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Osteopontin-dependent regulation of Th1 and Th17 cytokine responses in *Trypanosoma cruzi*-infected C57BL/6 mice

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ABSTRACT

Osteopontin (OPN) is a multifunctional protein participating in the regulation of different Th cell lineages and critically involved in the initiation of immune responses to diverse pathogens. Our study goal was to verify whether OPN helps modulate the protective Th1 and Th17 cytokine responses in C57BL/6 mice infected with *Trypanosoma cruzi*, the etiological agent of Chagas disease. Parasite infection induced OPN release from murine macrophages in vitro and acute Chagas mice displayed enhanced serum levels of this cytokine at the peak of parasitemia. Upon administration of a neutralizing anti-OPN antibody, recently infected mice presented lower Th1 and Th17 responses, increased parasitemia and succumbed earlier and at higher rates to infection than non-immune IgG-receiving controls. The anti-OPN therapy also resulted in reduced circulating levels of IL-12 p70, IFN-γ, IL-17A and specific IgG_{2a} antibodies. Furthermore, antibody-mediated blockade of OPN activity abrogated the ex vivo production of IL-12 p70, IFN-γ and IL-17A, while promoting IL-10 secretion, by spleen macrophages and $CD4^+$ T cells from *T. cruzi*-infected mice. Th1 and Th17 cytokine release induced by OPN preferentially involved the $\alpha_v\beta_3$ integrin OPN receptor, whereas concomitant down-modulation of IL-10 production would mostly depend on OPN interaction with CD44. Our findings suggest that, in resistant C57BL/6 mice, elicitation of protective Th1 and Th17 cytokine responses to *T. cruzi* infection is likely to be regulated by endogenous OPN.

1. Introduction

Chagas disease, caused by the intracellular protozoan parasite *Trypanosoma cruzi*, is one of the most important public health problems in Latin America. The overall prevalence of this human infection is estimated at 10 million cases, with 100 million at risk for infection [1]. The acute phase of infection is characterized by the presence of parasites in the bloodstream and diverse host tissues. Findings from several studies in experimental models of *T. cruzi* infection have suggested that a strong T helper type-1 (Th1) immune response is essential for the early control of parasitism [2]. Parasite replication is hampered by both innate and acquired immune responses mediated by macrophages, NK cells, B cells, $CD4^+$ and $CD8^+$ T cells [3]. Proinflammatory cytokines [e.g. interferon(IFN)-γ, tumor necrosis factor(TNF)-α and interleukin(IL)-12] also play a crucial role in protective immunity against *T. cruzi* [4]. IL-12 enhances IFN-γ production from NK cells and T cells. TNF-α cooperates with IFN-γ for induction of resistance to *T. cruzi* infection by activating phagocytes to release high levels of reactive nitrogen intermediates, such as NO, that are toxic to the parasite [3,5]. In addition, the proinflammatory cytokine IL-17A has recently been indicated as a necessary factor for the resolution of acute *T. cruzi* infection [6]. IL-17A is mainly produced by activated memory $CD4^+$ T cells, which are now classified as Th17 cells, and has been reported to elicit inflammatory responses through the organized production of inflammatory cytokines and chemokines, such as IL-1β, IL-6, TNF-α, granulocyte macrophage-colony stimulating factor (GM-CSF), and IL-8/CXCL8, leading to the induction of leukocyte migration [7,8].

Osteopontin (OPN) is a multi-faced glyco-phosphoprotein synthesized by a variety of non-immune and immune cells and is implicated in interactions with cells mediating signaling, migration, and attachment [9,10]. Within the immune system, OPN is produced by activated T cells, NK cells, dendritic cells, and macrophages [11,12]. The existence of variant forms of OPN as a secreted (sOPN) and intracellular (iOPN) protein and its modification through post-translational events and proteolytic cleavage explain its broad range of functions. sOPN interacts with integrins and CD44, mediates cell adhesion and migration, and exhibits Th1 cytokine activities [13]. iOPN has been described to modulate the recruitment of innate immune cells, the secretion of IFN-α in plasmacytoid dendritic cells and the development of autoimmunity [14]. Both sOPN and iOPN participate in the regulation of different Th cell lineages [12]. Recent reports demonstrated that OPN deficiency may result in impaired clearing of intracellular infections,

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likely to be caused by a defective Th1 response. Analysis of mice lacking OPN revealed that this pleiotropic protein contributes to protection against a panel of intracytoplasmic pathogens, including herpes simplex virus type 1, *Listeria monocytogenes*, *Mycobacterium bovis* and *Plasmodium falciparum* [13,15–17]. OPN has been implicated in the development of pathogenic Th17-linked pathways [18] but data on Th17-cell protective immune responses that are mediated by OPN is not yet available. To our knowledge, no report has so far addressed the question about the immunoregulatory ability of OPN in chagasic infection. The aim of this study was therefore to verify the occurrence of OPN-dependent regulation of the protective Th1 and Th17 cytokine responses in *T. cruzi*-infected C57BL/6 mice.

2. Materials and methods

2.1. Mice

Female wild-type (WT) and OPN-deficient ($OPN^{-/-}$) C57BL/6 mice (6–8 weeks) were purchased from the Jackson Laboratory (Bar Harbor, USA) and housed under specific pathogen-free conditions. Each test group consisted of five ($OPN^{-/-}$) to eight (WT) mice. The protocol was approved by the local ethics committee for animal studies and followed the guidelines on accommodation and care of animals used for scientific purposes formulated by the European Commission (EU Directive 2010/63/EU).

2.2. Parasites and experimental infection

Mice were infected intraperitoneally with 10^3 blood-trypomastigote forms of the Y strain of *T. cruzi* [19]. The levels of parasitemia were evaluated by counting parasites in 5 μl of blood from the tail vein, and mortality was recorded daily [20]. Individual serum specimens were collected before and at different times after *T. cruzi* infection. For in vitro experiments, trypomastigotes of the Y strain were grown and purified from a monkey fibroblast cell line (LLC-MK_2). The parasite lysate preparation was obtained from freeze–thawed trypomastigotes, centrifuged at 10,000 g for 30 min, and filtered through a 0.22-μm-pore-size membrane filter.

2.3. Antibody treatment

In vivo blockade of OPN was achieved by using a neutralizing IgG antibody (OPN Ab), as reported previously [21]. Mice were treated with goat anti-mouse OPN Ab (50 μg/mouse, R&D Systems, USA) or control goat IgG 2 h before infection and every other day thereafter for 7 days by tail-vein injection.

2.4. Spleen cell, T cell and macrophage cultures

Single-cell suspensions of splenocytes from both uninfected and *T. cruzi*-infected C57BL/6 mice were prepared. Spleens were dispersed by gently teasing the spleen tissue through a 100-μm-pore-size nylon cell strainer. Erythrocytes were lysed by 2-min incubation in lysis buffer (150 mM NH_4Cl, 1 M $KHCO_3$, 0.1 mM EDTA, pH 7.2). Splenic $CD4^+$ T cells and macrophages were isolated using paramagnetic beads (Dynabeads® FlowComp™ Mouse CD4 and CD14 kits, respectively, Dynal; Invitrogen Corp., USA) as suggested by the manufacturer. Briefly, positive selection of cells was accomplished by incubating total splenocytes with the antibody mix and beads, followed by magnetic separation of bead-bound cells. Cell survival was 95 ± 2% as judged by trypan blue exclusion test. Surface marker staining using FITC-conjugated antibody to mouse CD4 (BD PharMingen, USA) or PE-labeled antibody to mouse F4/80 (AbD SeroTec, UK) and flow analysis were used to ensure adequate enrichment of isolated cell subsets. The purity of enriched cell preparations was: $CD4^+$, 98.6 ± 2.1 (mean% ± SD); $F4/80^+$, 97.1 ± 3.2. Leukocytes were washed twice and adjusted to 2×10^6 cells/ml in complete medium RPMI containing 10% fetal bovine serum (FBS), 1 mM 2-mercaptoethanol, 1 mM sodium pyruvate, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were seeded by triplicate in 24-well plates (Sarstedt, Germany) in the presence of 10 μg/ml of parasite lysate or medium alone. All the media and reagents were endotoxin-free. For intracellular cytokine staining, splenic lymphocytes (5×10^5) were stimulated with parasite lysate (10 μg/ml) for 24 h. BD GolgiStop (BD Pharmingen) was added at the final 6 h. The cells were first stained extracellularly with FITC-conjugated anti-CD4 (RM4-5) and PE-Cy5-conjugated anti-CD3 (17A2), then fixed and permeabilized with BD Cytofix/Cytoperm solution (BD Pharmingen). The cells were then separately stained intracellularly with PE-conjugated anti-IFN-γ (XMG1.2), anti-IL-17A (TC11-18H10) or isotype control antibodies (all from BD Pharmingen). Samples were acquired on a FACSCalibur (BD Biosciences) and data were analyzed using BD CellQuest Pro software (BD Biosciences). To test the effect of OPN on splenocytes, purified $CD4^+$ T cells were cultured in the presence or absence of 1 μg/ml of mouse recombinant OPN (rOPN, R&D Systems) and infected with *T. cruzi* culture-derived trypomastigote forms at a 5:1 parasite:host cell ratio. In some experiments, $CD4^+$ T cells were stimulated with rOPN plus recombinant mouse IL-12 (100 ng/ml, R&D Systems) before infection. To study the involvement of OPN receptors, $CD4^+$ T cells and macrophages were treated in parallel experiments with 5 μg/ml of receptor-blocking monoclonal antibodies, including anti-CD44 and anti-β3 integrin (BD Biosciences). Supernatants were collected after 72 h at 37 °C and stored at −70 °C.

Normal mouse inflammatory macrophages were harvested from the spleens and peritoneal cavities 3 days after the injection of 1 ml of 3% sodium thioglycolate (Sigma–Aldrich, USA). The adherent cells were obtained after a 2- to 4-h incubation of single-cell suspensions in 96-well plates at 37 °C. The non-adherent cells were removed by exhaustive washing with Hank's medium. Moreover, primary cultures of bone marrow-derived macrophages (BMM) were obtained from femurs of naïve C57BL/6 mice as described [22] and cultured in BMM medium (DMEM supplemented with 20% FBS and 10% 3T3 fibroblast supernatant containing macrophage-CSF. The cells were differentiated for 7 days yielding > 98% $Mac3^+$, $F4/80^+$ and $CD11b^+$ BMM (data not shown). Macrophages were polarized with either 100 ng/ml LPS + 100 U/ml IFN-γ (M1) or 5 ng/ml IL-4 (M2). Non-polarized BMM (M0) were cultured in complete medium alone. Culture trypomastigotes were added in a 5:1 parasite-to-cell ratio and incubated for 48 h at 37 °C. The supernatants were then harvested, filtered and stored at −70 °C.

2.5. Measurement of murine cytokines and T. cruzi-specific antibodies

OPN, IFN-γ, IL-12 p70, IL-10, IL-13 and IL-17A measurements were done by sandwich ELISA (R&D Systems) according to the manufacturer's specifications. Supplied standards were used to generate the standard curves.

Serum specific IgG antibody response in parasite-infected mice was measured by use of a commercial ELISA kit (bioMérieux, France), as reported elsewhere [23]. To identify the antibody isotypes, the reaction was revealed with peroxidase-conjugated rat monoclonal antibodies for mouse IgG_{2a} and IgG_1 subclasses (BD Pharmingen), which are routinely used as an indirect measure of Th1 and Th2 immune responses, respectively [24].

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