibur flow cytometer (Becton Dickinson). For the detection of intracellular antigens, specific mAbs for CTLA-4 (BD PharMingen) and Foxp3 (PCH10 clone, eBioscience, San Diego, CA) were used. A double-labeling procedure was performed, first with an anti-CD4-FITC mAb, and then followed by fixation with 4% p-formaldehyde for 10 min at room temperature and permeabilization with 0.01% of saponine in PBS for 5 min on ice.

Finally, cells were additionally stained with anti-CTLA-4-PE and analyzed by flow cytometry. In separate experiments, cells were fixed, permeabilized (fixation-permeabilization buffers, eBiocience) and stained with an anti-foxp3-PE mAb, and then with an anti-CD4-FITC mAb. Results were expressed as the absolute number of positive cells/μL.

Cell proliferation assay. To analyze the suppressive function of CD4+CD25+ T lymphocytes on cell proliferation, we performed a fluorescent label cell partition assay, as described (50, 51). Briefly, by using a MACS separation column (Miltenyi Biotec), PBMCs were depleted or not of CD25+ T cells, and the depleted and non-depleted cells were labeled with 5 μM carboxyfluorescein succinimidyl ester (CFSE) (Molecular Probes, Invitrogen, OR). Then, cells were washed, and $2x10^5$ depleted and non-depleted cells were cultured in a flat-bottom, 24-well plates (Costar) that were pre-coated with 10μg/mL anti-CD3, and 10μg/mL anti-CD28 (BD PharMingen). After 3 days at 37°C and 5% CO2, cells were harvested and the percentage of divided cells was detected by flow cytometry analysis. Data were expressed as a percent of inhibition of cell proliferation according to the following formula: % inhibition cell proliferation = 100 (%non-depleted divided cells/% depleted divided cells).

Statistical analysis. Statistical analysis was performed using the Graph Pad program (Graph Pad software, San Diego, CA). The differences in absolute numbers of regulatory T cells were determined by parametric analysis using the student's T test. The association between absolute number of Treg and activated cells was determined by using the Pearson correlation analysis. Values of $p<0.05$ were considered as significant.

RESULTS

We first determined the levels of different lymphocyte subsets in the PBMC from parasitized and non-parasitized groups. Although levels of CD8+ and CD3+ T cells were similar in these groups, we found a significant enhancement in the levels of CD4+ T lymphocytes in parasitized children ($p<0.05$, Table 1). In addition, we found significant higher levels of activated CD3+CD69+ T cells in parasitized children compared to control group (Fig. 1b). In contrast, similar numbers of CD3+HLA-DR+ T cells were found in both groups studied (Fig. 1b) Based on these results, we considered as children with chronic immune activation those children that showed levels of CD69+ or HLA-DR+ T cells above of the value of the median of the control group.

Since it has been reported that parasitized individuals with chronic immune activation show enhanced levels of CTLA-4+ lymphocytes and TGF-β secretion (27), we decided to determinate the number of regulatory T cells in our groups of study. Flow cytometry analysis revealed that although the number of $CD4+CD25^{high}$ and CD4+Foxp3+ cells tended to be higher in parasitized children with chronic immune activation, no significant difference were found when compared to control group (Figs. 2b,c, and 3a,b). In contrast, parasitized children showed significant higher levels of Ts (CD8+CD28-) lymphocytes compared to non-parasitized children (Fig. 3c).

An association analysis between the levels of regulatory cells (both Treg and Ts), and the number of activated (both HLA-DR+ and CD69+) lymphocytes revealed a significant correlation of levels of $CD4+CD25^{high}$ or CD4+Foxp3+ lymphocytes and the number activated T cells ($r=0.5591$ and 0.5992, respectively, $p<0.05$ in both cases, Fig. 3). In contrast, no significant correlation between the levels of Ts and activated lymphocytes was found (Fig. 3f, and data not shown).

Finally, we detected an enhanced number of CTLA-4+ lymphocytes in children infected with helminths compared to both, controls and children infected with protozoa (Fig. 4).

In order to assess the immunocompetence of parasitized children, we stimulated their PBMC through CD3 and CD28, and determined their proliferative response. Even though these parasitized children tended to show lower levels of divided cells compared to control group, no significant differences were detected (Fig. 5a). In addition, the assays to determine the suppressive function of Treg cells showed similar levels of this activity in parasitized and control children (Fig. 5b, c). Finally, we did not detect a significant association, both in parasitized and control children. between the percentage of divided cells and the levels of activated lymphocytes ($r=0.2732$, $p=0.1679$, data not shown).

DISCUSSION

It has been reported that the persistent infections caused by parasites result in a chronic immune activation that is associated to a diminished responsiveness of T cells, and a defective immunocompetence (7, 29, 40, 41, 42). This condition has been associated with an increased number of CTLA-4+ lymphocytes in peripheral blood, and an enhanced secretion of TGF-β (29). As expected, the immunosuppressive state associated with chronic parasitic infection, may limit the success of preventive strategies based on vaccination. In addition, it is very feasible that the antigens or factors released by parasites that induce activation of immune cells and diminish the immunocompetence may favor the persistence of the infectious process.

In order to further explore the possible mechanisms of immunosuppression induced by chronic helminthic/protozoan infection, we decided to assess the status of regulatory T cells in children with persistent parasite infection. In this regard, the important role of regulatory T cells in different infectious diseases has been clearly demonstrated (12, 19, 28). These data indicate that regulatory T cells exert a key effect on the balance between the generation of an effective immune response and the prevention of tissue damage by the effector mechanisms of immune system (12, 13). Thus, regulatory T cells are able to inhibit the proliferation of effector and naïve lymphocytes as well as to suppress the synthesis of pro-inflammatory cytokines. In addition, these regulatory T cells release immunomodulatory cytokines, mainly IL-10 and TGF-β (11,39). It has been also reported that, as expected, an excessive function of regulatory T cells may result in the abrogation of an immune response and a high risk for infection. Although it has been proposed that natural regulatory cells mainly recognize self-antigens, and that thus these cells are preferentially involved in the tolerance to these antigens, it has been described their participation in infectious diseases (8, 19, 12). In addition, other regulatory T lymphocytes, including CD8+CD28- Ts cells as well as type 1 regulatory (Tr1) lymphocytes, and Tr1-like cells may participate in the modulation

of the immune response, and the inflammatory phenomenon induced by helminthes and protozoan (8, 29, 44).

Although it has been previously reported that natural regulatory T cells ($CD4+CD25^{high}$, CD4+CD25+Foxp3+) are involved in the modulation of the immune response against parasites (8, 7, 29, 31), our results shown that parasitized children with chronic immune activation have normal levels of Treg cells, with no apparent abnormalities in the suppressive function of these cells. However, these children tended to have higher levels of both $CD4+CD25^{high}$ and CD4+Foxp3+ cells in their peripheral blood, and it is feasible that by increasing the number of children studied, a significant difference could be reached. A more clear-cut difference was detected in the case of Ts cells, and these data strongly suggest that these regulatory Tcells may be involved in the diminished immunocompetence observed in parasitized children. In this regard, it has been reported that CD8+ T suppressor cells play an important role in the control of intestinal mucosa inflammation, and that epithelial cells may participate in its induction (28, 33, 34). Likewise, CD8+CD28- T cells appear to be involved in the pathogenesis of pulmonary tuberculosis in adults and infectious mononucleosis in children (35, 36). Unfortunately, the assays that detect the regulatory function of CD8+CD28- lymphocytes (e. gr., induction of expression of ILT3/4 by autologous DCs) requires a large number of PBMC, which was not feasible to obtain from the children included in our study. Therefore, it will be interesting to assess, through future studies, the regulatory function of Ts cells in parasitized children with chronic immune activation.

In contrast with the results reported by Leng, et al (29), we have found in our study that only a fraction of chronic parasitized individuals have evidence of chronic immune activation (high levels of CD3+HLA-DR+ cell in peripheral blood). It is feasible that these apparent contradictory results may be due to differences in age, degree of helminths/protozoan infection, and genetic background. In this regard, we have studied native children from a Tenek-Huasteco indian community, which

must have a different MHC and genetic background than those studied by Leng, et al. In any case, it is of interest that a significant proportion of children studied by us do not show evidence of persistent immune activation and diminished immunocompetence, despite chronic and infection by different helminthes/protozoan. We consider of interest to study other indian and mestizo Mexican communities in order to assess whether they have a different or similar behavior than Tenek children.

The presence of increased levels of CD3+CD69+ cells in a significant fraction of our parasitized children is of interest. Although it very feasible that these cells correspond to activated T lymphocytes, it is also possible that they may exert a regulatory function. In this regard, it has been described that, in addition to its role as an activation molecule, CD69 may confer regulatory activity to T cells (45, 47, 48, 49). This effect appears to be mainly mediated by TGF-β (46), and thus it is feasible that at least a fraction of the CD3+CD69+ cells detected by us may contribute to the diminished immunocompetence observed in some chronic parasitized children.

In summary, our results suggest that the apparently diminished immunocompetence observed in some parasitized children is associated with enhanced levels of peripheral blood CD8+CD28- Ts cells, and CD3+CD69+ lymphocytes. In contrast, our data suggest that natural regulatory T cells (CD4+Foxp3+ and CD4+CD25^{high}) do not seem to be significantly involved in this condition.

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FIGURE LEGENDS

Figure 1. Quantitative analysis of activated T cells in peripheral blood from parasitized and control children. PBMC were isolated from children with chronic helminthic infection (n= 53) and controls (n=29) and immunostained for CD3, HLA-DR, and CD69, as stated in Materials and Methods. Representative histograms of children with high levels of activated cells are shown in a), and data in b) correspond to the arithmetic mean ± SEM. The p value is indicated.

Figure 2. Quantification of regulatory T cells in peripheral blood from parasitized and control children. PBMC were isolated from parasitized children with chronic immune activation (high levels of CD3+CD69+ cells) and controls children, and the number of CD4+CD25^{high}, CD4+Foxp3+ and CD8+CD28- cells was determined by using specific mAb and flow cytometry analysis, as indicated in Material and Methods. Representative histograms are shown in a) and data in b), c), and d) correspond to the arithmetic mean ± SEM. The p value is indicated. n.s., not significant.

Figure 3. Quantification of regulatory T cells in peripheral blood from parasitized and control children. a-c) PBMC were isolated from parasitized children with chronic immune activation (high levels of CD3+HLA-DR+ cells) and the number of CD4+CD25^{high}, CD4+Foxp3+ and CD8+CD28- T cells was determined by flow cytometry analysis, as indicated in Material and Methods. Data correspond to the arithmetic mean and SEM, and the p value is indicated. n.s., non-significant. d-e) Correlation between the levels of CD3+HLA-DR+ lymphocytes and the number of CD4+CD25^{high} d) or CD4+Foxp3+ cells e) in parasitized children with chronic immune activation.

Figure 4. Levels of expression of CTLA-4 in peripheral blood lymphocytes from parasitized children. PBMC were isolated from parasitized children and controls, and the expression of CTLA-4 was assessed by flow cytometry, as stated in

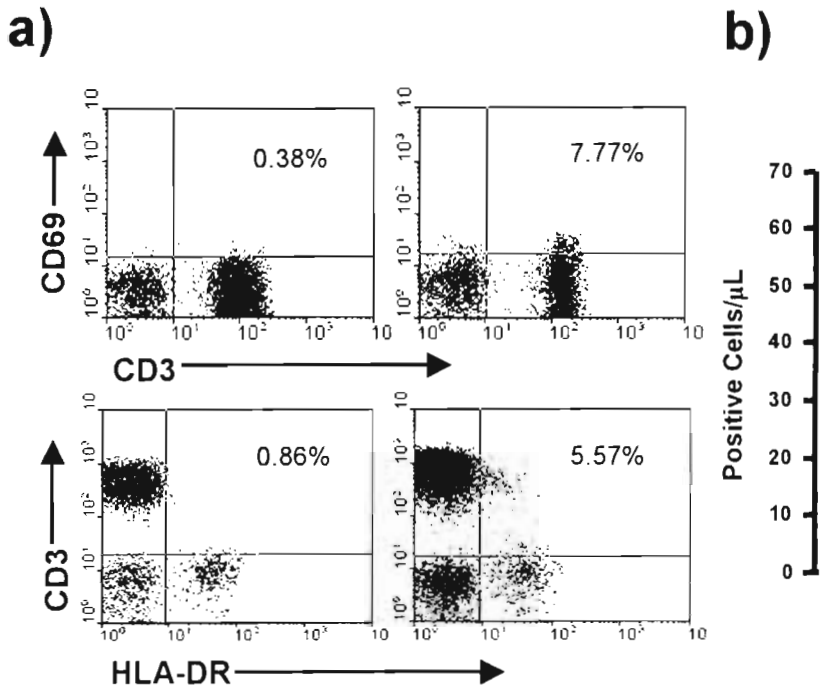
Materials and Methods. A representative histogram is shown in (a). Data correspond to the arithmetic mean ± SEM, and the p value is indicated. n.s., non-significant.

Figure 5. Functional analysis of Treg cells in parasitized children and control group. a) PBMC from parasitized and non-parasitized children were stimulated through CD3/CD28 for three days, and then cell proliferation was determined by a CFSE dilution assay and flow cytometry analysis, as described in Material and Methods. b) PBMC from parasitized and non-parasitized children were depleted or not of CD25+ T cells, and then stimulated through CD3/CD28 for three days. Finally, cell proliferation was assessed as in a). Data correspond to the arithmetic mean ± SEM. n.s., non-significant. Representative histograms of non-depleted (middle panel) and depleted (right panel) cells cultures are shown in c).

Table 1. Main data of children included in the study.

	Parasitized	Non- parasitized
Weight (Kg)	29±7.96	28± 8.11
Height (m)	1.35± 0.11	1.31± 0.14
Age (years)	8.7±1.70	8.5± 1.72
CD4+ T cells/μL	967.5±285.8	760.7±159.1 *
CD8+ T cells/μL	643.6±387.2	623.4±201.4
CD3+ T cells/μL	1859±635.9	1571±414.7

* $p<0.05$


a)
b)
CD69
CD3
0.38%
7.77%
CD3
HLA-DR
0.86%
5.57%
Positive Cells/µL
70
60
50
40
30
20
10
0

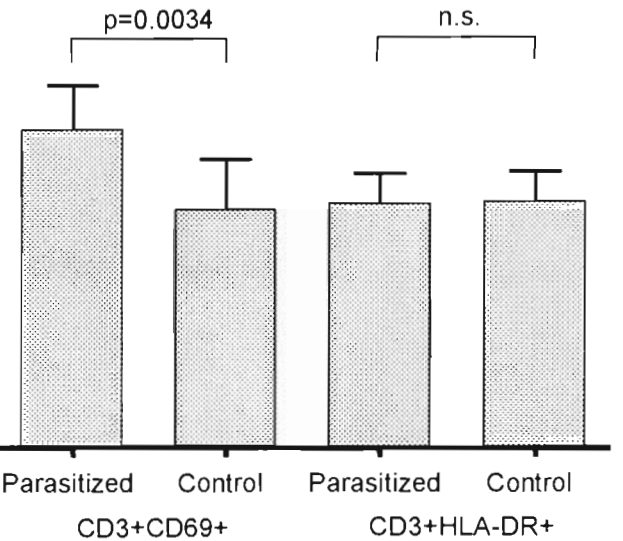

p=0.0034
n.s.
Parasitized
Control
Parasitized
Control
CD3+CD69+
CD3+HLA-DR+

Fig. 1

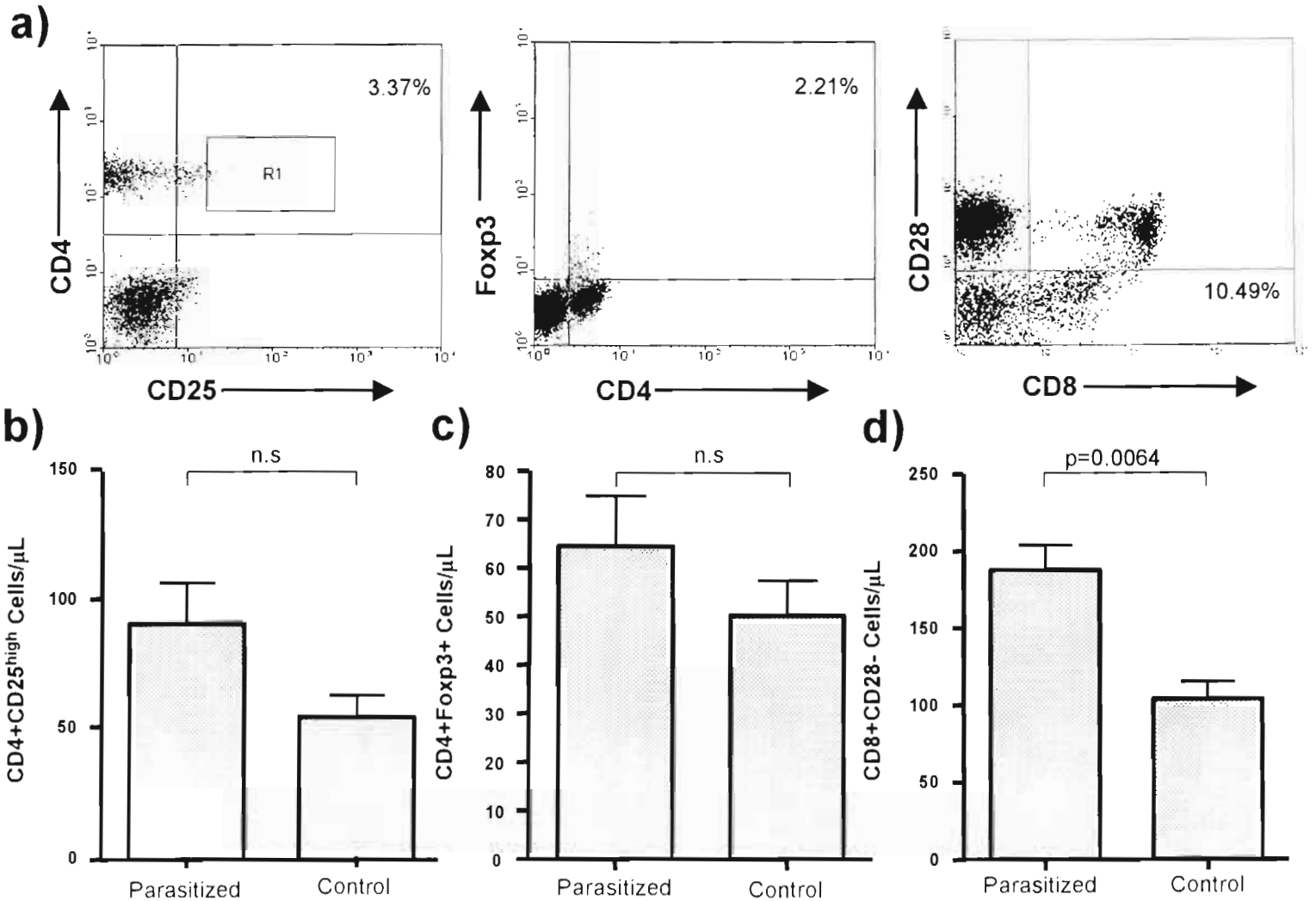

a)
3.37%
R1
CD4
CD25
2.21%
Foxp3
CD4
10.49%
CD28
CD8
b)
n.s
CD4+CD25high Cells/µL
150
100
50
0
Parasitized
Control
c)
n.s
CD4+Foxp3+ Cells/µL
80
70
60
50
40
30
20
10
0
Parasitized
Control
d)
p=0.0064
CD8+CD28- Cells/µL
250
200
150
100
50
0
Parasitized
Control

Fig. 2

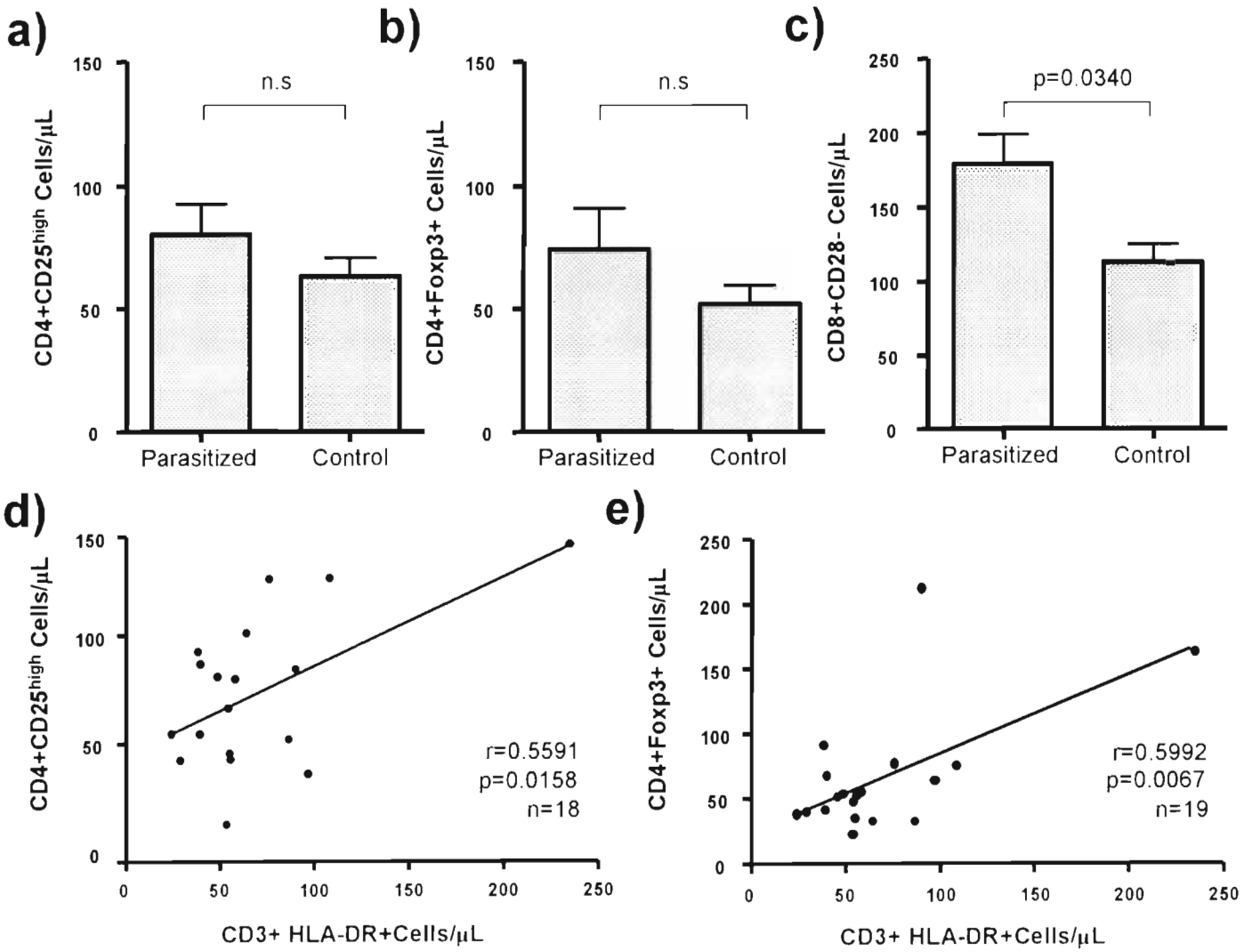

a)
b)
c)
d)
e)
CD4+CD25high Cells/µL
CD4+Foxp3+ Cells/µL
CD8+CD28- Cells/µL
n.s
n.s
p=0.0340
Parasitized
Control
Parasitized
Control
Parasitized
Control
0
50
100
150
200
250
CD4+CD25high Cells/µL
CD3+ HLA-DR+Cells/µL
r=0.5591
p=0.0158
n=18
CD4+Foxp3+ Cells/µL
CD3+ HLA-DR+Cells/µL
r=0.5992
p=0.0067
n=19

Fig. 3

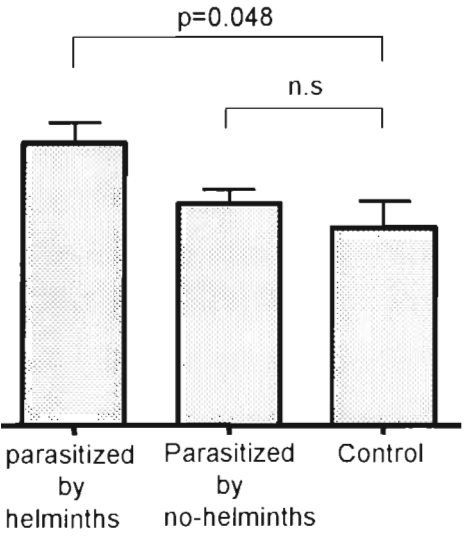

p=0.048
n.s
parasitized by helminths
Parasitized by no-helminths
Control

Fig 4

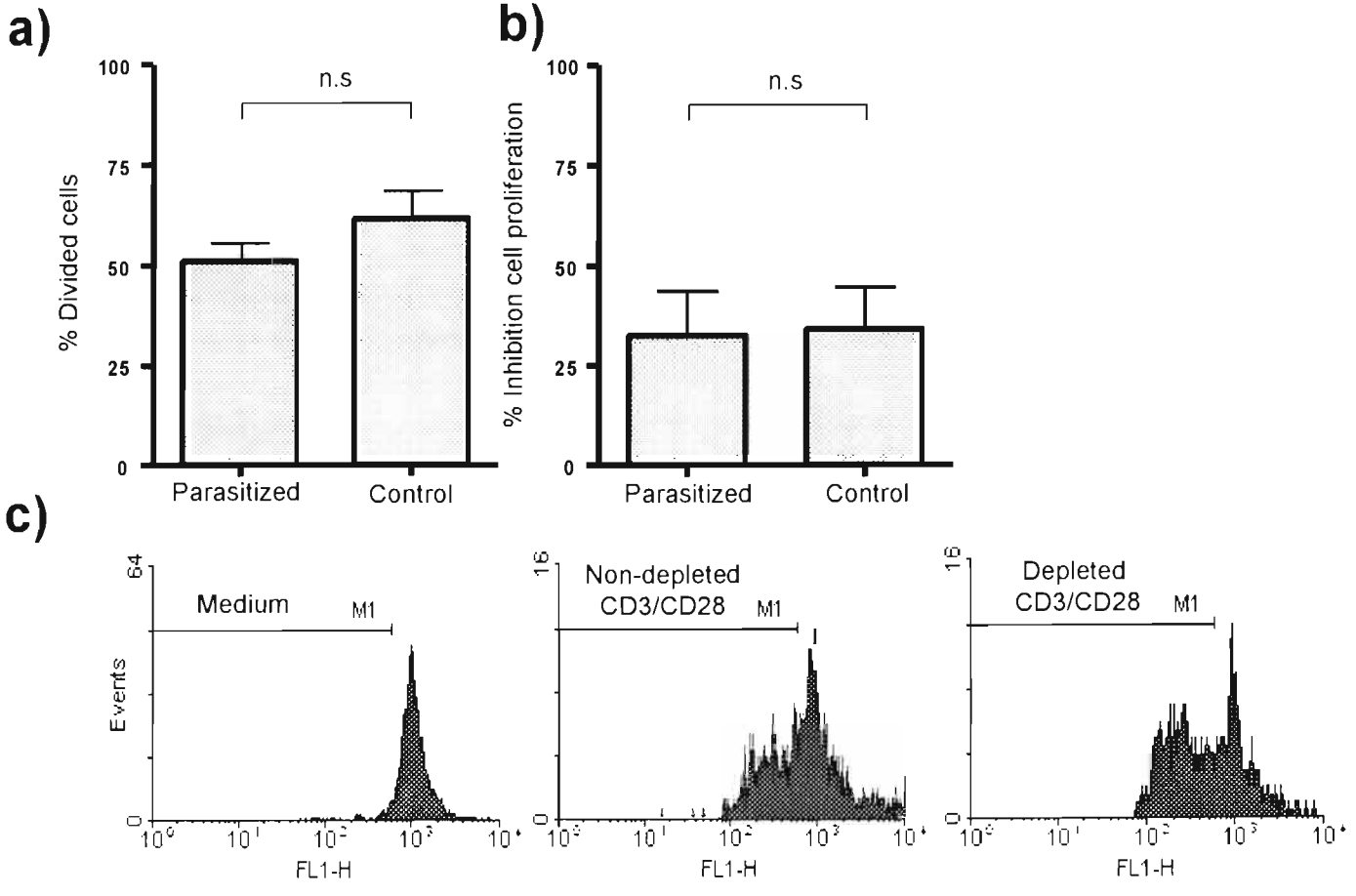

a)
b)
c)
n.s
n.s
% Divided cells
% Inhibition cell proliferation
100
75
50
25
0
Parasitized
Control
Medium
M1
Non-depleted
CD3/CD28
Depleted
CD3/CD28
Events
64
16
FL1-H

Fig 5