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Research paper

Excretory-secretory products (ESP) from *Fasciola hepatica* induce tolerogenic properties in myeloid dendritic cells

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ABSTRACT

Fasciola hepatica is a helminth trematode that migrates through the host tissues until reaching bile ducts where it becomes an adult. During its migration the parasite releases different excretory-secretory products (ESP), which are in contact with the immune system. In this study, we focused on the effect of ESP on the maturation and function of murine bone marrow derived-dendritic cells (DC). We found that the treatment of DC with ESP failed to induce a classical maturation of these cells, since ESP alone did not activate DC to produce any cytokines, although they impaired the ability of DC to be activated by TLR ligands and also their capacity to stimulate an allospecific response. In addition, using an *in vitro* ovalbumin peptide-restricted priming assay, ESP-treated DC exhibited a capacity to drive Th2 and regulatory T cell (Treg) polarization of $CD4^+$ cells from DO11.10 transgenic mice. This was characterized by increased IL-4, IL-5, IL-10 and TGF-β production and the expansion of $CD4^+CD25^+Foxp3^+$ cells. Our results support the hypothesis that ESP from *F. hepatica* modulate the maturation and function of DC as part of a generalized immunosuppressive mechanism that involves a bias towards a Th2 response and Treg development.

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1. Introduction

Immature dendritic cells (DC) can endocyte antigens in the periphery and undergo maturation after recognition of the conserved pathogen-associated molecular patterns present in microorganisms through pattern-recognition receptors (PRR) (Steinman and Swanson, 1995). These cells then migrate to lymphoid organs where they prime T cells. The DC activation and maturation processes are conventionally associated with upregulation of the major histocompatibility complex (MHC) class II and co-stimulatory molecule surface expression (Langenkamp et al., 2000). However, not all stimuli induce the same type of DC maturation. The maturation process depends upon the type of microbial stimulus and can result in the differentiation of DC to distinct phenotypic states (de Jong et al., 2002; MacDonald et al., 2001; Sher et al., 2003). Associated with this differential maturation is the acquisition by DC of the capacity to prime polarized Th cell responses. Thus, depending upon the initial maturation signal, DC can prime Th cells to differentiate towards either the Th1, Th2, Th17 or T regulatory (Treg) types (Cools et al., 2007; Manickasingham et al., 2003; Rutella et al., 2006; Sher et al., 2003; Zhu and Paul, 2008). In addition, feedback from Th cells also plays an important role in the phenotypic state of DC and is likely to be critical in determining the characteris-

Abbreviations: CD40L, CD40 ligand; DC, dendritic cells; ESP, *Fasciola hepatica* excretory-secretory products; MFI, Mean fluorescent intensity; MLR, mixed lymphocyte reaction; nTreg, naturally regulatory T cells; OVA, ovalbumin; PRR, pattern-recognition receptors; Teg, tegumental antigen from *F. hepatica*; Tg, transgenic; TLR, toll like receptor; Treg, regulatory T cells.

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tics of the ensuing immune response (Rutella et al., 2006). Although some of the factors that promote Th1 development are well defined, those that promote Th2 responses or Treg development are less well understood. In this respect, in the sensing of pathogens such as viruses, bacteria, fungi and protozoa by DC, the TLR ligation induces phenotype maturation with increased expression of co-stimulatory molecules and secretion of pro-inflammatory cytokines that together promote a Th1 response (Kane et al., 2004; Roses et al., 2008; Sun et al., 2005; Zhu and Paul, 2008). In contrast, chronic diseases caused by helminth infections, such as filariasis and schistosomiasis, are generally associated with strong Th2-type responses (Maizels and Yazdanbakhsh, 2003). The interaction of filarial nematode or schistosomal Ags with DC does not induce conventional maturation in these cells (Goodridge et al., 2005), drives the differentiation of Th cell responses towards the Th2 pole (Kane et al., 2004; Whelan et al., 2000), and leads to the development of Treg cells (McKee and Pearce, 2004; Segura et al., 2007). Thus, the long-term survival of helminth parasites in the host is associated with the development of the Th2 response, which prevents the protective Th1 response and controls excessive inflammation that could be dangerous to the host (McKee and Pearce, 2004).

Fasciolosis, caused by the liver fluke *Fasciola hepatica*, is a chronic infection that affects >600 million animals, such as cattle, sheep and goats, in the world and causes economical losses estimated at >UD$ 2000 million per year by reducing the production of milk, wool and meat, especially in cattle and sheep (McManus and Dalton, 2006). In addition, fasciolosis is now an emerging disease in humans, with an increasing number of reported cases, particularly in certain regions of the world where the prevalence is very high (100% in some areas such as the high altitudes in the Andean rural regions) (McManus and Dalton, 2006). During its life cycle, *F. hepatica* migrates across the body cavity, penetrates host tissues and settles in the bile ducts, where the flukes can then live for many years (McManus and Dalton, 2006). During the migration, the rapid turnover of the outer glycocalix prevents an attack from granulocytes, due to the antibodies being unable to bind to the surface of the parasite (Hanna, 1980). The glycoproteins released by this parasite are called excretory-secretory products (ESP), which contain antioxidants which are secreted by the shedding of tegument as well as proteases from the caecal content that allow the digestion of tissue proteins such as collagen and fibronectin (Berasain et al., 1997; Robinson et al., 2009). The long survival time of *F. hepatica* in the liver suggests that the parasite induces an immunosuppressive effect in order to prevent the innate immune response, and consequently suppresses the effective prime of an adaptive immune response. Because the interaction between ESP and the immune system occurs not only during larvae migration, but also in bile ducts (McManus and Dalton, 2006), ESP could be critical molecules involved in the sensing of *F. hepatica* by the immune system. Related to this, we and others have demonstrated the important role of ESP in the induction of immunoevasion mechanisms (Cervi et al., 1998, 1999; Zhang et al., 2005). It has been reported that ESP are able to down modulate the proliferation of spleen mononuclear cells (Cervi and Masih, 1997; Cervi et al., 1996). In addition, ESP induce a population of spleen mononuclear cells able to suppress the delayed type hypersensitivity response to parasite antigens as well as to non-related antigens (Cervi et al., 1996). Moreover, ESP can inhibit nitric oxide (NO) production and the phagocytic activity of peritoneal cells (Cervi et al., 1998, 1999) and also induce alternative activation of bovine macrophages (Flynn and Mulcahy, 2008).

In this paper we focus on studying the effect of ESP on the maturation and functionality of murine bone marrow derived DC. We found that ESP from *F. hepatica* modulate DC maturation inducing a phenotype able to promote the development of Th2 and Treg cells.

2. Materials and methods

2.1. Animals and ESP preparation

Six- to 8-week-old inbred female BALB/c and C57BL/6 were purchased from Ezeiza Atomic Center (CNEA, Buenos Aires, Argentina). OVA TCR-transgenic DO11.10 mice were kindly provided by Dr. Oscar Campetella at the Biotechnological Research Institute, National University of San Martín, Buenos Aires, Argentina. The Institutional Experimentation Animal Committee (authorization no. 15-01-44195) approved animal handling and experimental procedures. ESP was prepared as described previously with slight modifications (Cervi et al., 1996). Adult worms were removed from infected bovine livers and cultured in PBS (1 worm/2 ml of PBS) at 37 °C for 2 h. The supernatant was collected, centrifuged at 10,000 × *g* for 30 min at 4 °C and then concentrated using a high-flow YM 10 membrane filter (Millipore, Billerica, MA). Endotoxin levels in ESP preparation were <90 UE/ml and were detected using the Limulus amebocyte lysate test (EndosafeTimes, Charles River, laboratories,Wilmington, Delaware). In some experiments, proteinase inhibitors (2 mM PMSF, 5 mM EDTA, Sigma–Aldrich, St. Louis, MO) and 10 μg/ml polimixin B (Sigma–Aldrich) were added to the supernatant before centrifugation. Protein concentration was quantified by Bio-Rad Protein assay (CA, USA). The protein concentration of ESP varied from 500 to 700 μg/ml.

2.2. DC generation and stimulation

DC were generated as previously described (MacDonald et al., 2001), with slight modifications. Briefly, bone marrow was collected from femurs of mice and the cells were seeded into bacteriological Petri dishes at 2×10^5/ml in 10 ml of complete medium supplemented with 2 mM L-glutamine (Life Technologies, Gaithersburg, MD), 1% of supernatant from GM-CSF producing J558 cells (20 ng/ml final concentration), 10% heat-inactivated endotoxin free and filtered fetal calf serum (Gibco, Invitrogen), 50 μM 2-mercaptoethanol (Sigma–Aldrich) and 50 μg/ml gentamicin (Gibco). At day 3, a further 10 ml of medium containing GM-CSF was added. At day 6, 10 ml culture supernatant was removed and replaced with 10 ml fresh culture medium containing GM-CSF. At day 8, plates were fed as day 6, but only 25% of GM-CSF was added in fresh media (5 ng/ml final concentration), and cells were har-

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