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Abstract

Studies have shown that CD1 double negative mice (CD1d-/-) develop larger liver abscesses due to their inability to present amebic antigens to NK T lymphocytes. Therefore, we conducted flow cytometry studies to determine the frequency of NK T, CD4+ T, CD8+ T and B lymphocytes in mice with amebic colitis. The frequency of NK T, CD8+ T and B lymphocytes was reduced in the MLN of mice in the CTRL-CD1-/- and Eh-CD1-/- groups compared to the CTRL-WT and Eh-WT groups. There was also a significant decrease in the frequency of B lymphocytes in the spleens of the animals in the Eh-WT group when compared with the CTRL-CD1-/-, Eh- CD1-/- and CTRL-WT groups. The results of the flow cytometry analysis highlight the importance of NK T lymphocytes in the immune response of mice to amebic intestinal infection and the importance of CD1 molecules in the activation of T and B lymphocytes.

Index terms— amebic colitis, Entamoeba histolytica, natural killer T lymphocytes.

1 I. Introduction

ntamoeba histolytica is a protozoan of the genus Entamoeba and the causative agent of amoebiasis, a disease that produces approximately 50 million cases of two major clinical syndromes worldwide per year, amoebic colitis and amoebic liver abscess [1,2]. Amoebiasis is the most serious protozoiasis that affects the human intestine and comes only after malaria in deaths resulting from parasitic diseases [3]. The estimated mortality rate of this disease is approximately 100,000 deaths per year, and the majority of deaths occurs as a result of severe complications associated with invasive intestinal or extra-intestinal disease [1].

Entamoeba histolytica exhibits a complex glycoconjugate anchored by glycosylphosphatidylinositol (GPI), a lipopeptidophosphoglycan (LPPG) of E. histolytica (EhLPPG) on its surface; this has also been observed in other protozoa. Differences in the quantity and antigenicity of EhLPPGs in pathogenic and nonpathogenic amoebae have indicated that this glycoconjugate is associated with the pathogenicity of E. histolytica [4,5]. EhLPPG is involved in the immune response against E. histolytica infection by the activation of natural killer T lymphocytes (NK T) [6].

Some authors have demonstrated that NK T cells constitute an important barrier to the development of amoebic liver abscesses in their initial stages [6]. They found that CD1 deficient mice (CD1d -/-) develop larger liver abscesses due to their inability to present antigens derived from amebic lipopeptidophosphoglycan to NK T lymphocytes. The results obtained by our group reinforce the idea that CD1 molecules are involved in the resistance of mice with experimentally induced amoebic colitis to Entamoeba histolytica trophozoites, possibly due to the presentation of antigens to invariant natural killer T (iNK T) lymphocytes and the stimulation of MUC-2 production [7]. Likewise, the reduction in activated NK T lymphocyte populations in CD1d-deficient mice results in an increased susceptibility of the mice to Toxoplasma gondii infection [8].

NK T lymphocytes appear to be related to both types of secondary immune responses, Th1 and Th2, due to their ability to initiate the production of large quantities of IFN-? and IL-4 [6,9,10]. Due to the rapidonset

of the effector functions of iNK T lymphocytes after their activation, it have been described their participation in a wide variety of immune reactions, from the response against pathogens and neoplastic cells to autoimmune mechanisms [11,12].

The production of IFN-? by iNK T lymphocytes activated with EhLPPG can initiate a Th1-type adaptive response, which is able to increase the secretion of IFN? and contribute to an increase in the efficiency of the immune response against E. histolytica; this reduces the number of trophozoites and the expansion of amebic abscesses [6]. Recent studies have suggested that CD1d and iNK T lymphocytes are involved in controlling bacterial colonization in the gastrointestinal tract of mice. Intestinal colonization by both Gram-negative and Gram-positive bacteria has been shown to be higher in CD1d-deficient mice. In the same study, the authors observed that NK T lymphocytes were able to stimulate Paneth cells, which also express CD1d, to secrete antimicrobial peptides [13]. Thus, mice deficient in NK T lymphocytes, especially iNK T lymphocytes, have an increased susceptibility to infections [14,15].

Amoebiasis is one of the most important parasitic diseases affecting the world's population, making the understanding of the mechanisms and events related to its pathogenicity increasingly necessary. The present study demonstrated the importance of NK T lymphocytes via the identification and proliferation of these lymphocytes using flow cytometry in the spleen and mesenteric lymph nodes in an experimental model of E. histolytica-induced colitis.

2 II. Methods

3 a) Animals

In total, 32 female mice approximately 70 days old were used in this experiment, including 16 C57BL/6 wild-type (WT) (8 Eh-WT infected and 8 CTRL-WT controls) and 16 C57BL/6CD1 -/mice (8 Eh-CD1 -/infected and 8 CTRL-CD1 -/controls). The animals were obtained from the vivarium at the Institute of Biological Sciences (Instituto de Ciências Biológicas -ICB) of the UFMG and the vivarium at FIOCRUZ/Belo Horizonte. The C57BL/6CD1 -/mice were kindly provided by Professor Ricardo Tostes Gazzinelli. All procedures involving animals were conducted according to the guidelines of the Ethics Committee in Animal Experimentation (CETEA/UFMG) (266/2008).

4 b) Culture and inoculation of trophozoites

The EGG axenic strain of E. histolytica, which was isolated in 1988 in the Amoebiasis Laboratory of the Department of Parasitology of the ICB-UFMG from a patient with dysenteric colitis and amebic liver abscesses, was used for this study. A serological analysis via ELISA and zymodeme and PCR analyses were all positive for E. histolytica [16,17]. Trophozoites were thawed in a water bath and maintained in Pavlova medium at 37°C; they were subcultured every three days. Sixteen mice (8 Eh-CD1 -/and 8 Eh-WT) divided into subgroups of 4 animals each were anaesthetized with a 2% xylazine (10 mg/kg) and 5% ketamine (150 mg/kg) solution. Subsequently, an approximately 2-cm horizontal incision in the abdomen was performed, and 10 6 trophozoites in 0.1 mL of YI-S-32 culture medium were inoculated intracecally. The sixteen control mice (8 CTRL-CD1 -/and 8 CTRL-WT) were also divided into groups of 4 animals each and intracecally inoculated with sterile YI-S-32 culture medium.

5 c) Preparation of cellular suspensions of the spleen and mesenteric lymph nodes (MLN)

The animals were sacrificed 48 hours postinfection via cervical dislocation under general anesthesia with a 2% xylazine (10 mg/kg) and 5% ketamine (150 mg/kg) solution prior to removing the spleen and MLN. The spleen cell suspensions were washed with water and 10x PBS to remove red blood cells via hemolysis. After the washes, the spleen and MLN cell suspensions were maintained in a RPMI complete medium to count the viable cells using a Neubauer chamber and erythrocin as a marker of cell viability. The concentrations of each suspension were then standardized to 5 x 10 6 cells/mL.

6 d) Flow cytometry analysis

Following isolation of cells from the spleen and MLN, the cells were resuspended in PBS (pH 7.2) containing 0.2% fetal bovine serum and 0.1% sodium azide at a concentration of 2 x 10 7 cells/mL. Then, 25 µL of the cell suspension was added to a 96-well U bottom plate and incubated for 30 minutes at 4°C with 10 µL of a solution of phenotypic anti-marker monoclonal antibodies, including CD3, NK1.1, CD4, CD8, CD19 and CD69 (PharMingem, San Diego, CA, USA), diluted in PBS and conjugated with the fluorochromes phycoerythrin (PE), fluorescein (FITC) and CyChrome (Cy). The plates were subsequently centrifuged for 10 minutes at 1200 rpm and 4°C, and the supernatant was discarded by rapid inversion. The pellet was then washed twice with PBS-azide. The pellet was resuspended in 200 ?L of the fixative Mac Facs Fix. The suspensions were stored at 4°C and protected from light until data were acquired using a three color FACScan (Becton Dickinson, Mountain View, California, USA). IgG2a-FITC and IgG2b-PE antibodies were used as negative controls for cells incubated

with immuneglobulins of the same isotype used in the labeled antibody. The samples were analyzed using the program Cell Quest. During acquisition, 30,000 events were collected for analysis. The identification of the cell populations of interest and the determination of the percentage of cellular populations and subpopulations were performed using a computer system coupled to the flow cytometer.

7 e) Statistical analyses

The program Prism 5.0 was used to perform the statistical analyses. One-way ANOVA followed by the Tukey test, as a post-test, were used when analyzing more than two groups, and the unpaired t-test was used when comparing two groups. A Gaussian distribution was assumed for all groups when they were subjected to the Shapiro-Wilk test for normality. The results were expressed as means ± SEM, and differences were considered significant at p ? 0.05. The experimental model used in this study allowed for a phenotypic analysis by flow cytometry of NK T, CD4 + T, CD8 + T and B lymphocytes in the spleen and MLN. They were also used to define the cell frequency profiles of wild-type and CD1-deficient mice in response to E. histolytica infection.

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At 48 hours post-infection, spleens and mesenteric lymph nodes were collected to determine the frequency of NK T, CD4 + T, CD8 + T and B lymphocytes by flow cytometry in the CTRL-WT, Eh-WT, CTRL-CD1 -/and Eh-CD1 -/mice.

The frequency of CD3 + NK1.1 + NK T lymphocytes was significantly reduced in the MLN of mice in the CTRL-CD1 -/-(4.76 ± 1.59%) and Eh-CD1 -/-(3.19 ± 1 57%) groups compared to the Eh-WT (20.24 ± 6.09%) and CTRL-WT (13.00 ± 1.45%) groups ($p < 0.05$) (Figure 1A). This reduction was expected, as CD1 molecules are required for the activation and proliferation of NK T lymphocytes.

In the spleen, there was no significant difference in the frequency of NK T lymphocytes for the CTRL-WT (8.18 ± 1.12%), Eh-WT (8.16 ± 1.16%) CTRL-CD1 -/-(7.60± 0.8563%) and Eh-CD1 -/-(8.46 ± 1.39%) groups (Figure 1B). There was also no significant difference in the frequency of CD4 + CD69 + T lymphocytes in the MLN among the CTRL-WT (13.68 ± 3.57%), Eh-WT (14.84 ± 2.0%), CTRL-CD1 -/-(11.08 ± 0.27%) and Eh-CD1 -/-(11.05 ± 0.39%) groups (Figure 2A). The frequency of CD4 + CD69 + T lymphocytes in the spleen also did not vary significantly among the CTRL-WT (6.97 ± 0.54%), Eh-WT (7.78 ± 0.45%), CTRL-CD1 -/-(7.66 ± 0.57%) and Eh-CD1 -/-(7.08 ± 0.39%) groups (Figure 2B). A significant reduction was observed in the frequency of CD8 + CD69 + T lymphocytes in the MLN of the CTRL-CD1 -/-(7.99 ± 0.43%) and Eh-CD1 -/-(6.74 ± 0.84%) groups compared to the CTRL-WT (31.64 ± 9.29%) and Eh-WT (35.68 ± 3.71%) groups ($p < 0.05$) (Figure 3A).

In the spleen, there was no significant difference in the frequency of CD8 + CD69 + T lymphocytes for the CTRL-WT (17.50 ± 2.54%), Eh-WT (13.57 ± 2.71%), The MLN of the animals in the CTRL-CD1 -/-(1.55 ± 0.25%) and Eh-CD1 -/-(0.87 ± 0.11%) groups showed a significant reduction in the frequency of CD19 + CD69 + B lymphocytes compared to the CTRL-WT (38.70 ± and Eh-WT (64.88 ± 10.46%) groups ($p <0.05$). There was also a significant increase in the frequency of CD19 + CD69 + B lymphocytes in the MLN of the Eh-WT group (64.88 ± 10.46%) compared to the CTRL-WT group (38.70 ± 6.97%) ($p < 0.05$) (Figure 4A).

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The frequency of CD19 + CD69 + B lymphocytes in the spleen of animals in the Eh-WT group (3.52 ± 0.23%) was lower than in the CTRL-CD1 -/-(7.60 ± 0.85%), Eh-CD1 -/-(8.46 ± 1.39%) and CTRL-WT (4.74 ± 0.44%) groups ($p < 0.05$) (Figure 4B).

10 IV. Discussion

The aim of this study was to analyze, using flow cytometry, the frequency of NK T, CD4 + T, CD8 + T and B lymphocytes in the spleens and MLN of wild-type mice (C57BL/6 WT) and mice genetically deficient for CD1d molecules (C57BL/6CD1 -/-) in response to E. histolytica infection. It is important to note that, to date, this is the only study that has used flow cytometry to analyze NK T lymphocytes and their involvement in amebic colitis.

In previous study, we demonstrated that CD1deficient mice (CD1 -/-), which consequently have a lower number of NK T lymphocytes, are more susceptible to amebic infection and to the development of cecal lesions. Furthermore, we found that a decrease in the production of the mucin MUC-2 in C57BL/6CD1 -/mice is associated with a reduction in the number of NK T lymphocytes and to the appearance of more severe cecal lesions [7].

The frequency of CD3 + NK1.1 + NK T lymphocytes in MLN was significantly lower in mice from the CTRL-CD1 -/and Eh-CD1 -/groups compared to the Eh-WT and CTRL-WT groups. NK T lymphocytes are activated directly through the recognition of glycolipidic antigens by CD1 molecules [12]. Thus, this reduction in the CD3 + NK1.1 + NK T lymphocytes in the MLN of the C57BL/6CD1 -/mice was expected because CD1 molecules are required for the activation and proliferation of iNK T lymphocytes. This analysis confirmed that C57BL/6CD1 -/mice actually have a reduced frequency of NK T lymphocytes relative to wildtype mice, making them appropriate for our study.

The significant increase in CD3 + NK1.1 + NK T lymphocytes in the MLN of the animals of the Eh-WT group indicated that these lymphocytes play a major role in the immune response to amebic trophozoites. In a previous study, we found that the mice in the Eh-WT group had fewer trophozoites and less cecal lesions than the Eh-CD1 -/group [7].

The higher frequency of NK1.1 + lymphocytes in the submucosa and lamina propria of the mice in the Eh-WT group, combined with the smaller numbers of trophozoites observed in these mice compared to the control mice, suggest that these lymphocytes may act in the immune response to amebic intestinal infection. This hypothesis is strengthened by the observation that the mice in the Eh-CD1 -/group had reduced numbers of NK1.1 + lymphocytes, elevated tissue parasitism and more severe lesions [7].

In our study, no significant difference in the frequency of CD4 + CD69 + T lymphocytes in the MLN was observed among the CTRL-WT, Eh-WT, CTRL-CD1 -/and Eh-CD1 -/groups. The frequency of CD4 + CD69 + T lymphocytes in the spleen also did not vary significantly among the CTRL-WT, Eh-WT, CTRL-CD1 -/and Eh-CD1 -/groups. However, in the MLN of the animals in the CTRL-CD1 -/and Eh-CD1 -/groups, there was a lower frequency of CD8 + CD69 + T lymphocytes compared to the CTRL-WT and Eh-WT groups. This reduction may have occurred because of the deficiency in activated NK T cells. CD1d-deficient NK T cells produce a variety of cytokines when activated, and the absence of these cells can lead to a decrease in the proliferation of CD8 + CD69 + T cells. Some authors have observed that in the mesenteric, inguinal, axillary and cervical lymph nodes and spleens of J?18 -/and CD1d -/mice, the activation of NK T cells mediated by ?-galactosylceramide increases the homeostatic proliferation of CD8 + T cells but not CD4 + T cells [18]. In our study, no significant difference in the frequency of CD4 + CD69 + lymphocytes in the MLN or spleen was observed among the CTRL-WT, Eh-WT, CTRL-CD1 -/and Eh-CD1 -/groups. The increase in the homeostatic proliferation of CD8 + T cells has been shown to be related to the production of IL-4 by activated NK T cells. Thus, IL-4 acts directly on CD8 + T cells to induce their proliferation [18]. However, with respect to the cytotoxic T lymphocytes, studies by other authors have shown that there is a lack of these cells in mice with amebic colitis or liver abscesses. The immunohistochemical characterization of CD8 + and CD4 + T lymphocytes in humans with amebic colitis did not find significant numbers of these cells or contact between these cells and trophozoites in either lesioned regions or intact areas of the intestinal tract [19]. Some authors propose that the main immune mechanisms used in intestinal E. histolytica infections occur during the first days following infection and are mediated by innate immunity, which is independent of T lymphocytes [20]. We did not find significant differences in the frequencies of CD8 + CD69 + T cells in the spleens of mice in the CTRL-WT, Eh-WT, CTRL-CD1 -/and Eh-CD1 -/groups. However, the results showed a trend toward a reduction in the frequency of CD69 + CD8 + T lymphocytes in the spleens of the CTRL-CD1 -/and Eh-CD1 -/groups compared to the CTRL-WT and Eh-WT groups.

CD1d molecules are constitutively expressed in dendritic cells, B lymphocytes and macrophages in both humans and mice, although the levels of expression may vary among cell types **??**21]. The quantitative analysis of lymphocytes showed a significant reduction in the frequency of B lymphocytes (CD19 + CD69 +) in the MLN of animals in the CTRL-CD1 -/and Eh-CD1 -/groups compared to the CTRL-WT and Eh-WT groups. It is possible that this reduction in recently activated B lymphocytes in the MLN is related to the scarcity of The results of other studies are consistent with our findings and have shown that NK1.1 + lymphocytes are an important barrier against the development of amebic liver abscesses in their early stages [6]. These authors reported that CD1-deficient mice developed larger liver abscesses due to their inability to present antigens derived from amebic lipopeptidophosphoglycan to NK T lymphocytes. Likewise, the reduction in the activated NK T lymphocyte population in CD1ddeficient mice resulted in an increased susceptibility to Toxoplasma gondii infection [8]. To verify whether NK T lymphocytes secrete IFN-? following EhLPPG stimulation, lymphocytes were removed from CD1d -/and J?18 -/mice deficient in iNK T lymphocytes or in all NK T lymphocyte subpopulations and cultured with antigen presenting cells (APCs) stimulated by EhLPPG. The authors observed a great reduction in IFN-? secretion in CD1d -/and J?18 -/mice, indicating that iNK T lymphocytes are an important source of IFN-? when exposed to EhLPPG. IFN-? production by EhLPPGactivated iNK T lymphocytes may initiate a Th1-type adaptive response that is able to amplify the secretion of IFN-? and increase the efficiency of the immune response to E. histolytica, thus reducing the number of trophozoites and the expansion of amebic abscesses [6]. Previous research has also shown that NK T lymphocytes are important in controlling bacterial colonization of the gastrointestinal tract of C57BL/6 mice [13]. In that study, the authors showed that intestinal colonization by both Gram-negative and Gram-positive bacteria was higher in C57BL/6CD1 -/mice.

Participation of T, B and NKT Lymphocytes and CD1 Molecule in the Infection by Entamoeba histolytica in Mice activated iNK T lymphocytes in CD1-deficient mice. The MLN drain directly into the lymph of the cecal mucosa, where there are many iNK T lymphocytes. Some authors have shown that the in vivo activation of murine iNK T lymphocytes with ?-galactosylceramide induces the production of IL-4 by these lymphocytes and leads to the expression of activation markers, such as CD69, B7-2 and I-A b in B lymphocytes [22]. In humans, the in vitro activation of NK T lymphocytes with ?galactosylceramide induces the production of IL-4 and IL-13, which stimulate B lymphocyte proliferation and the total production of IgG1 and IgM antibodies [23].

When we compared the number of recently activated B lymphocytes in the MLN of mice in the Eh-WT group to that of mice in the CTRL-WT group, we observed a significant increase in these lymphocytes in the E. histolytica infected mice. In addition to participating in antigen presentation via MHC and CD1d, B lymphocytes also act in the immunity to E. histolytica through the production of IgA and IgG. The humoral response to E.

histolytica in mice with amebic colitis may act both locally and systemically depending on the level of intestinal and extra-intestinal invasion caused by the protozoan [24]. The significant increase in the number of recently activated B lymphocytes in the MLN of the Eh-WT suggests the activation of humoral immunity and its likely participation in the resistance against trophozoites, although the time of infection in this study did not allow for a more detailed analysis. The ability of MLN to drain directly into the lymph from the intestine, where the inflammatory focus is located, could also aid in the delivery of antigens and the activation of B lymphocytes.

In contrast to our observations in the MLN, there was a reduction in the frequency of recently activated B lymphocytes in the spleens of the animals in the Eh-WT group compared to those in the CTRL CD1 -/-, Eh-CD1 -/and CTRL-WT groups. B lymphocytes appear to participate in immunity to E. histolytica. Thus, the recruitment of recently activated B lymphocytes from the spleen to the MLN or other organs, where these cells would have increased exposure to antigens from the site of inflammation and would be activated to produce IgA and IgG, may be occurring. This migration would explain the reduction in the frequency of these cells in the spleen.

11 V. Conclusions

Combined with the pathological study that we performed previously, the results of this flow cytometry analysis reinforce the importance of NK T lymphocytes in immunity against intestinal amebic infection and of CD1 molecules in the activation of T and B lymphocytes. The direct involvement of these cells in experimental amebic colitis still requires further study.

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Figure 1: Figure 1 :

[1] [2]

[1] © 2015 Global Journals Inc. (US) Participation of T, B and NKT Lymphocytes and CD1 Molecule in the Infection by Entamoeba histolytica in Mice

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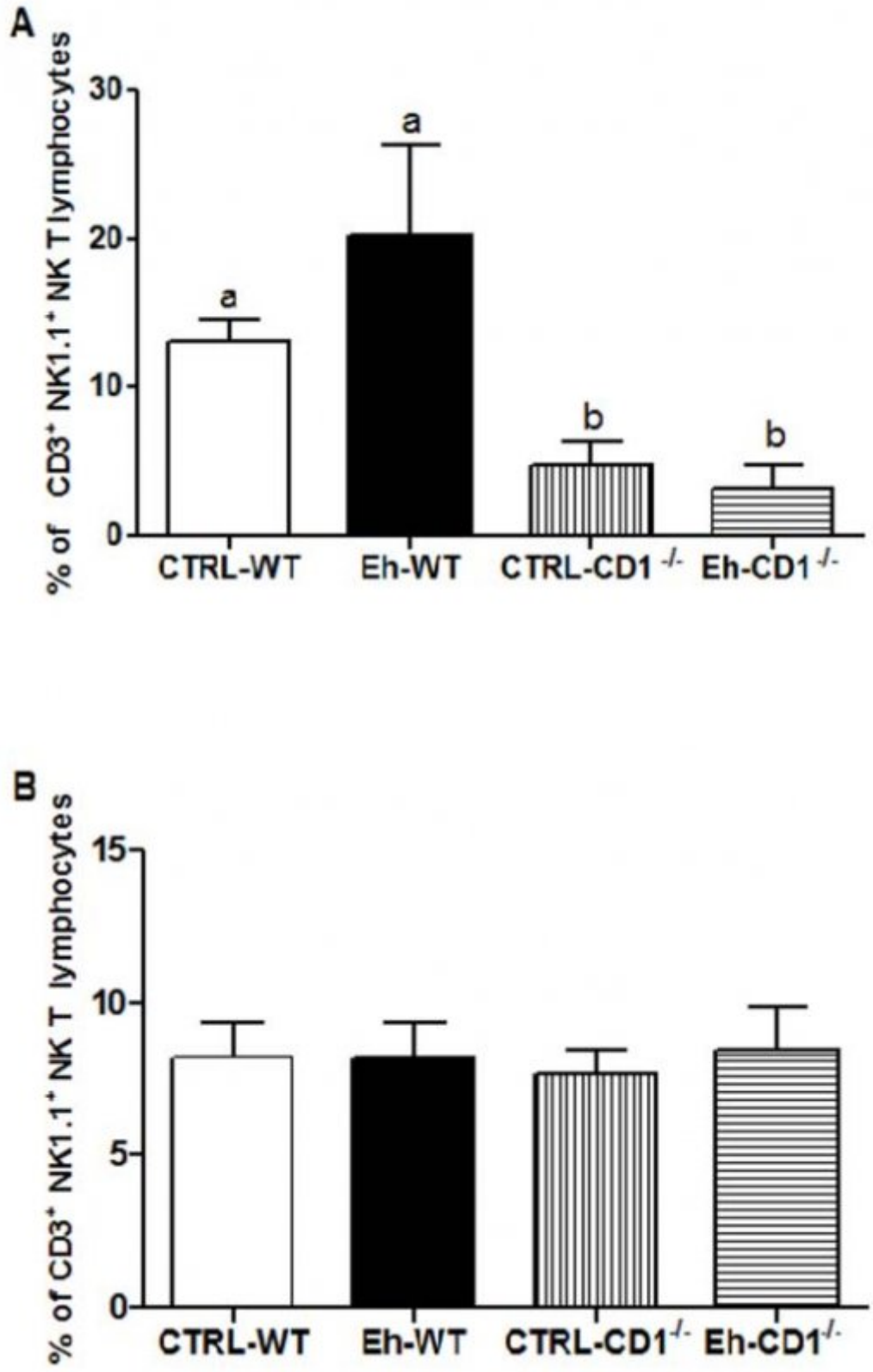

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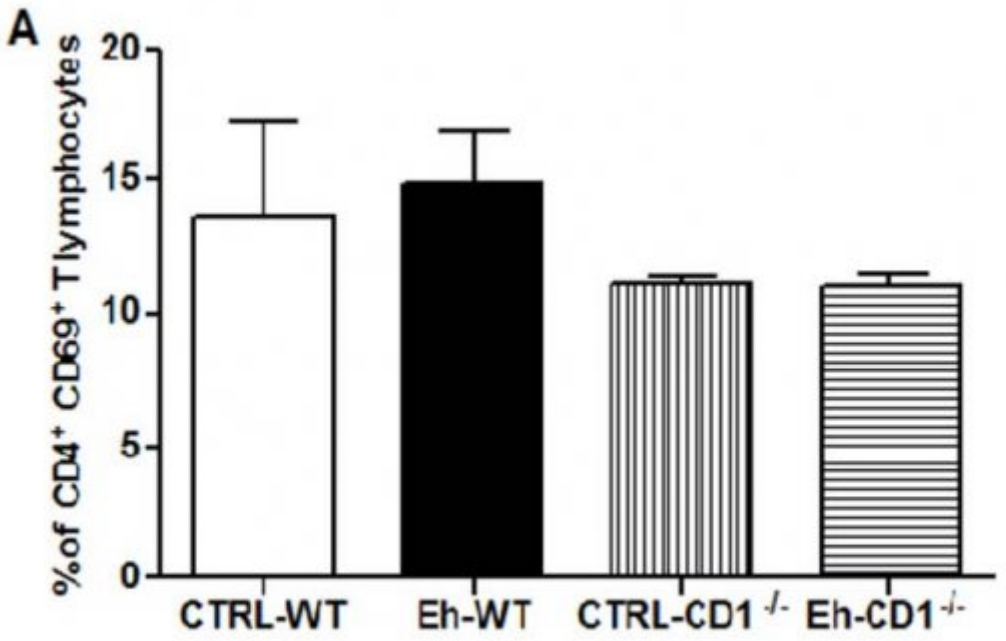

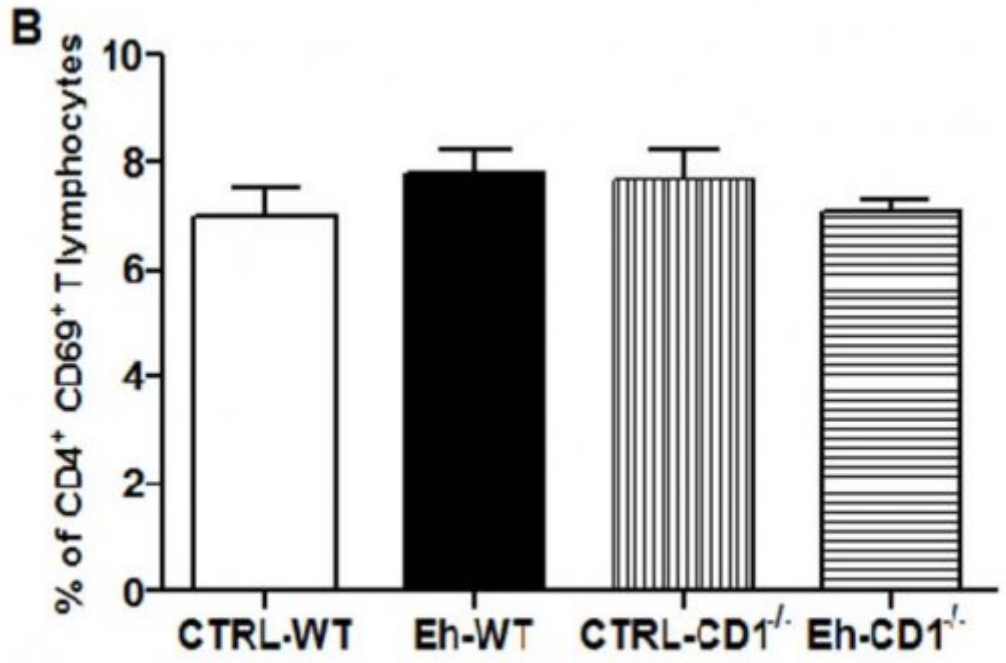

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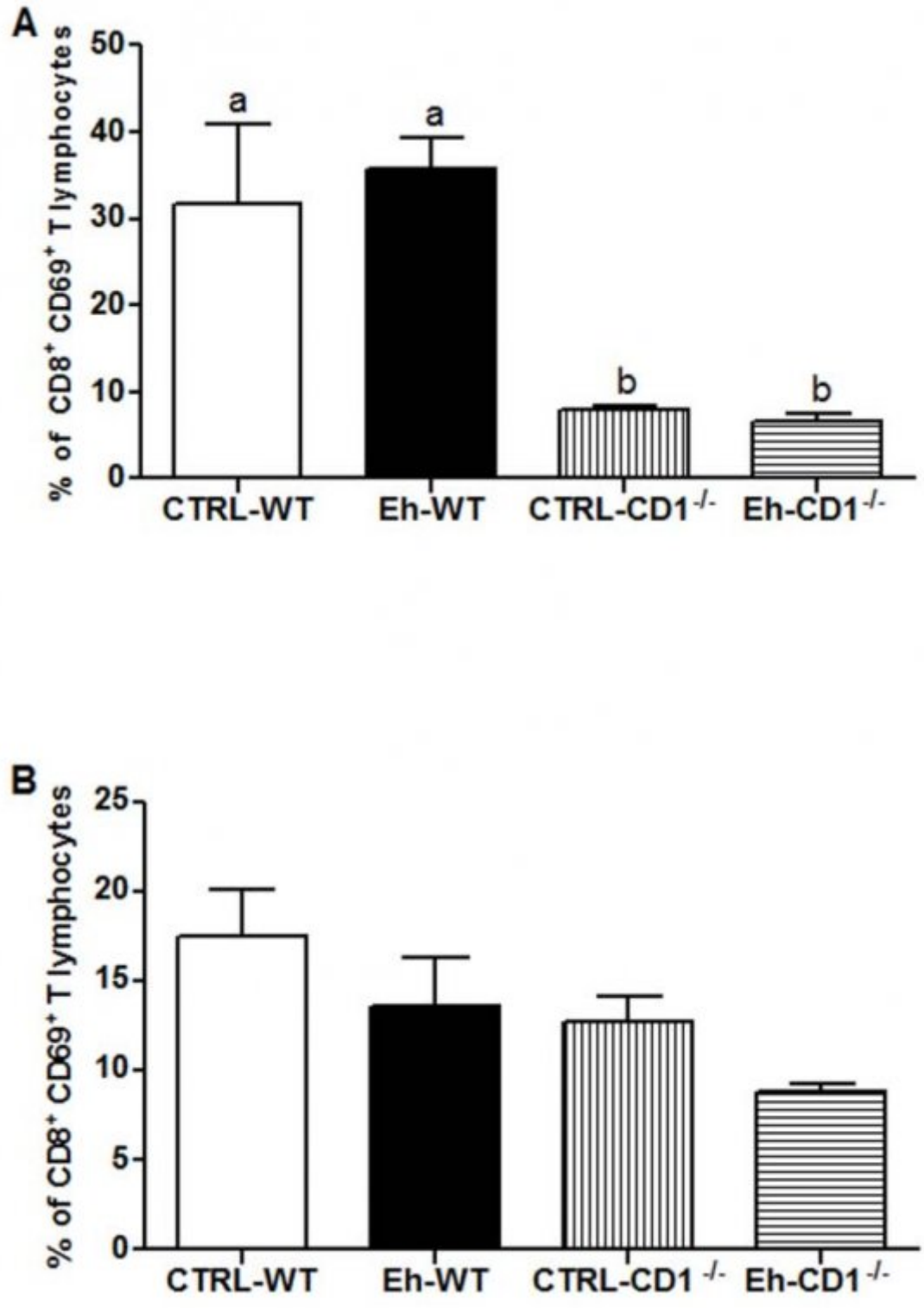

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Figure 4: Figure 4 :

.1 Competing interests

The authors declare that they have no competing interests.

.2 VI. Acknowledgments

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