Experimental Parasitology 136 (2014) 74–78

Contents lists available at ScienceDirect

Experimental Parasitology

journal homepage: www.elsevier.com/locate/yexpr

Effect of cytotoxic T-lymphocyte-associated protein 4 on $CD4^+CD25^+$ regulatory T cells in murine *Schistosomiasis japonica*

Chun-Lian Tang [a,*], Jia-Hui Lei [b], Fei Guan [b], Yong-Long Li [b], Rong Liu [c], Christoph G. Grevelding [d], Ming-Sen Jiang [e], Hui-Fen Dong [e]

[a] Department of Clinical Laboratory, Wuchang Hospital, No. 116 Yangyuan Street, Wuhan 430063, China
[b] Department of Parasitology, Tongji Medical College, Huazhong University of Science and Technology, No. 13 Hangkong Road, Wuhan 430030, China
[c] School of Public Health, Peking University, Beijing 100191, China
[d] Institute for Parasitology, Justus-Liebig-University, 35392 Giessen, Germany
[e] Department of Parasitology, Medical College of Wuhan University, Wuhan 430071, China

HIGHLIGHTS

- Anti-CTLA-4 mAb favors the host to clear *S. japonicum*.
- Anti-CTLA-4 mAb may bias and enhance Th1-type host immune responses.
- Egg granuloma sizes in anti-CTLA-4 mAb-treated animals were significantly larger.
- Tregs and CTLA-4 may exert synergistic effect on the immune evasion of *S. japonicum*.

ARTICLE INFO

Article history:
Received 27 March 2011
Received in revised form 3 March 2012
Accepted 11 November 2013
Available online 20 November 2013

Keywords:
Schistosoma japonicum
$CD4^+CD25^+$ Tregs
CTLA-4
Cytokines
Immune evasion

ABSTRACT

In a previous study we demonstrated that $CD4^+CD25^+$ regulatory T cells (Tregs) contributed to the escape of *Schistosoma japonicum* (*S. japonicum*) from the host's immune responses. In this paper, we studied the effect of cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) on $CD4^+CD25^+$ Tregs in murine *Schistosomiasis japonica* and its corresponding role in the immune evasion of *S. japonicum* in mice. The results showed substantial reductions of worm burden and egg production in worm groups treated with anti-CD25 or anti-CTLA-4 monoclonal antibodies (mAb) compared to an infected but untreated control. The reduction effect was even enhanced in an experimental group co-treated with both mAbs. Compared to the control group, the percentage of $CD4^+CD25^+$ Tregs was very much lower in the anti-CD25 mAb group as determined by FACS analyses and higher in the anti-CTLA-4 mAb group. ELISA analyses showed that both the anti-CTLA-4 mAb and the co-treated groups had higher levels of cytokines compared to the control group as well as larger egg granuloma sizes as determined by microscopical analyses of liver sections of infected mice. These results suggest that treatment with an anti-CTLA-4 mAb allows the host to clear *S. japonicum*, but at the cost of elevated pathological damage. The latter indicated a role of CTLA-4 in granuloma formation. Moreover, $CD4^+CD25^+$ Tregs and CTLA-4 may exert synergistic effects during immune evasion processes by enhancing Th1-type immune response.

1. Introduction

Regulatory T cells (Tregs) are important molecules mediating inflammation and specific immune responses. During infectious diseases, Tregs lessen inflammatory processes to limit tissue damage, but they are also able to inhibit effector immunity, thus impairing pathogen clearance (Belkaid et al., 2006). Infections by pathogens such as parasites can induce both CD4(+) and CD8(+) Tregs (Joosten and Ottenhoff, 2008). Parasites have evolved mechanisms influencing the Treg population of the host to generate conditions allowing their survival in the hostile environment (Maizels, 2007).

In our studies on *Schistosoma japonicum* (*S. japonicum*) we demonstrated in a mouse model recently that $CD4^+CD25^+$ regulatory T cells (Tregs) contributed to the escape from the host's immune responses upon infection. Injecting anti-CD25 monoclonal antibodies (anti-CD25 mAb) blocking $CD4^+CD25^+$ Tregs promoted the clearing of the parasites by the host (Tang et al., 2011). In studies of other groups $CD4^+CD25^+$ Tregs were described to suppress both the activation and proliferation of effector T cells and the maintenance of peripheral self-tolerance and immune homeostasis

* Corresponding author. Fax: +86 27 68758766.
E-mail address: tangchunlina@yahoo.com.cn (C.-L. Tang).

0014-4894/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.exppara.2013.11.001

(Shen et al., 2011; Sakaguchi, 2011). Apart from CD25, cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), tumor necrosis factor receptor (GITR), and CD103 were identified as further molecules controlling the development and activation $CD4^+CD25^+$ Tregs (Mariano et al., 2008). CTLA-4 has been shown to be present on the surface and in the cytoplasm of peripheral $CD4^+CD25^+$ Tregs (Verhagen et al., 2008). Within 3–4 weeks of birth mice deficient in CTLA-4 develop spontaneous lethal lympho proliferative disease with multi organ infiltration, suggesting that CTLA-4 plays a primary role in T cell homeostasis and peripheral tolerance (Hoff et al., 2010). The role of CTLA-4 in parasitic infections has been explored in nematodes (McCoy et al., 1997) and *Trypanosoma cruzi* (Graefe et al., 2004) infection models, indicating its role during the escape from the host's immune responses. Walsh et al. (2007) identified a marked expansion of the $CTLA\text{-}4^+$ population during *Schistosoma mansoni* infection. Blocking of CTLA-4 during acute infection caused significant weight loss of infected mice and altered their Th2-type cytokine response. However, there has been no report of the effect of CTLA-4 on the infection of *S. japonicum*.

To examine the effect of blocking $CD4^+CD25^+$ Tregs in more detail we used anti-CTLA-4 monoclonal antibody (anti-CTLA-4 mAb) alone or in combination with anti-CD25 mAb in this study to explore the effect of CTLA-4 on $CD4^+CD25^+$ Tregs in murine *Schistosomiasis japonica*.

2. Materials and methods

2.1. Animals and parasites

BALB/c female mice, 6–8 weeks old, were obtained from Hubei Province Center for Disease Control and Prevention (China) and fed with pathogen-free food and water. The experiment was approved by the Committee on Animal Research of Medical College of Wuhan University. Mice were divided randomly into 5 groups, normal group, infected control group, anti-CD25 mAb group, anti-CTLA-4 mAb group, and co-treated group with both anti-CD25 mAb and anti-CTLA-4 mAb. *Oncomelania* snails infected with *S. japonicum* were supplied by Jiangxi Province Institute of Parasitosis Control and Prevention (China). *S. japonicum* cercarie were shed from the snails. Each anesthetized mouse was percutaneously infected with 40 cercariae through the shaved abdomen. Two weeks after infection, mice received 300 μg anti-CD25 mAb, or 300 μg of anti-CTLA-4 mAb, or a combination of both by intraperitoneal injection. As control, an equivalent volume of PBS was injected. Mice were sacrificed 6 weeks after infection.

2.2. Assessment of worm burden and eggs per gram liver

To assess worm burden, infected mice were perfused from the portal vein 6 weeks after infection according to procedure introduced by Ruppel et al. (1990). The number of worms was counted under a dissecting microscope. The reduction rate in parasite burden was calculated. To determine egg burden, livers were removed, and 0.5 g of the large left lobe of each liver was digested at 37 °C for 3 h in 20 ml of 5% potassium hydroxide (KOH). Four times 250 μl of each sample were counted, and a mean value was determined according to Cheng et al. (2008). The number of eggs per gram liver was calculated. The remaining livers were used for pathological evaluation.

2.3. Flow cytometric analysis

Mice were sacrificed 6 weeks after administration of anti-CD25 mAb. A single-cell suspension of splenocytes was prepared according to Mo et al. (2007). To determine the frequency of $CD4^+CD25^+$ Tregs, a three-color cytometry analysis was performed. Cells were stained using Mouse regulatory T cell staining Kit (eBioscience) and analyzed on FACS Calibur (Becton Dickinson, Mountain View, CA) with CellQuest software. The following conjugated antibodies were incubated with lymphocyte populations, fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD4, allophycocyanin (APC)-conjugated anti-mouse CD25, and phycoerythrin (PE)-conjugated anti-mouse Foxp3 (forkhead box P3). PE-conjugated rat IgG2α was reserved as isotype control.

2.4. ELISA for the detection of cytokines

Mice splenocyte suspensions were prepared 6 weeks after infection and splenocytes (5×10^6 cells/well) were cultured in RPMI-1640 supplemented with 10% FCS, 1% penicillin, and 1% streptomycin. Cultures were incubated with 5 μg/ml Con A for 72 h at 37 °C in 5% CO_2 (all from Sigma). A sandwich ELISA was used to measure IFN-γ, IL-4, IL-5, and IL-10 in cell culture supernatants according to the instruction of the ELISA manufactures (eBioscience). In brief, mouse IFN-γ, IL-4, IL-5, and IL-10 were detected by biotinylated monoclonal antibodies, which were subsequently displayed by avidin-conjugated horseradish peroxidase followed by incubation with TMB substrate. OD values at 450 nm were recorded using MK3 microplate reader.

2.5. Histologic evaluation of granuloma formation

The remaining right liver lobe of each infected mouse was fixed in 10% phosphate-buffered formaldehyde, embedded in paraffin wax. The sections (5 μm) were cut and stained with hematoxylin and eosin (H & E). Representative H & E-stained liver sections from each animal were scanned under 200× magnification with a compound microscope (Olympus, Japan).

2.6. Statistical analysis

All data were expressed by mean ± S.D, and analyzed with SPSS 13.0. Comparison among different groups was made with ANOVA. Value of $P < 0.05$ was considered as significant.

3. Results and discussion

3. 1 The effect of anti-CD25 mAb, anti-CTLA-4 mAb or both on worm burden and egg production

In previous studies on immunological aspects of parasite-host interaction clear evidence had been found that Tregs play a crucial role during this interplay. Among these, CD4(+)Tregs were found to be involved in immune evasion strategies. Besides CD25, CD4(+) Tregs also express further surface proteins such as CTLA-4 (Mariano et al., 2008). In an independent study for *S. mansoni* it was shown that the $CTLA\text{-}4^+$ population of Tregs increased, and that its inhibition altered physiology and cytokine profiling of mice during an infection (Walsh et al., 2007). To investigate whether this observation is a more general effect during schistosome infections or a species-specifically induced one, we performed CD4(+) CD25(+) Tregs determination and anti-CD25 blocking experiments in a mouse infection model of *S. japonicum*. The results showed that the percentages of CD4(+)CD25(+) Tregs also increased upon infection, an effect which was reversed by blocking with anti-CD25 mAb. Furthermore, worm burden was lowered in anti-CD25 mAb treated animals as well as the level of interleukin 10, whereas the level of interferon gamma was increased upon blocking