Vaccine 28 (2010) 7407–7413

Contents lists available at ScienceDirect

Vaccine

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

Dendritic cells devoid of IL-10 induce protective immunity against the protozoan parasite *Trypanosoma cruzi*

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ARTICLE INFO

Article history:
Received 15 June 2010
Received in revised form 29 August 2010
Accepted 31 August 2010
Available online 16 September 2010

Keywords:
Dendritic cells
IL-10
Trypanosoma cruzi
Vaccine
Mouse model
Adoptive transfer
IL-10 deficient mice

ABSTRACT

In diverse models of microbial infections, protection is improved by immunization with dendritic cells (DC) loaded with whole pathogen lysate. However, pathogens that modulate DC function as a way to evade immunity may represent a challenge for these vaccination strategies. Thus, DC must be instructed in a particular manner to circumvent this issue and drive an effective immune response. *Trypanosoma cruzi* or its molecules alter DC function and, as we demonstrated, this phenomenon is associated with the parasite-driven stimulation of IL-10 production by DC. Here, we show that DC from IL-10-deficient mice pulsed *in vitro* with trypomastigote lysate secreted increased amounts of Th1-related cytokines and stimulated higher allogeneic and antigen-specific lymphocyte responses than their wild-type counterparts. In a model of DC-based immunization, these antigen-pulsed IL-10-deficient DC conferred protection against *T. cruzi* infection to recipient mice. Efficient immunity was associated with enhanced antigen-specific IFN-gamma production and endogenous DC activation. We illustrate for the first time a DC-based vaccination against *T. cruzi* and evidence the key role of IL-10 produced by sensitizing DC in inhibiting the induction of protection. These results support the rationale for vaccination strategies that timely suppress the effect of specific cytokines secreted by antigen presenting DC.

1. Introduction

The actual challenges in vaccine development against infectious diseases are focused on the characterization of relevant antigen candidates but also on the understanding of factors that determine protective immunity. In this sense, vaccines for chronic or recurrent parasitic diseases are still lacking. In fact, immune responses to natural infection are sufficient to control parasite burden but fail to achieve total pathogen clearance and/or long-lasting memory. Thus, vaccines against these pathogens should stimulate stronger immune responses than active infections do [1]. It is now well acquainted that effective vaccine formulations must provide not only the antigen of interest but also the proper adjuvant. In this sense, activation of dendritic cells (DC) has gained interest as one of the major targets of adjuvants to enhance immune responses.

DC are highly specialized antigen presenting cell (APC) populations responsible for the initiation of primary immune responses. They are endowed with a unique capacity to sense pathogens or pathogen derived molecules through receptors with specificity for pathogen-associated molecular patterns [2]. They react to pathogen specific activation with a sequence of signals involving antigen presentation, costimulation and cytokine secretion [3–5] thus controlling the scale and quality of this response. Owing to these properties, DC have been employed as "natural adjuvants" by loading them *ex vivo* with the antigens of interest and injecting them back into animals or humans to manipulate the immune response. The capacity of these cells to serve as adjuvants and vaccine carriers in cancer and infectious diseases has been extensively documented [6,7]. The final goal of these *ex vivo* approaches is to disclose features that contribute to induce protection by appropriate targeting of DC and manipulation of their functions *in vivo*. Despite the promise of "DC-based immunization", many individual pathogens that trigger alterations of DC function as a way to evade immunity represent a challenge for this vaccination strategy [8]. Therefore, DC must be educated in a specific manner to circumvent this failure and drive an efficient immune response.

Trypanosoma cruzi is a complex protozoan parasite with intra and extra cellular stages that causes persistent infection in the

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doi:10.1016/j.vaccine.2010.08.105

mammalian host. Humoral and cellular arms of the immune response are known to control *T. cruzi* infection but fail to achieve total pathogen clearance. Different studies show that *T. cruzi* infection triggers regulatory mechanisms able to curb immunity [9–11]. In this sense, we demonstrated previously the capacity of this parasite to modulate *in vitro* and *in vivo* DC function. Experimental murine infection with *T. cruzi* down-regulates the expression of MHC class II and costimulatory molecules as well as impairs T cell stimulatory capacity of splenic DC [12]. *In vitro*, live or heat-killed trypomastigotes plus LPS induce DC with tolerogenic properties that produce high levels of IL-10 with an impaired capacity to induce lymphoproliferation [13,14]. Furthermore, we demonstrated that this enhanced IL-10 production by DC is independent of TLR2 and involves TLR4 through parasite–DC interactions which have not been characterized yet [14]. Noticeably, neutralization of IL-10 produced by these DC partially reverts this parasite-driven functional impairment [13]. IL-10 was initially described as a Th2 T cell clone product that regulated cytokine synthesis and proliferation of Th1 cells. At present, it is known that the anti-inflammatory action of this cytokine is also the result of its direct impact on DC and that production of IL-10 by these cells may limit the induction of antimicrobial effector mechanism [15–17]. Thus, modulation of DC function through the induction of IL-10 appears to be a *T. cruzi* mechanism leading to the stimulation of an immune response unable to eradicate the parasite.

We propose that strategies capable of instructing antigen presenting DC to avoid parasitic stimulation of endogenous IL-10 could improve immunity against *T. cruzi* infection. As a proof of concept, we employed DC-based immunization to study the impact of DC derived IL-10 on the capacity of these cells to induce protection against *T. cruzi* infection. For this, DC from IL-10 deficient (IL-$10^{-/-}$) and WT mice were pulsed *in vitro* with *T. cruzi* antigen before being transferred into WT mice. Next, recipient mice were challenged with *T. cruzi* trypomastigotes to test the efficacy of the response primed by transferred DC.

2. Materials and methods

2.1. Animals

IL-$10^{-/-}$ and WT mice of Balb/c background were obtained from the animal facilities of the Fundación Instituto Leloir (Buenos Aires, Argentina) and C57BL/6J mice from the animal facilities of the Academia Nacional de Medicina (Buenos Aires, Argentina). Mice, 6- to 8-week old and sex-matched within each experiment, were housed in our animal facilities (Departmento de Microbiología, Parasitología e Immunología, Facultad de Medicina, Universidad de Buenos Aires). All procedures requiring animals were performed in agreement with institutional guidelines.

2.2. Parasites and T. cruzi antigen

Bloodstream forms of the RA strain of *T. cruzi* were maintained by weekly intraperitoneal passages through mice. Sterile blood trypomastigotes, obtained by cardiac puncture, were enriched from blood by differential centrifugation ($200 \times g$, 15 min at room temperature) and used to infect Vero cell monolayers in a 10 to 1 parasite to cell ratio. Trypomastigotes of a maximum of 3 passages were obtained from culture supernatants, separated from cell debris ($3000 \times g$, 10 min, 20 °C) and washed twice by centrifugation. A pellet of pure trypomastigotes was obtained by centrifugation ($10{,}000 \times g$, 30 min, 20 °C). *T. cruzi* antigen was prepared by 5 freeze-thawing cycles and sonication (3 cycles of 30-sec at 40 Hz at 0 °C) the trypomastigote pellet and stored at −80 °C until used.

2.3. DC generation and antigen pulsing

Bone marrow derived DC were generated in the presence of recombinant GM-CSF by the method described by Inaba et al. [18] with few modifications [13]. On day 7 of culture, cells displayed a myeloid phenotype (>95% CD11b+) and were highly enriched in DC (>80% CD11c+). Cultures showed a low percentage of cells expressing CD11b+ Gr-1+ (myeloid precursors) and were B220− CD8α−. Cells were harvested by gentle pipetting, washed and plated (10^6 cells/ml) in 24-well plates (Nunc), and pulsed for 24 h in medium containing *T. cruzi* antigen (300 μg/ml).

2.4. Adoptive transfer and T. cruzi infection

Six-week-old female mice ($n = 5$; each group) were injected with 10^6 *T. cruzi* antigen-pulsed or unpulsed IL-$10^{-/-}$ DC and 10^6 *T. cruzi* antigen-pulsed or unpulsed WT DC in 50 μl of PBS, or 50 μl of PBS alone by intravenous route. For challenge of immunized mice, 2 weeks following adoptive transfer, mice were infected with 200 bloodstream trypomastigotes by intraperitoneal route. For infection of IL-$10^{-/-}$ and WT mice, 6-week-old female mice were infected with bloodstream trypomastigotes by intraperitoneal route (IP) or intradermal (ID) route at the left foot hind pad. Parasitemia levels were assessed twice a week by counting in a haemocytometer.

2.5. Immunostaining and flow cytometry

Cells suspensions were stained for surface markers using conventional protocols. Previously optimized amounts of one or more of the following anti-mouse monoclonal antibodies were used: PE- or biotin-labeled CD11c (HL3), FITC-labeled IAd (39-10-8) or biotin-labeled CD80 (16-10A1), CD86 (GL-1), CD40 (3/23), and CD3 (1415c11) (BD Bioscience, San Jose, CA). In the case of biotinylated antibodies, Streptavidin-PerCP (BD Bioscience, San Jose, CA) was used as a second-step reagent. As controls, cells were stained with the corresponding isotype-matched antibodies. Three-color flow cytometry was performed using a FACScalibur flow cytometer (BD Biosciences). Data were analyzed using the WINMDI software (Joseph Trotter, The Scripps Research Institute).

2.6. Cytokine analysis

Cell culture supernatants were collected after 24 h of culture and stored at −80 °C until used. Mouse IL-10, IL-12p70, TNF-alpha, IFN-gamma, TGF-beta and IL-6 levels were assayed by enzyme linked immunosorbent assay (ELISA; R&D Systems, Minneapolis, MN) following manufacturer's protocol.

2.7. Mixed lymphocyte reaction

Pulsed and unpulsed IL-$10^{-/-}$ and WT DC were co-cultured with nylon enriched T cells suspensions (>90% CD3+ cells) prepared from lymph nodes of C57BL/6 H-2^b mice (allogeneic) or Balb/c H-2^d mice (syngeneic) chronically infected with *T. cruzi* (4 months post-infection). Cells were plated at 1/5; 1/10 and 1/20 DC/lymphocyte ratios using 2×10^5 lymphocytes/well in triplicate in flat-bottom 96-well microtiter plates in 200 μl of IMDM for 3 days (for allogeneic) and 5 days (for syngeneic) cultures at 37 °C under 5%CO_2. Cultures were pulsed with 0.25 μCi/well [^{3}H] thymidine (ICN, Costa Mesa, CA) for the last 24 h. Cell proliferation was measured by liquid scintillation counting (Rack Beta Scintillation Counter). The results are expressed as mean counts per minute (cpm) of triplicates.