

# Susceptibility to *Entamoeba histolytica* intestinal infection is related to reduction in natural killer T-lymphocytes in C57BL/6 mice

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## Abstract

*Entamoeba histolytica* is a protozoan that causes amoebiasis. Recent studies demonstrated that natural killer T lymphocytes (NKT) are critical for preventing the development of amoebic liver abscess. In spite of that, there are only a handful of studies in the area. Herein, we explored the role of NKT cells in *E. histolytica* infection using C57BL/6 wild-type and CD1<sup>-/-</sup> mice. Animals were inoculated with *E. histolytica* and sacrificed 48 hours later to collect caecum samples that were used for quantitative analyses of lesions, trophozoites, NK1.1<sup>+</sup> T lymphocytes and expression of the mucus protein MUC-2 by immunohistochemistry technique. Quantitative analyses confirmed that the frequency of NK1.1<sup>+</sup> T cells was significantly lower in samples from C57BL/6 CD1<sup>-/-</sup> mice as compared to their wild type (WT) counterparts. The extension of necrotic mucosa was larger and the number of trophozoites higher in *Entamoeba* (Eh)-infected CD1<sup>-/-</sup> mice when compared with Eh-infected WT mice. In mice from both groups, non-infected (CTRL) and Eh-infected CD1<sup>-/-</sup>, there was a reduction in the thickness of the caecal mucosa and in the MUC-2-stained area in comparison with CTRL- and Eh-WT mice. Our results showed that NKT lymphocytes contribute to resistance against *Entamoeba histolytica* infection and to the control of inflammation in the colitis induced by infection. The presence of a normal epithelial layer containing appropriate levels of mucus had also a protective role against infection.

## Introduction

*Entamoeba histolytica* is a protozoan that causes amoebiasis, a disease with a prevalence of 50 millions of cases and responsible for up to 100 thousands of deaths per year worldwide.<sup>1</sup> Amoebiasis is the most severe protozooisis that infects the human gut and it is the third main cause of death among parasitic diseases, only overcome by schistosomiasis and malaria.<sup>2,3</sup> *E. histolytica* trophozoites live in the large intestine of infected hosts, being able to penetrate through the intestinal mucosa, to reach the blood circulation and to spread to other organs occasionally.<sup>4</sup> Intestinal invasion is associated with production of different molecules such as galactose- and N-acetylgalactosamine-binding lectins, cysteine proteinases (CPs) and amoebapores that cause direct or indirect damage to the gut wall.<sup>5</sup> As observed in other protozoans, *E. histolytica* expresses a glycosyl-phosphatidylinositol(GPI)-anchored glycoconjugated protein (LPPG) called *E. histolytica* lipopeptide phosphoglycan (EhLPPG). EhLPPG has been associated to *E. histolytica* pathogenicity since differences in quantity and antigenicity of this lipopeptide glycan have been observed in pathogenic *vs* non-pathogenic amoebas.<sup>6</sup> There are reports showing that EhLPPG has also a role in immune response to *E. histolytica* infection by the activation of natural killer T (NKT) lymphocytes. CD1d<sup>-/-</sup> and J 18<sup>-/-</sup> mice that lack *i*NK T lymphocytes have a deficient production of INF-  when challenged with EhLPPG.<sup>7</sup> INF-  production by EhLPPG-activated *i*NK T cells can initiate a Th1 adaptive response contributing for an augmented efficiency in the immune response to *E. histolytica*. Interestingly, it has also been demonstrated that natural T killer lymphocytes constitute an important barrier against the development of amoebic hepatic abscess in its initial stages.<sup>7</sup> CD1-deficient mice developed larger hepatic abscesses and this outcome was associated with to the absence of CD1-presentation of lipopeptide phosphoglycan amoebic derived antigen to NKT lymphocytes. Although more studies are needed, NKT lymphocytes can be related to the production of IL-10 and of mucin MUC-2 which is the main component of intestinal mucus. CD1<sup>-/-</sup> mice produce low levels of IL-10 mRNA.<sup>8</sup> On the other hand, IL-10-deficient mice present a reduced synthesis of MUC-2, gut inflammation and increased susceptibility to infection by *E. histolytica* trophozoite.<sup>9</sup>

Amoebiasis is an important parasitic disease that is related to high morbidity and mortality all over the world. Attempts to control its expansion will require a better understanding of disease pathogenesis. The present work addressed the role of NKT lymphocytes in the development of amoebic lesions using an

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Key words: Amoebic colitis, *Entamoeba histolytica*, natural killer T lymphocytes, MUC-2 mucin, innate immunity.

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Contributions: FMSO, MAG, LOP, performed the culture of amoebas, made the inocula and intracecal inoculation of mice; FMSO, LOP, AFS, ACA, BCH, AMCF, MVC, made the necropsy of mice, histopathologic procedures, immunohistochemical and histochemical assays, morphometry and statistical analysis.

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experimental model of colitis induced by infection with *E. histolytica*.

## Materials and Methods

### Animals

Thirty-two mice were used in the experiment, 16 C57BL/6 wild-type (WT) (8 infected Eh-WT and 8 control CTRL-WT) and 16 C57BL/6CD1<sup>-/-</sup> mice (8 infected Eh-CD1<sup>-/-</sup> and 8 control CTRL-CD1<sup>-/-</sup>) with approximately 70 days of age. Animals were obtained from the animal facility of the Instituto de Ci ncias Biol gicas (UFMG) and FIOCRUZ Institute, Belo Horizonte.

### Trophozoite culture and inoculation

Axenic EGG strain from *E. histolytica*, isolated in 1988 in the Amoebiasis Laboratory in Departamento de Parasitologia (ICB-UFMG)

from patient presenting dysenteric colitis and amoebic hepatic abscess was used. Presence of *E. histolytica* infection in these individuals was confirmed by serum antibody test by ELISA (Enzyme-linked immunosorbent assays) as well as by polymerase chain reaction and zymodeme<sup>10</sup>. Trophozoites were thawed in water bath and maintained in Pavlova medium at 37°C with passages every three days. Sixteen mice (8 Eh-CD1<sup>-/-</sup> and 8 Eh-WT) were anesthetized with 2% xylazine solution (10 mg/Kg) and 5% ketamine (150 mg/Kg) for abdominal incision procedure of approximately 2 cm and inoculation of 10<sup>6</sup> trophozoites in 0.1 mL tissue culture medium. Sixteen control mice (8 CTRL-CD1<sup>-/-</sup> and 8 CTRL-WT) were inoculated with sterile culture medium YI-S-32 by intracaecal route.

### Necropsy and histopathology

Animals were sacrificed 48 hours after infection by cervical dislocation under general anesthesia to collect *caeca* that were fixed in 10% buffered formaldehyde 7.2 pH. After fixation each *caecum* was divided into 3 slices that were dehydrated, diaphanized, infiltrated and included in paraffin. Histological sections of 4 µm of thickness were obtained for Haematoxylin and Eosin (H&E) staining, schiff periodic acid (PAS) staining and immunohistochemistry reactions. Lesions were described according to their intensity, topography and presence of parasites.

### Quantitative analyses of intestinal lesions (ulcer and erosion)

Erosions are small destructives lesions in the *caecal* mucosa, not reaching the sub-mucosal layer, containing or not a mixed inflammatory infiltrate. Intestinal ulcers are larger and deeper lesions, always reaching the mucosa, the sub-mucosa and occasionally the muscular layers. For total area measurement of intestinal lesions, histological sections were analyzed by H&E staining. All erosions and ulcers were digitalized using a JVC TK-1270/RGB camera (Tokyo, Japan) and manually analysed for necrosis (in µm) measurement using the KS300 program contained in the Carl Zeiss image analyzer (Oberkochen, Germany).

### Immunohistochemistry reaction and quantitative analyses of trophozoites

Caecal sections were deparaphinized, hydrated and washed in pH 7.2 phosphate buffer solution (PBS). Endogenous peroxidase activity was eliminated by section incubation in H<sub>2</sub>O<sub>2</sub> 40vv solution at 0.2% PBS and unspecific binding was blocked by goat serum diluted 1:40. Next, sections were incubated with anti-*E. histolytica* polyclonal serum 1:1000 (produced in the Amoebiasis and Protozoosis

laboratories/ICB/UFMG) for 18 hours, with biotin IgG 1:200 (Zymed Laboratories Inc., San Francisco, USA) and peroxidase conjugated streptavidin 1:200 for 1 hour (Zymed Laboratories Inc., San Francisco, USA). Color reaction was developed by diaminobenzidine solution (DAB) 0.05% in H<sub>2</sub>O<sub>2</sub> 40vv at 0.2%. Histological sections obtained from amoebic lesions rich in trophozoites were used as positive control. As negative control, PBS-treated sections were used. All sections were stained with Harris haematoxylin, hydrated and diaphanized for entelan assembly. Trophozoites identified by immunohistochemistry were visualized and quantified using the 40x objective of Axiolab Carl Zeiss microscopy.

### Immunohistochemistry reaction and quantitative analyses of natural killer 1.1 lymphocytes

Tissue sections were deparaphinized, hydrated and washed in pH 7.2 PBS. Endogenous peroxidase activity was eliminated by treatment with H<sub>2</sub>O<sub>2</sub> 40vv solution at 0.2% PBS and unspecific binding was blocked by incubation with mice serum diluted at 1:40. Next, sections were incubated with biotin-labeled anti-NK1.1 antibody (Pharmingen, San Diego, CA, USA) solution at 1:40 for 18 hours; then incubated with peroxidase conjugated streptavidin at 1:100 for 1 hour (Zymed Laboratories Inc., San Francisco, USA). NK-1.1 marker is expressed on Natural Killer (NK) cells and on NKT lymphocytes from selected strains of mice (such as C57BL/6). Color reaction was developed using DAB 0.05% in H<sub>2</sub>O<sub>2</sub> 40vv at 0.2%. Histological sections of *caeca* obtained from CTRL-WT mice were used as positive control. As negative control, PBS-treated sections were used. NK1.1+ lymphocytes identified by immunohistochemistry were visualized and quantified using the 40x objective of Axiolab Carl Zeiss microscopy.

### Immunohistochemistry reaction to detect mucin MUC-2

Tissue sections were deparaphinized, hydrated and washed in pH 7.2 PBS. Endogenous peroxidase activity was eliminated by treatment with H<sub>2</sub>O<sub>2</sub> 40vv solution at 0.2% PBS and unspecific binding was blocked by incubation with goat serum diluted at 1:40. Next, sections were incubated with monoclonal anti-MUC-2 antibody (Santa Cruz Biotechnology, Santa Cruz, USA) diluted at 1:150 for 18 hours, then incubated with biotin-labeled IgG at 1:100 (Bethyl Laboratories Inc, Montgomery, USA) and peroxidase-conjugated streptavidin at 1:100 for 1 hour (Zymed Laboratories Inc., San Francisco, USA). Color reaction was developed using DAB 0.05% in H<sub>2</sub>O<sub>2</sub> 40vv at 0.2%. Histological sections of *caeca* obtained from CTRL-WT mice were used

as positive control. As negative control, PBS-treated sections were used. All sections were stained with Harris haematoxylin, hydrated and diaphanized for entelan assembly.

### Quantitative analyses of caecal mucosal area, mucus and mucin MUC-2

A histological section of each caecum sample from both CTRL-WT and CTRL-CD1<sup>-/-</sup> groups was stained by PAS, visualized by the 40X objective for digitalization of 20 randomized images by a JVC TK-1270/RGB microcamera (Tokyo, Japan). For each animal, 11×10<sup>3</sup> µm<sup>2</sup> of *caecal* mucosa were analyzed for calculation of the mucosal areas that were PAS positive. Pixels with magenta hues of the regions stained by PAS were selected for generation of a binary image, digital processing and obtaining the area in µm<sup>2</sup>. The total caecal mucosal area was calculated using the same 20 digitalized images from each animal by the selection of all types of pixels present in the mucosa and by the generation of the respective binary image.

The MUC-2 stained area was calculated from 20 images of the caecal mucosa of animals from all groups by selecting the pixels with brown hues and utilizing the same methodology for the analysis of PAS<sup>+</sup> regions. Microscopic imaging, image segmentation, and all measures were obtained by software KS300 of the Carl Zeiss image analyzer (Oberkochen, Germany).<sup>11</sup>

### Statistical analyses

Statistical analyses were performed utilizing Prism 5.0 program and One-way ANOVA test when more than two groups were analyzed, followed by Tukey as post test. Unpaired t-test was utilized when two groups were compared. Data had a Gaussian distribution when submitted to the Shapiro-Wilk test of normality. Results were expressed as mean ± standard error of measurement and differences were considered significant at P≤0.05. Some results with a normal distribution among variables were correlated with each other using the Pearson correlation test. For results that did not show a linear distribution, Spearman's correlation test was used.

## Results

### Macroscopy, histopathology and quantitative analyses of caecal lesions

After 48 hours post infection, hyperemia was observed macroscopically in *caeca* of all mice from group Eh-CD1<sup>-/-</sup>. Two animals from

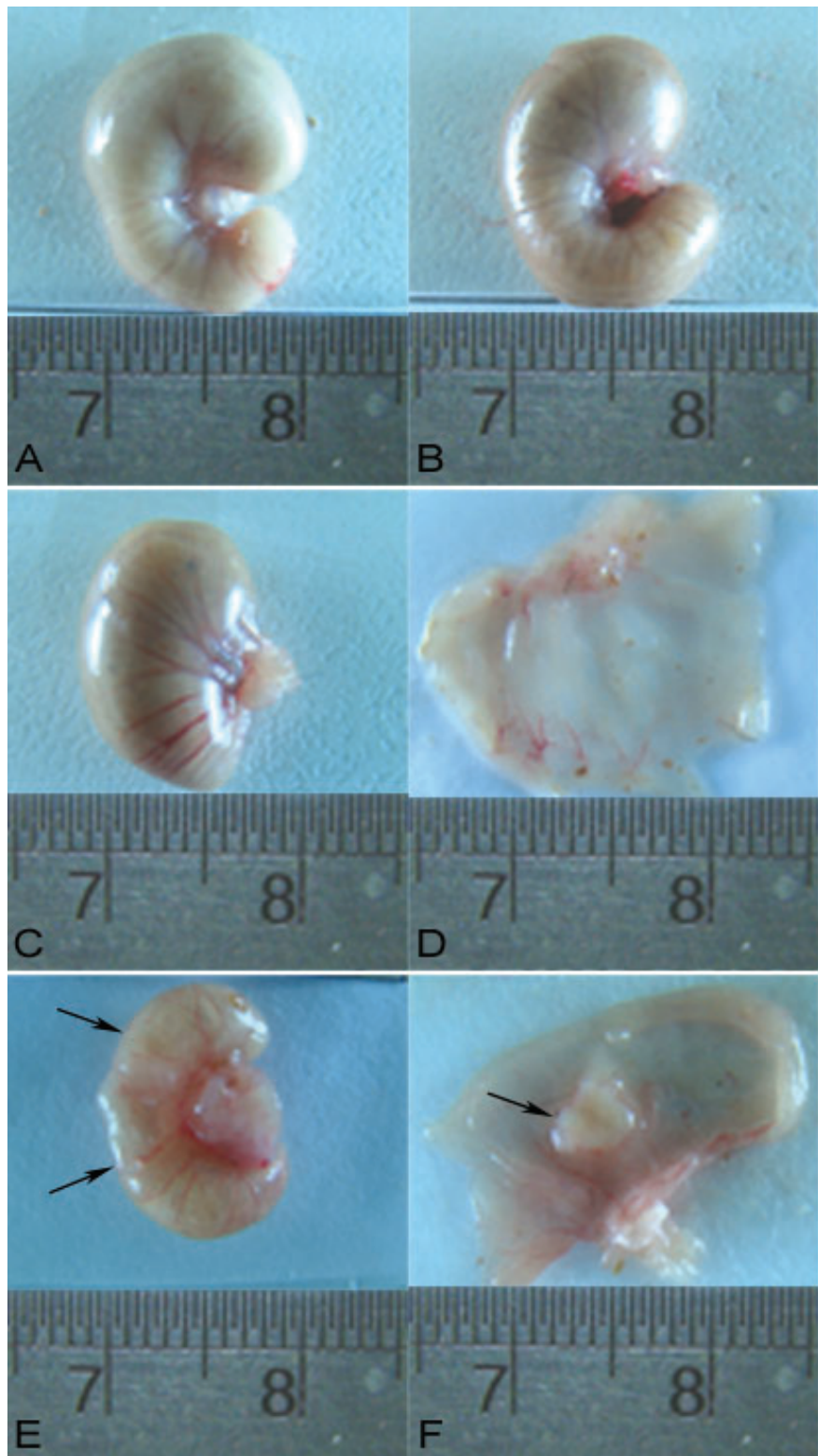
this group also exhibited caecal ulcers with a protruded aspect, round shape, whitish color and they were visible both in the mucosa surface and in the serosa layer (Figure 1E, F). Macroscopic analysis of the *caeca* of mice from Eh-WT group had no lesion and only hyperemia was seen (Figure 1C, D). *Caeca* of mice from Eh-CD1<sup>-/-</sup> group were larger and more expanded by gases when compared to *caeca* from CTRL-CD1<sup>-/-</sup> control mice (*data not shown*). This was not observed in mice from Eh-WT group when compared to CTRL-WT controls.

Histopathological analysis showed that the caecal mucosa of CTRL-WT mice were thicker, with more elongated crypts and, apparently, with higher production of mucus by caliciform cells when compared to the mucosa of CTRL-CD1<sup>-/-</sup> mice (Figure 2A, B).

Amoebic caecal lesions were observed in all mice of Eh-CD1<sup>-/-</sup> and Eh-WT groups. The amoebic ulcers were characterized by necrotic lesions in the mucosa and in the submucosa, sometimes reaching the muscular layer, with the presence of cellular debris and a mixed inflammatory infiltrate of neutrophils, macrophages, lymphocytes and rare eosinophils (Figure 2E, F). Ulcers were observed in seven out of ten animals from Eh-CD1<sup>-/-</sup> group, in contrast with animals from Eh-WT group in which ulcers were not observed. In all animals of both Eh-WT and Eh-CD1<sup>-/-</sup> groups, superficial necrotic zones (erosions) were also found and they were detected under the optical microscope. These erosions were characterized by a small zone of mucosal destruction sometimes reaching the submucosa and accompanied by an inflammatory infiltrate (Figure 2C, D). Quantitative analysis showed that the extension of the necrotic areas in the mucosa was greater in Eh-CD1<sup>-/-</sup> mice (2713±566.6 µm) than in Eh-WT mice (575.4±69.73 µm; P<0.05; Figure 3A).

### Qualitative and quantitative immunohistochemical analysis of trophozoites

There was no positive reaction for trophozoites in control PBS-treated histological sections (*data not shown*) as opposed to the sections that had received the anti-*E. histolytica* antibody (Figure 4A). *Entamoeba histolytica* trophozoites, identified by immunohistochemistry using anti-*E. histolytica* antibodies, were found mainly in necrotic areas and on the border of caecal ulcers in mice from the two infected groups (Figure 4B, C). Although in smaller numbers, trophozoites were observed in the submucosa, immediately below the ulcer, and also away from it, which indicates that the parasite has the ability to disseminate (Figure 4C, D). Quantitative analysis showed that trophozoites were more frequent in mice from Eh-CD1<sup>-/-</sup> group (14.3±3.95) than in Eh-WT mice (5.6±1.19; P<0.05; Figure 3B).



**Figure 1.** Caeca of C57BL/6 wild type and C57BL/6 CD1<sup>-/-</sup> mice inoculated or not with *Entamoeba histolytica* trophozoites. A) non-infected-wild type and B) non-infected-CD1<sup>-/-</sup> groups: normal aspect of the caecum; C) *Entamoeba*-wild type group: caecum with hyperemia; D) aspect of the caecal mucosa of Figure 1C after longitudinal section showing some hyperemic regions; E) *Entamoeba*-CD1<sup>-/-</sup> group: whitish ulcer (arrows indicate ulcers), hyperemic serosal surface; F) aspect of the caecal mucosa of Figure 1E after longitudinal section allowing visualization of an ulcer (arrow) in the mucosal surface surrounded by areas of hyperemia.

### Qualitative and quantitative immunohistochemical analysis of natural killer 1.1<sup>+</sup> lymphocytes

NK1.1<sup>+</sup> cells were identified by the presence of cytoplasmic dark brown staining, resulting from the binding of anti-NK1.1 antibodies and precipitation by diaminobenzidine. All positive cells showed small and round nuclei and their cytoplasmic regions were frequently easy to visualize (Figure 5A). In mice from CTRL-WT and CTRL-CD1<sup>-/-</sup> groups, NK1.1<sup>+</sup> cells were predominantly found in the *lamina propria* of the mucosa and, more rarely, in the submucosa (Figure 5A, D). However, in Eh-CD1<sup>-/-</sup> and Eh-WT mice, NK1.1<sup>+</sup> cells were more frequently observed equally in both regions (Figure 5B, C, E, F).

Quantitative analysis showed that NK1.1<sup>+</sup> cells were significantly less frequent in CTRL-CD1<sup>-/-</sup> (26.3±2.95) and Eh-CD1<sup>-/-</sup> (22.4±4.23) mice when compared to CTRL-WT (69.5±7.17) and Eh-WT (121.1±21.98) mice (P<0.05) (Figure 3A). Cell suspensions from caecal lymph nodes were analyzed by flow cytometry using antibodies to CD3 and to NK1.1 molecules to distinguish between NK cells (CD3<sup>+</sup>NK1.1<sup>+</sup>) and NKT lymphocytes (CD3<sup>+</sup>NK1.1<sup>+</sup>) (*data not shown*). Such quantitative analyses showed significant reduction in the NKT lymphocyte population in CTRL-CD1<sup>-/-</sup> and Eh-CD1<sup>-/-</sup> mice when compared with Eh-WT and CTRL-WT mice suggesting that most of the cells identified by immunohistochemistry were NK T lymphocytes.

In addition, a significant increase in the number of NK1.1<sup>+</sup> cells was observed in Eh-WT mice when compared to animals from all other groups (P<0.05). There was no difference between NK1.1<sup>+</sup> cells from CTRL-CD1<sup>-/-</sup> and Eh-CD1<sup>-/-</sup> mice (Figure 3E).

### Qualitative and quantitative immunohistochemical analysis of the caecal mucosa, mucus and MUC-2 mucin

The caecal mucosa consists of glands or tubular crypts formed mainly by caliciform cells and enterocytes. At the optical microscope, the mucosa of CTRL-WT mice were thicker (Figure 2A) and showed a higher production of mucus by caliciform cells when compared to the mucosa of CTRL-CD1<sup>-/-</sup> mice (Figure 2B).

Next, schiff periodic acid histochemical staining (PAS) was used to evaluate putative differences in the expression of sulphate and carboxylated polysaccharides and glycoproteins that constitute the mucus produced by caliciform cells and also to measure the mucosa area. Quantitative analysis showed that the mucosa areas of CTRL-WT mice (31,254±1,273 μm<sup>2</sup>) was larger than the ones

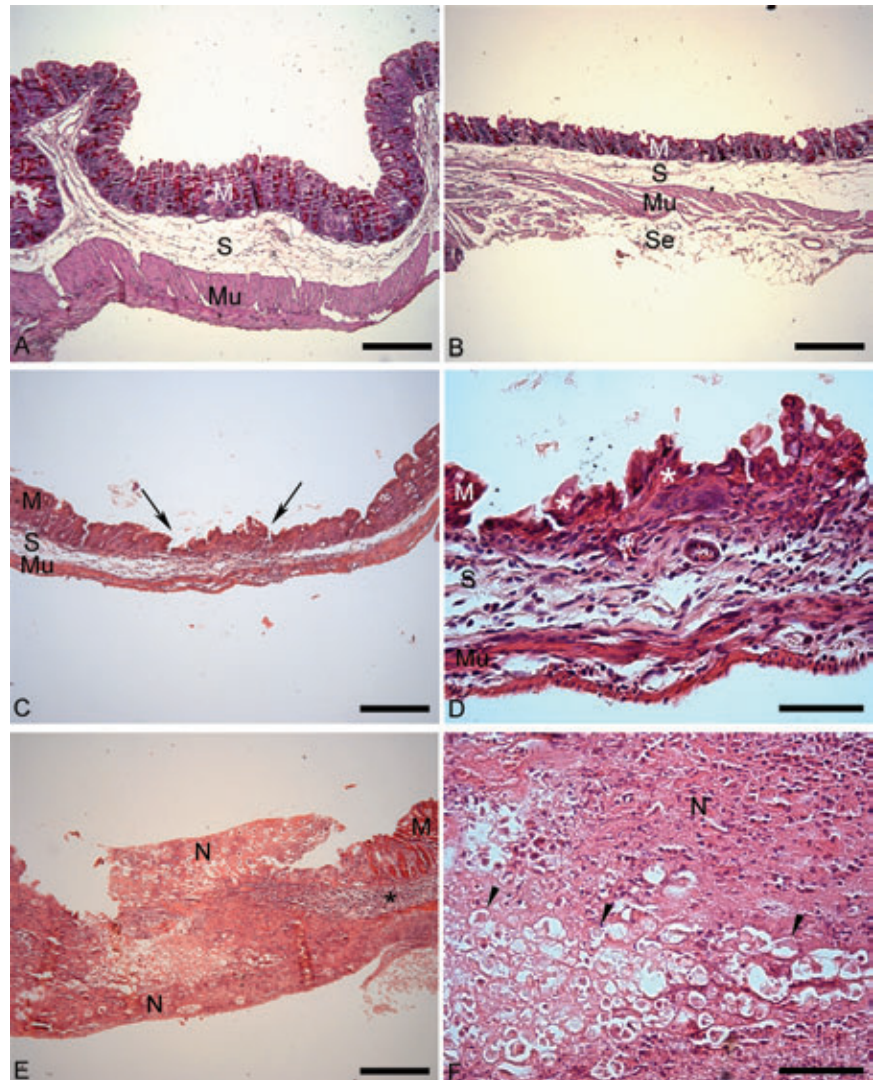
observed for CTRL-CD1<sup>-/-</sup> mice (20,641±1,885 μm<sup>2</sup>) (P<0.05) (Figure 3C). The area stained by PAS was also larger in CTRL-WT mice (5,820 ±237.1 μm<sup>2</sup>) than in CTRL-CD1<sup>-/-</sup> controls (3,525±496.2 μm<sup>2</sup>; P<0.05; Figure 3D). Because of the differences observed, we decided to evaluate the immunohistochemical expression of MUC-2 mucin in the caecal mucosa of all animals.

In both CTRL-CD1<sup>-/-</sup> (391.0±47.34 μm<sup>2</sup>) and Eh-CD1<sup>-/-</sup> (750.0±68.44 μm<sup>2</sup>) mice, a significant decrease in the MUC-2 stained area was

detected when compared to the stained areas found for CTRL-WT (1066±192.9 μm<sup>2</sup>) and Eh-WT (2,168±152.9 μm<sup>2</sup>) mice (P<0.05) (Figure 6A, B, C, D). There was no difference between mice from CTRL-CD1<sup>-/-</sup> and Eh-CD1<sup>-/-</sup> groups. In the Eh-WT group, MUC-2 positive areas were significantly greater than the ones observed in mice from the CTRL-WT group (P<0.05) (Figure 3F).

### Correlation analyses

To evaluate the probable correlations between the area of MUC-2 and the extension



**Figure 2.** Photomicrograph of caeca of C57BL/6 wild type and C57BL/6CD1<sup>-/-</sup> mice inoculated or not with *Entamoeba histolytica*. A) non-infected-wild type group: mucosal, submucosal and muscular layers with normal aspect. Periodic Acid Schiff (PAS) stain. Bar = 100 μm; B) non-infected-CD1<sup>-/-</sup> group: caecum showing a thin mucosal layer. PAS. Bar = 100 μm; C) Entamoeba-wild type group: caecum with erosion (arrows) in the mucosa. Haematoxylin and Eosin (H&E). Bar = 100 μm; D) higher magnification of the previous figure showing partial destruction (\*) of the mucosa and discrete inflammatory infiltrate in the submucosa. H&E. Bar = 30 μm; E) Entamoeba-CD1<sup>-/-</sup> group: extensive necrosis (N) in all layers of the caecal wall. Inflammatory infiltrate in the submucosa (\*). H&E. Bar = 100 μm; F) higher magnification of the previous figure showing necrosis (N) and high number of trophozoites (arrowheads). H&E. Bar = 30 μm. Mucosa (M), Submucosa (S), Muscular (Mu) and Serosa (Se).

of necrosis, Pearson correlation test was applied after confirmation that the variables followed a normal distribution. In animals inoculated with *E. histolytica*, a negative correlation between the MUC-2 positive areas and the extension of necrosis ( $r=0.4168$ ) was observed, *i.e.*, the smallest the MUC-2<sup>+</sup> area, the highest the necrosis extension ( $P<0.05$ ) (Figure 7A).

To analyze putative correlations between MUC-2 positive area and number of NK1.1<sup>+</sup> cells, we have used Spearman's correlation test since a linear correlation between variables could not be assumed. In animals inoculated with *E. histolytica*, a positive correlation was observed between MUC-2<sup>+</sup> area and number of NK1.1<sup>+</sup> cells ( $r^2=0.5956$ ), *i.e.*, the smaller the MUC-2<sup>+</sup> area, the lower the number of NK1.1<sup>+</sup> cells ( $P<0.05$ ) (Figure 7B).

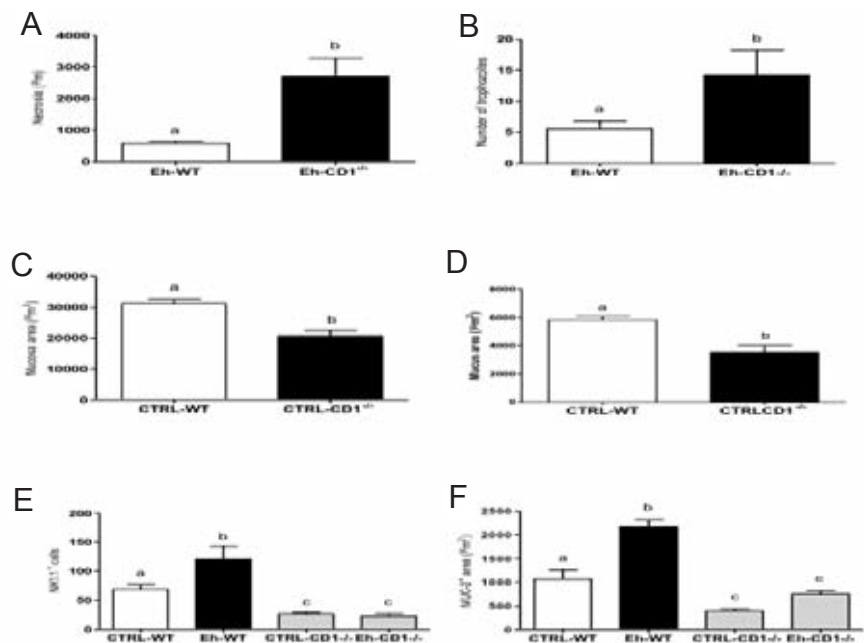
Spearman's correlation test was also used to analyze possible correlations between extension of necrosis and number of NK1.1<sup>+</sup> cells. In animals inoculated with *E. histolytica*, a negative correlation between extension of necrosis and number of NK T lymphocytes was found ( $r^2=0.6159$ ), *i.e.*, the lower the NK1.1<sup>+</sup> cell number the greater the extension of necrosis ( $P<0.05$ ) (Figure 7C).

## Discussion

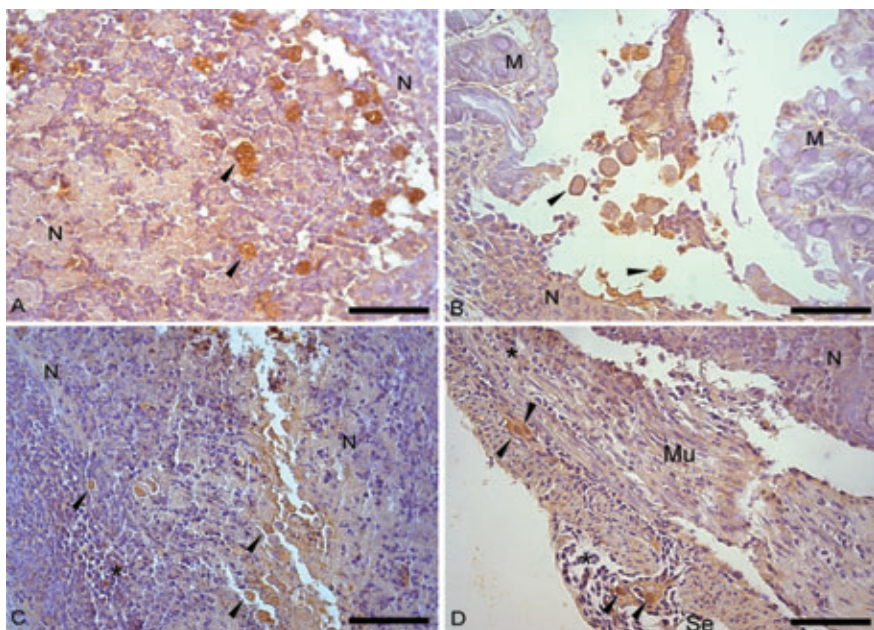
The objective of this study was to evaluate the role of NKT lymphocytes in amoebiasis. Our strategy was to analyze, in a comparative basis, the frequency of NKT lymphocytes in caeca of wild type (C57BL/6WT) vs CD1d-deficient (C57BL/6CD1<sup>-/-</sup>) mice infected with *E. histolytica* and to correlate it with the intensity of amoebic intestinal lesions, number of trophozoites, mucosal area and mucosal MUC-2 expression. It is important to emphasize that, up to this moment, this was the only work that has investigated the involvement of NKT lymphocytes in amoebic colitis.

One of the most important observations of our study was that CD1d-deficient mice that have lower number of NKT lymphocytes are more susceptible to amoebic infection and to the development of caecal lesions. In addition, they had a lower production of MUC-2 mucin and this reduction also correlated to a decrease in the number of NK1.1<sup>+</sup> cells and to the development of more intense caecal lesions.

Only the group of Lotter and coworkers (2009) has so far reported the participation of NK T lymphocytes in amoebiasis.<sup>7</sup> These authors observed that C57BL/6CD1<sup>-/-</sup> mice were more susceptible to development of hepatic abscesses than wild type C57BL/6 mice, demonstrating the critical role of iNK T lymphocytes in the modulation of the hepatic disease triggered by *E. histolytica* infection.



**Figure 3.** Morphometric analysis of mucosa, trophozoite, natural killer 1.1<sup>+</sup> T cell and MUC-2 in caecal mucosa of C57BL/6 wild type and C57BL/6CD1<sup>-/-</sup> mice inoculated or not with *Entamoeba histolytica*. A) Extension of necrotic mucosa; B) Number of trophozoites; C) Area of caecal mucosa; D) Area of mucus in the caecal mucosa; E) Number of natural killer 1.1<sup>+</sup> lymphocytes; F) Expression of MUC-2 in the caecal mucosa of C57BL/6WT and C57BL/6CD1<sup>-/-</sup> control mice and infected with *Entamoeba histolytica*. (n=10 per group). Data are shown as the mean  $\pm$  standard error.  $P<0.05$ .



**Figure 4.** Immunohistochemical reaction for *Entamoeba histolytica* trophozoites. A) Liver parenchyma of hamster with large numbers of trophozoites (arrowheads) used as positive control of immunohistochemical reaction for trophozoites, necrosis (N); B) Entamoeba-wild type group: positive trophozoites (arrowheads) in erosion surrounded by well preserved caecal mucosa. Necrosis (N) in the submucosa; C) Entamoeba-CD1<sup>-/-</sup> group: extensive necrosis (N) with high number of trophozoites (arrowheads) and inflammatory infiltrate (\*); D) Entamoeba-CD1<sup>-/-</sup> group: trophozoites (arrowheads) in the serosa. Necrosis (N) in the submucosa and inflammatory infiltrate (\*) in muscular and serosal layers. Bar = 30  $\mu$ m. M, mucosa; Mu, muscular; Se, serosa; S, Submucosa.

Similarly, in our study, quantitative analysis of caecal mucosa necrotic areas has shown that C57BL/6-CD1<sup>-/-</sup> mice infected with *E. histolytica* exhibited a larger area of necrotic mucosa than the infected group of C57BL/6WT mice. In fact, caecal ulcers were observed in seven out of ten mice from the Eh-CD1<sup>-/-</sup> group whereas they were absent in wild type Eh-infected mice. Also, a significant increase in the number of NK1.1<sup>+</sup> cells was observed in animals of the Eh-WT group when compared to mice from all other groups. Correlation analysis between extension of necrosis and number of NK1.1<sup>+</sup> cells of mice inoculated with *E. histolytica* was positive, demonstrating that the lower the number of NK1.1<sup>+</sup> cells, the higher the extension of necrosis.

Another important finding was obtained by the immunohistochemical identification and quantitative analysis of *E. histolytica* trophozoites which were significantly more abundant in the caeca of mice from the Eh-CD1<sup>-/-</sup> group when compared to those from the Eh-WT group. These results suggest that NK T lymphocytes are effective in the resistance against infection by trophozoites in amoebic experimental colitis. These cells have been also described as important players in the control of bacterial colonization in the gastrointestinal tract of mice. C57BL/6CD1<sup>-/-</sup> mice are more susceptible to colonization by both Gram-negative and Gram-positive bacteria than their wild type counterparts.<sup>12</sup>

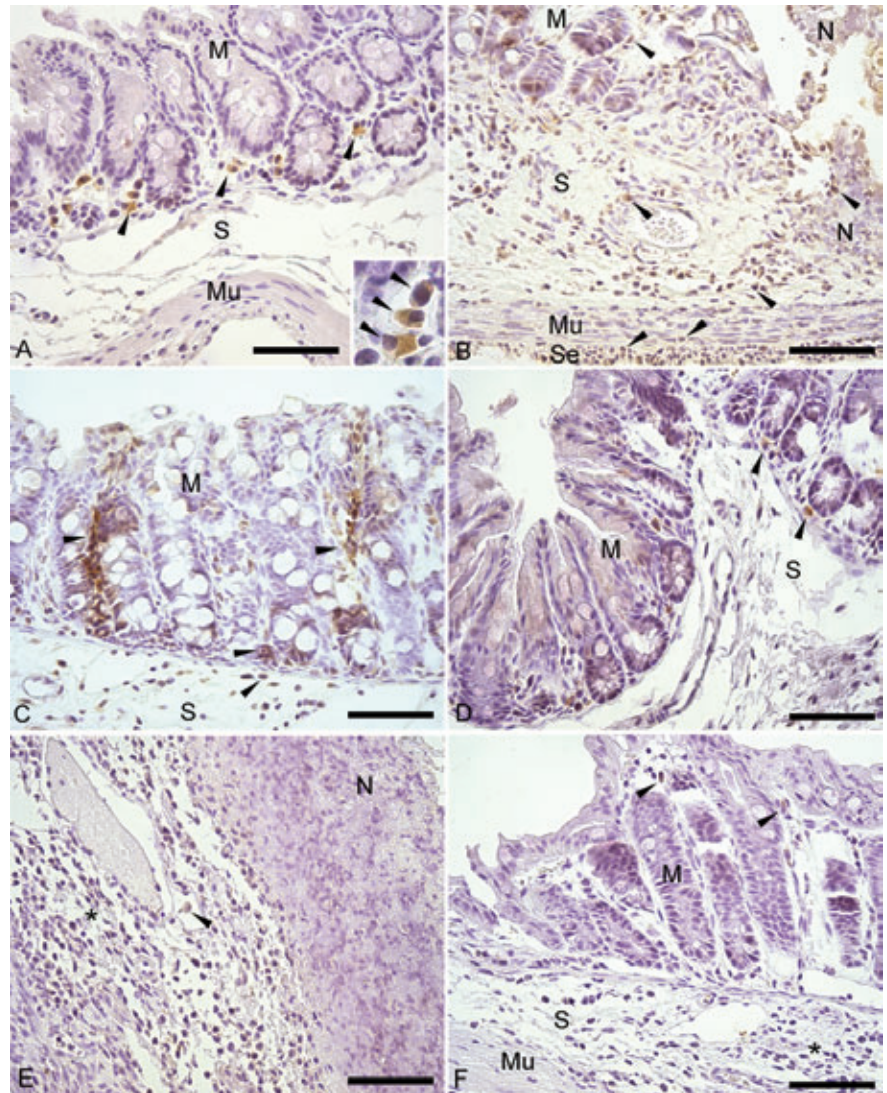
The CD1d molecule is known for its ability to present glycolipid antigens to NK T lymphocytes.<sup>13</sup> Thus, cells restricted to CD1d could respond to infection by recognizing antigenic lipids and carbohydrates of different pathogens.<sup>14-15</sup> We have found, in CTRL-WT and CTRL-CD1<sup>-/-</sup> mice, that NK1.1<sup>+</sup> cells were predominantly found in the lamina propria and, more rarely, in the submucosa. Conversely, in Eh-WT and Eh-CD1<sup>-/-</sup> mice, they were frequently observed both in the mucosal lamina propria and submucosa. These differences in location of NK1.1<sup>+</sup> cells between infected and non-infected mice can be attributed to the immune response against trophozoites present in the caecal wall, which triggers migration of neutrophils and other inflammatory cells to the submucosa and mucosal lamina propria. During the evolution of amoebiasis, *E. histolytica* trophozoites move around the submucosa, increasing the areas of necrosis and giving rise to the classical amoebic colonic lesion known as flask-shaped ulcer.<sup>16</sup> The higher frequency of NK1.1<sup>+</sup> cells and the lower number of trophozoites in the caeca of Eh-WT mice suggest that these lymphocytes are recruited and may act efficiently in the response to amoebic intestinal infection. This hypothesis is reinforced by the observation that Eh-CD1<sup>-/-</sup> mice had a low number of NK1.1<sup>+</sup> cells and a high tissue parasitism.

The lower number of NK1.1<sup>+</sup> cells in the caeca of CTRL-CD1<sup>-/-</sup> and Eh-CD1<sup>-/-</sup> mice was expected since iNK T lymphocytes are restricted to CD1d for antigen-presentation and therefore development of these cells are severely affected in CD1d-deficient mice.<sup>8</sup>

It is known that innate immunity mechanisms, such as the barriers of epithelial mucosa, are the first reaction of the host

organism when facing a pathogen; they act by limiting the damage caused by infection at the initial stages of the exposure to microorganisms. Innate immunity to pathogens also contributes to the formation of acquired immunological responses, and could influence their character and efficiency.

Histopathological analysis of wild type mice inoculated with *E. histolytica* (Eh-WT) showed that their caecal mucosa was thicker, with



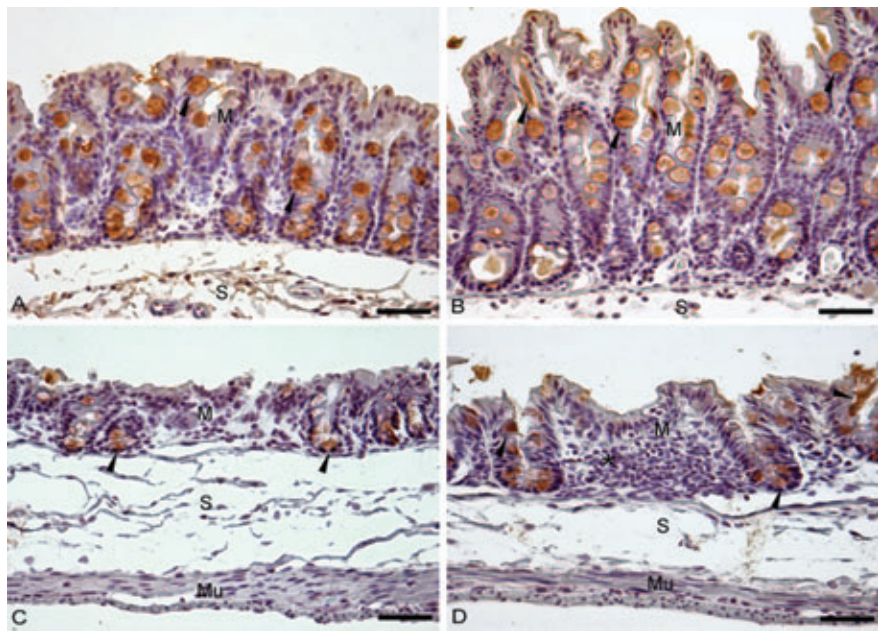
**Figure 5.** Immunohistochemical reaction for natural killer (NK) 1.1<sup>+</sup> lymphocytes in the caeca of C57BL/6 wild type and C57BL/6CD1<sup>-/-</sup> mice inoculated or not with trophozoites of *Entamoeba histolytica*. A) non-infected-wild type group: NK1.1<sup>+</sup> lymphocytes with brown cytoplasm indicating positive staining (arrowheads). The insert shows NK1.1<sup>+</sup> lymphocytes (arrowheads); B) Entamoeba-wild type group: note the presence of several NK1.1<sup>+</sup> lymphocytes in mucosal, submucosal, muscular and serosal layers, necrosis (N); C) Entamoeba-wild type group: large number of NK1.1<sup>+</sup> lymphocytes in preserved mucosa and submucosa; D) non-infected-CD1<sup>-/-</sup> group: scarce NK1.1<sup>+</sup> lymphocytes (arrowheads) in the mucosa; E) Entamoeba-CD1<sup>-/-</sup> group: NK1.1<sup>+</sup> lymphocytes (arrowhead) among the inflammatory infiltrate of the submucosa. Necrosis (N); F) Entamoeba-CD1<sup>-/-</sup> group: area of preserved mucosa with scarce NK1.1<sup>+</sup> lymphocytes (arrowheads). Inflammatory infiltrate (\*) in the submucosa. Counter-staining with Harris haematoxylin. Bar = 30  $\mu$ m. M, Mucosa M; S, Submucosa, Mu, Muscular; Se, Serosa.

elongated crypts and greater production of mucus when compared to C57BL/6 CD1<sup>-/-</sup> mice inoculated with *E. histolytica*. Compelled by these observations and by lack of data in the literature about the normal caecum of CTRL-CD1<sup>-/-</sup> mice, we decided to perform a PAS staining procedure to evaluate putative alterations in the expression of sulphate and carboxylated polysaccharides and glycoproteins that constitute the mucus produced by caliciform cells and also in their mucosa. Quantitative analysis of the mucosa and PAS<sup>+</sup> areas confirmed the histopathological findings and suggest that the mucosal area and the mucus production of wild type (CTRL-WT) mice were significantly greater than that observed in CD1-deficient (CTRL-CD1<sup>-/-</sup>) mice.

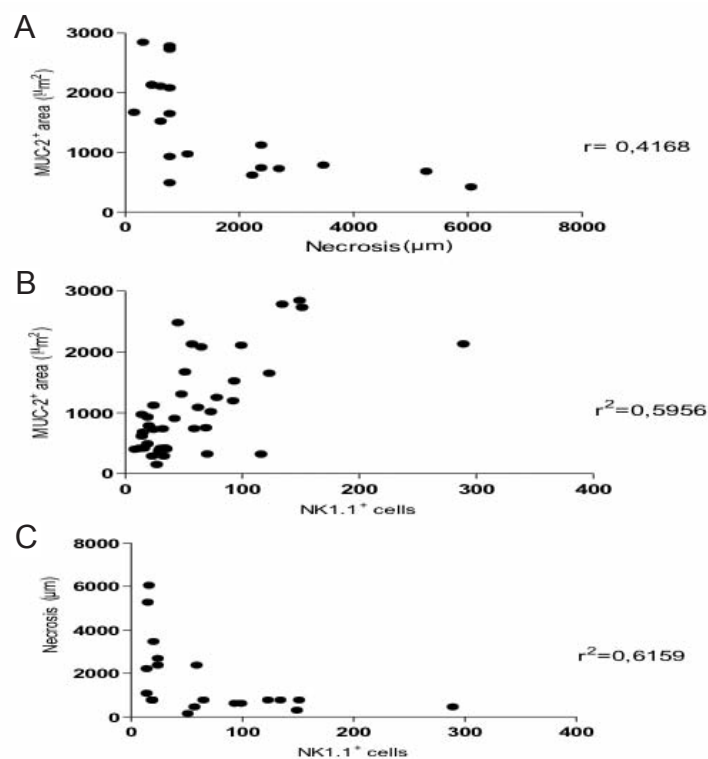
It is possible that the thickness and mucus content of the mucosal epithelial barrier have contributed to the lower levels of lesion and parasitism in Eh-infected wild type (Eh-WT) mice. The paucity of these elements in infected CD1<sup>-/-</sup> (Eh-CD1<sup>-/-</sup>) mice may have partially favored the higher frequency of caecal ulcers and higher number of trophozoites. Some studies corroborate our findings by showing that, in different mice lineages, resistance against *E. histolytica* occurs in the first days after infection mediated by innate immunity and it is independent of T lymphocytes.<sup>17</sup>

Since wild type mice had a greater PAS stained area as compared to CD1<sup>-/-</sup> mice, expression of MUC-2 mucin by immunohistochemistry was performed in the caecal mucosa of all animals. *In vitro* studies showed that *E. histolytica* trophozoites secrete a family of cysteine proteinases (CP) able to disturb the production of MUC-2 by adenocarcinoma cells of the large intestine.<sup>18</sup> In CD1-deficient mice of both control (CTRL) and infected (Eh) groups, there was a significant decrease of MUC-2-stained areas in comparison to the ones found in wild type (CTRL and Eh) mice. Such result was expected, since MUC-2 is the main mucin of the intestinal mucus and quantitative analysis of mucus stained by PAS showed that CD1<sup>-/-</sup> mice had a smaller mucosa area and less mucus than WT mice.

Some studies have shown that murine NKT lymphocytes isolated from thymus, spleen, bone marrow and liver have an increased cytotoxic activity and produce either Th1 type cytokines, such as IFN- $\gamma$  and TNF- $\alpha$ , or Th2 type, such as IL-4, IL-10 and IL-13.<sup>19,20</sup> Mice lacking activated NK T lymphocytes should exhibit a significant decrease in the production of these cytokines, including IL-10. Indeed, it has been demonstrated that mice lacking CD1d have lower levels of IL-10 mRNAs.<sup>8</sup> On the other hand, mice that are genetically deficient for IL-10 exhibit reduced MUC-2 synthesis and, thus, a weaker defense barrier against trophozoites.<sup>9</sup> In animals inoculated with *E. histolytica*, a positive correlation



**Figure 6.** Immunohistochemical reaction for MUC-2 mucin in the caeca of C57BL/6 wild type and C57BL/6CD1<sup>-/-</sup> mice inoculated or not with *Entamoeba histolytica*. A) non-infected-wild type group: note the presence of MUC-2 staining (arrowheads) in the mucosa as seen by brown staining; B) Entamoeba-wild type group: increase of the MUC-2<sup>+</sup> area (arrowheads) in the mucosa compared to the previous figure; C) non-infected-CD1<sup>-/-</sup> group: small areas of MUC-2 staining (arrowheads); D) Entamoeba-CD1<sup>-/-</sup> group: increase of the MUC-2 staining (arrowheads) in the mucosa in relation to Figure 6C; inflammatory infiltrate (\*). Counter-staining with Harris haematoxylin. Bar = 20  $\mu$ m. M, Mucosa M; S, Submucosa, Mu, Muscular; Se, Serosa.



**Figure 7.** Correlation between expression of MUC-2, necrosis and natural killer 1.1<sup>+</sup> lymphocytes. A) Correlation between MUC-2<sup>+</sup> areas and extension of necrosis. Data shown as determination coefficient  $r$ .  $P < 0.05$ ; B) Correlation between MUC-2<sup>+</sup> area and number of NK1.1<sup>+</sup> lymphocytes. Data shown as determination coefficient  $r^2$ .  $P < 0.05$ ; C) Correlation between extension of necrosis and number of NK1.1<sup>+</sup> lymphocytes. Data shown as determination coefficient  $r^2$ .  $P < 0.05$ .

between MUC-2<sup>+</sup> area and number of NK1.1<sup>+</sup> cells was observed indicating that the decrease in the number of NK1.1<sup>+</sup> cells has also reduced MUC-2<sup>+</sup> expression. The negative correlation found between MUC-2 area and extension of necrosis in infected animals suggests that this mucin is an important mechanism of innate immunity against *E. histolytica*.

In addition, MUC-2<sup>+</sup> areas of infected wild type (Eh-WT) mice were significantly larger than the ones found in mice of non-infected wild type (CTR-WT) mice. It is possible that this increase has occurred in response to the infection by *E. histolytica*, and the consequently triggered inflammatory process. Cells from the gut epithelium damaged by amoebic products, such as amoebapores and cysteine proteinases, are stimulated to produce high amounts of inflammatory mediators, including IL-8, IL-1, GM-CSF, TNF- $\alpha$  and cyclooxygenase.<sup>21</sup> Thus, in mice of Eh-WT group, the positive correlation between the MUC-2 area and extension of necrosis suggests that the greater area of MUC-2 may have favored the lower frequency of lesions. This would support previous reports on the protective role of this mucin in amoebic colitis.<sup>9</sup>

Our results showed that reduction of NK T lymphocytes in CD-deficient (CD1<sup>-/-</sup>) mice correlated with a lower production of mucus, especially MUC-2, which reduced the capacity of the host to control infection. At the same time, these lymphocytes seem to have a role in controlling the inflammatory reaction triggered by *E. histolytica* infection since CD1-deficient mice develop a more intense amoebic colitis. The presence of a normal mucosa with appropriate levels of mucus in wild type mice also correlated with protection against infection and colitis. Therefore, our data suggests that NK T lymphocytes and the gut epithelial barrier are two related elements of the innate immunity sharing a critical protective role in the colitis induced by *E. histolytica* infection.

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