Induction of Protective Immunity against Experimental *Eimeria tenella* Infection using Serum Exosomes

Emilio del Cacho^{a,*}, Margarita Gallego^a, Hyun Soon Lillehoj^b, Joaquin Quilez^a,
Erik P. Lillehoj^c, Caridad Sánchez-Acedo^a

^aDepartment of Animal Pathology, Faculty of Veterinary Sciences, University of Zaragoza, Zaragoza, Spain

^bAnimal Parasitic Diseases Laboratory, Animal and Natural Resources Institute, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, MD, 20705, USA

^cDepartment of Pediatrics, University of Maryland School of Medicine, Baltimore, MD 21201, USA

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*Corresponding author: Parasitología y Enfermedades Parasitarias, Facultad de Veterinaria, Miguel Servet 177, 50013-Zaragoza, Spain

Tel.: +34 976 7615 56; Fax: +34 976 76 16 12

E-mail: edelcach@unizar.es (E. del Cacho)

Abstract

Avian coccidiosis is caused by *Eimeria*, a unicellular, apicomplexan protist which primarily infects intestinal epithelia resulting in nutrition malabsorption and reduced growth of commercial poultry. Vaccination of chickens with exosomes isolated from antigen presenting cells and containing parasite antigens (Ags) represents a promising alternative strategy to control avian coccidiosis, but is restricted in its commercial application due to limitations on production scale-up for mass immunization programs. Here, we report the biochemical and physiologic characteristics of exosomes derived from serum of *E. tenella*-infected chickens and their feasibility for inducing protective immunity to experimental coccidiosis. Exosomes isolated from the serum of *E. tenella*-infected chickens contained a subset of protein Ags found in the intact parasite. Serum-derived exosomes containing these *E. tenella* Ags localized to the intestine and spleen following intramuscular injection into naïve chickens. *In vitro* ELISPOT assays revealed increased numbers of IL-2-, IL-4-, IL-6-, and IFN- γ -secreting cells in the intestine and spleen of exosome-administered chickens, compared with vehicle controls. Pre-immunization of chickens with serum exosomes from *E. tenella*-infected chickens increased both body weight gain and feed conversion efficiency, and reduced both fecal parasite shedding and gut lesion scores following parasite infection, compared with vehicle controls. Finally, immunization with CD80⁺ serum exosomes stimulated greater numbers of cytokine-producing cells, and higher levels of protective immunity to *E. tenella* infection, compared with CD80⁻ exosomes. These results suggest the possibility of producing an effective, parasite-free vaccine against avian coccidiosis under field conditions using serum-derived CD80⁺ exosomes containing parasite Ags.

Introduction

Eimeria is the etiologic agent of avian coccidiosis, a major parasitic disease of poultry that is indigenous to most regions of the world (Dalloul and Lillehoj, 2006). Infection by coccidial parasites has an enormous impact on worldwide poultry production due to the morbidity, mortality, and reduced body weight gain that the infection produces. Conventional disease control methods have relied on prophylactic administration of drugs with anticoccidial activity. However, alternative methods of disease mitigation are needed due to increasing government restrictions on the use of coccidiostats, the high costs of new drug development, and the emergence of drug-resistant parasites (Landman and Peek, 2005). Vaccination with live attenuated parasites is a practical alternative to drugs for coccidiosis control. However, vaccination is limited by the lack of cross-protection against *Eimeria* species absent in the vaccine formulation. Consequently, there is an urgent need to develop a field vaccine against avian coccidiosis that is safe and effective against all relevant parasites.

Exosomes are cell-derived, phospholipid-enclosed vesicles less than 100 nm in diameter released into the extracellular milieu that can be taken up by recipient cells at sites distal to their release. Exosomes from different cellular sources contain proteins specific for their cell type of origin, and these proteins, as well as those of their target cells, mediate exosome uptake (Théry et al., 2002). Exosomes secreted from antigen-presenting cells (APCs) play a functional role in mediating innate and adaptive immune responses to microbial pathogens (Moll, 2003), and recent reports suggest that such an exosome-based approach may be feasible for immunization against infections by *Toxoplasma gondii* (Beauvillain et al., 2007), *Leishmania major* (Schnitzer et al., 2010), *Mycobacterium tuberculosis* (Bhatnagar et al., 2007), and *Salmonella enterica* serovar Typhimurium

(Bhatnagar et al., 2007). Dendritic cell (DC)-derived exosomes expressing major histocompatibility complex class I (MHC-I) and MHC-II molecules induce and enhance antigen (Ag)-specific T cell responses *in vitro* and *in vivo* (Denzer et al., 2000). DC-derived exosomes also express on their surface co-stimulatory molecules, such as CD80, necessary for Ag presentation (Clayton et al., 2001; Lamparski et al., 2002; Viaud et al., 2010).

Our previous work demonstrated a potent T cell-dependent protective immune response against *Eimeria tenella*, *E. maxima*, and *E. acervulina* infection of chickens following *in vivo* administration of exosomes isolated from DCs of the intestinal cecal tonsils and pulsed *in vitro* with soluble *Eimeria* Ags (del Cacho et al., 2012). Current technologies, however, limit the practicality of isolating chicken DCs exosomes on a scale sufficient for mass vaccination against avian coccidiosis in the field. Alternatively, exosomes from serum might constitute a more convenient source for such an application. It is well known that exosomes not only act locally, as it is the case in cecal tonsils during coccidiosis, but also circulate systemically through the vasculature and lymphoid systems (Caby et al., 2005). Highly purified exosomes can be isolated from serum with high efficiency and reproducibility, and relatively nominal cost compared with cellular sources. Therefore, this study was designed to investigate the feasibility of isolating exosomes from serum of chickens infected with *E. tenella* for conferring protective immunity against experimental avian coccidiosis.

Materials and Methods

Animals. Cobb 500 broilers (Grupo Sada, Zaragoza, Spain) were hatched and reared under *Eimeria*-free conditions, with access to feed and water provided *ad libitum*. All

experiments were performed in accordance with the guidelines approved by the University of Zaragoza Institutional Animal Care and Use Committee.

Parasites. The Houghton strain of *E. tenella* was kindly provided by Dr. R. Marshall (Veterinary Laboratories Agency, Weybridge, UK). Oocysts were propagated, isolated, and sporulated using standard procedures (Raether et al., 1991). Sporulated oocysts stored less than 4 weeks were used for infection.

Purification of CD80⁺ and CD80⁻ exosomes. Fifty chickens received 3 successive oral doses of 1.5×10^4 *E. tenella* oocysts each, given on days 10, 20, and 30 after hatching. At 10 days following the third inoculation, blood was collected from the brachial vein of each chicken, and serum was separated from clotted blood and pooled. Exosomes were isolated using the Total Exosome Isolation Reagent (from serum) (Life Technologies, Carlsbad, CA) according to the manufacturer's guidelines. CD80⁺ and CD80⁻ exosomes were separated using magnetic beads coated with the anti-chicken CD80 antibody (Ab) (Life Technologies) as described (Lee et al., 2011).

Western blot analysis. Exosomes were sonicated and centrifuged at 100,000 x g for 20 min, the pellets were dissolved in reducing SDS-PAGE sample buffer, and proteins were resolved on a discontinuous gel system using standard protocols (Laemmli, 1970). Proteins were electrophoretically transferred to nitrocellulose at room temperature for 18 h at 40 mV. The membrane was blocked in PBS containing 4% non-fat dry milk and incubated with rabbit anti-*E. tenella* polyclonal Ab (1:100) (del Cacho et al., 2009) or mouse anti-chicken CD80 monoclonal Ab (1:100) (Lee et al., 2011) for 1 h at room temperature. Immunoblots were

developed with peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG secondary Abs and diaminobenzidine substrate.

Exosome immunization. One-day-old chickens (n = 224) were distributed into 4 groups of 56 chickens each (7 cages/group, 8 birds/cage) based on body weight. Treatment groups were randomly assigned using Microsoft Excel 2007. At 7 d of age, chickens in groups 1 and 2 were injected intramuscularly (i.m.) with 100 μ l of sterile PBS alone, and chickens in groups 3 and 4 were injected i.m. with 10 μ g/bird of CD80⁺ or CD80⁻ serum exosomes from *E. tenella*-infected birds in 100 μ l of sterile PBS, respectively.

***Eimeria* Ag immunofluorescence in tissue sections.** At 8 d post-immunization with exosomes, 6 chickens from each of the 4 treatment groups were euthanatized, and cecal tonsils (CT) and spleens were harvested and snap frozen in liquid nitrogen. Cryostat tissue sections were blocked with normal horse serum for 10 min, followed by sequential incubations with rabbit anti-*E. tenella* Ab (del Cacho et al., 2009) (1:100) for 18 h at 4°C and Alexa Fluor 350-conjugated goat anti-rabbit IgG secondary Ab for 30 min (Molecular Probes, Eugene, OR). Nuclei were stained with mouse anti-DNA Ab (Santa Cruz Biotechnology, CA) followed by fluorescein-conjugated goat anti-mouse IgG secondary Ab for 30 min (Vector Laboratories, Burlingame, CA). As a negative control, tissue sections were stained with normal rabbit serum.

ELISPOT quantification of cytokine-secreting cells. CT and spleens were harvested at 8 d post-immunization with exosomes, pressed through a 250 μ m mesh screen, and washed in GKN buffer (1.0 mg/ml glucose, 0.4 mg/ml KCl, 8.0 mg/ml NaCl, 0.35 mg/ml NaHCO₃) containing 5.0 mM EDTA. Single cell suspensions were obtained by filtering

through a 70 µm cell strainer (BD Falcon, Franklin Lakes, NJ) and were purified from dead cells, erythrocytes, and epithelial cells by Percoll density gradient centrifugation (1.075 g/ml, high density; Amersham Pharmacia Biotech, Uppsala, Sweden) at 950 x g for 20 min. Since Cobb chickens are allogenic, quantification of cytokine-producing cells was performed by culturing 1.0×10^5 cells from individual chicken in the absence or presence of 100 µg/ml of *E. tenella* Ag in 96-well microplates with 0.2 ml of RPMI 1640 medium for 3 d (del Cacho et al., 2011). For ELISPOT assays, microtiter plates were coated overnight at 4°C anti-chicken IL-2, IL-4, IL-6, or IFN-γ Abs, blocked with 10 µg/ml of PBS containing 2% bovine serum albumin and 0.05% Tween 20 (PBS/BSA/T) at room temperature for 2 h, and washed 3 times with PBS. The cells were added to Ab-coated microplates and ELISPOT assays were performed as described (del Cacho et al., 2011).

***In vivo* protection against *E. tenella* infection.** At 8 d post-immunization with CD80⁺ or CD80⁻ serum exosomes from *E. tenella*-infected birds (groups 3 and 4), or PBS control (groups 1 and 2), chickens in groups 2, 3 and 4 were infected by oral gavage with a single dose of 1.0×10^4 sporulated oocysts of *E. tenella* and chickens in group 1 were given PBS alone. Body weight gains and feed conversion ratios were measured at 0 and 10 d post-infection as described (del Cacho et al., 2009) (Fig. 1). Fecal oocyst shedding was measured between 5 and 10 d post-infection as described (Lee et al., 2007). At 6 d post-infection, intestinal lesion scores were determined on a graded scale from 0 (none) to 4 (high) in a blinded fashion by 2 independent observers as described (Johnson and Reid, 1970). The percent mortality in all groups was recorded at 10 d post-infection.

Statistical analysis. All data were expressed as mean ± standard deviation (SD) values. Duncan's multiple-range test was used to evaluate the differences between treatment

groups. Differences between mean values were considered statistically significant at $P < 0.05$.

Results

CD80 and *Eimeria* Ag expression by serum exosomes. Immunoblot analysis of total proteins from CD80⁺ exosomes using an anti-chicken CD80 Ab revealed a band with an apparent molecular weight of 35.0 kDa, identical to that reported for chicken CD80 (Lee et al., 2011) (Fig. 2). A similar protein band was not detected using CD80⁻ exosomes (Fig. 2). Immunoblot analysis using an anti-*E. tenella* rabbit Ab revealed an identical pattern of 3 protein bands with apparent molecular weights between 42.0 and 55.0 kDa in both CD80⁺ and CD80⁻ serum exosomes isolated from *E. tenella*-infected chickens (Fig. 3) However, not all of the protein bands identified in *E. tenella* sporozoites were seen in either of the exosomes (Fig. 3).

Immunofluorescence localization of *E. tenella* Ags in tissue sections. *E. tenella* Ags in cells morphologically resembling follicular dendritic cells (FDCs) in the intestinal CT and spleen were seen in chickens immunized with CD80⁺ serum exosomes from *E. tenella*-infected chickens by immunofluorescence microscopy (Figs. 4A, 4C). Immunization of chickens with CD80⁻ exosomes did not reveal Ag-containing cells in the CT or spleen (Figs. 4B, 4D).

Ag-stimulated cytokine-producing cells. Immunization of chickens with CD80⁺ or CD80⁻ serum exosomes increased the number of IL-2-, IL-4, IL-6-, and IFN- γ -producing cells in the CT and spleen compared with unimmunized chickens (Fig. 5). While the numbers of IL4- and IL6-producing cells were identical following immunization with either CD80⁺ or

CD80⁻ exosomes, chickens immunized with CD80⁺ exosomes had increased numbers of IL-2- and IFN- γ -producing cells compared with birds immunized with CD80⁻ exosomes.

***In vivo* protection against *E. tenella* infection.** Chickens immunized with CD80⁺ (group 3) or CD80⁻ (group 4) serum exosomes isolated from *E. tenella*-infected chickens and subsequently infected with *E. tenella* had increased body weight gains, reduced feed conversion ratios, diminished fecal oocyst shedding, and decreased intestinal lesion scores, compared with the unimmunized and infected controls (group 2) (Table I). Additionally, CD80⁺ group 3 chickens had greater body weight gains as well as decreased feed conversion ratios, fecal oocyst shedding, and intestinal lesion scores, compared with CD80⁻ group 4 animals. These results indicate that while immunization with either type of exosomes induces a protective immune response against *E. tenella* infection, a more robust immunity was generated against the parasite following immunization with CD80⁺ exosomes, compared with CD80⁻ exosomes.

Discussion

This study demonstrates that serum-derived exosomes from *E. tenella*-infected chickens express a subset of protein antigens present in the intact parasite, and that CD80⁺, but not CD80⁻, exosomes localize to the intestinal CT and spleen following i.m. injection into naïve chickens. Immunization of chickens with serum exosomes increased the numbers of IL-2-, IL-4, IL-6-, and IFN- γ -producing cells in the CT and spleen in response to *E. tenella* Ag exposure, compared with unimmunized chickens. Finally, CD80⁺ serum exosomes induced greater numbers of IL-2- and IFN- γ -secreting CT and spleen cells, and stimulated greater protective immunity to *E. tenella* challenge infection, compared with CD80⁻ exosomes. Our previous studies demonstrated successful vaccination against avian coccidiosis using DC-

derived exosomes incubated with *Eimeria* Ags (del Cacho et al., 2012). The current study now extends this vaccination strategy to serum exosomes, which when compared with cell-derived exosomes, potentially offers the advantages of reduced vaccine production costs and decreased manufacturing complexity.

Exosomes were initially described as a relatively homogeneous population of vesicles with diameters of 40-90 nm by electron microscopy (Raposo et al., 1996). Exosomes have been extensively studied for their contributions to physiology, immunity, cancer, and intercellular communication, revealing an impressive diversity of functions depending on their cellular source. Ultrastructural, Western blot, and mass spectrometry analyses have identified distinct subsets of exosomal proteins between different cell types (Graner et al., 2009; Ataya et al., 2011), which may explain their functional diversity. The current study demonstrated both CD80⁺ and CD80⁻ exosomes in the serum of chickens infected with *E. tenella*. Mammalian CD80 has been reported to be a specific marker of APC-derived exosomes (Raposo et al., 1996; Zitvogel et al., 1998; Clayton et al., 2001). The majority of CD80⁻ exosomes appear to be released from cells which do not have a role in the immune response (Ataya et al., 2011; Heijnen et al., 1999; Zhang and Grizzle, 2014). However, CD80⁻ exosomes released from the basolateral membrane of intestinal epithelial cells reportedly function in Ag transport, leading to either the induction of tolerance under noninflammatory conditions (Van Niel et al., 2001) or Ag-specific immunity under inflammatory conditions (Van Niel et al., 2003). Therefore, similar to CD80⁺ exosomes, some CD80⁻ exosomes are capable of transferring foreign Ags to intestinal DCs (Andre et al., 2004; Van Niel et al., 2003) for presentation to mucosal T lymphocytes to activate local and systemic immune responses (Skokos et al., 2003; Morelli et al., 2015). Considering the high degree of tissue tropism of *E. tenella* for the intestinal caecum of chickens, our results suggest that CD80⁻

exosomes containing parasite Ags are released from infected cecal epithelial cells and contribute, at least in part, to the host immune response against parasite infection.

Naïve chickens immunized with either CD80⁺ or CD80⁻ serum exosomes had increased numbers of intestinal and spleen cells secreting Th1 (IL-2, IL-6, IFN- γ) and Th2 (IL-4) cytokines, compared with unimmunized controls. However, compared with chickens immunized with CD80⁻ exosomes, CD80-immunized birds had (1) increased numbers of IL-2- and IFN- γ -producing gut and spleen cells, (2) greater localization in the CT and spleen, and (3) greater protective immunity following *E. tenella* infection, as measured by weight gain, feed efficiency, parasite shedding, and intestinal lesions. Given that localization of foreign Ags in FDCs has been associated with the induction of immunological memory (Szakal et al., 1989; Gallego et al., 1995; Banchereau and Steinman, 1998), taken together these findings suggest that immunization of chickens with CD80⁺ exosomes containing parasite Ags not only may be more efficient in conferring protection against parasite infection, but also may extend immunological memory to subsequent re-infection, compared with CD80⁻ exosomes.

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Figure Legends

Fig. 1. Schematic illustration of the experimental design. Chickens immunized with CD80⁺ or CD80⁻ serum-derived exosomes were used to assess *E. tenella* Ag localization and Ag-specific immune response in the absence of parasite infection. Exosome-immunized and *E. tenella*-infected chickens were used to measure *in vivo* parameters of protection against *E. tenella* infection.

Fig. 2. Immunoblot analysis of serum-derived exosomes using anti-chicken CD80 Ab. Lane 1, prestained molecular weight marker proteins. Lane 2, proteins extracted from CD80⁺ serum exosomes. Lane 3, proteins extracted from CD80⁻ serum exosomes. Lane 4, proteins extracted from chicken intestinal DCs. Lane 5, proteins extracted from *E. tenella* sporozoites. Note the presence (lane 2) or absence (lane 3) of the 35.0 kDa CD80 protein in serum exosomes isolated from chickens infected with three successive oral doses of 1.5×10^4 *E. tenella* oocysts that co-migrates with a similar protein in DCs (lane 4).

Fig. 3. Immunoblot analysis of serum-derived exosomes using rabbit anti-*E. tenella* Ab. Lane 1, prestained molecular weight marker proteins. Lane 2, proteins extracted from CD80⁺ serum exosomes. Lane 3, proteins extracted from CD80⁻ serum exosomes. Lane 4, proteins extracted from chicken intestinal DCs. Lane 5, proteins extracted from *E. tenella* sporozoites. Note the presence of *E. tenella* Ags in CD80⁺ (lane 2) and CD80⁻ (lane 3) serum exosomes isolated from chickens infected with three successive oral doses of 1.5×10^4 *E. tenella* oocysts that co-migrate with similar proteins in *E. tenella* sporozoites (lane 5).

Fig. 4. Immunofluorescence analysis of *E. tenella* Ag-binding cells in CT and spleen of *E. tenella*-immunized chickens. CT (A, B) and spleen (C, D) tissue sections were stained with

rabbit anti-*E. tenella* Ab revealing parasite Ag-binding cells (blue) in sections from chickens immunized with CD80⁺ serum exosomes (A, C) or CD80⁻ serum exosomes (B, D). Note that only tissues from chickens immunized with CD80⁺ serum exosomes display Ag-binding cells (arrows) within germinal centers where the labelled cells form a dense network distributed within the entire germinal center.

Fig. 5. Cytokine secreting cells in nonimmunized chickens, or chickens immunized with CD80⁻ or CD80⁺ serum exosomes isolated from *E. tenella*-infected chickens. *Eimeria* Ag-stimulated IL-2-, IL-4-, IL-6-, and IFN- γ -secreting cells in the spleen (open bars) and CT (closed bars) were quantified by ELISPOT assay at 8 days following exosome immunization. Each bar represents the mean $\pm$ SD value. Within each graph, bars with different letters are significantly different ($P < 0.05$) according to Duncan's multiple range test.

TABLE 1. Effect of immunization with serum exosomes on *in vivo* parameters of protective immunity against *Eimeria tenella*.

Treatment Group	Body wt gain (g/chicken)	Feed conversion ratio (g feed/g chicken)	Fecal oocyst output $\times 10^3$ per chicken	Lesion score (scale of 0–4)	Mortality (%)
Unimmunized, Uninfected	653 $\pm$ 12 ^a	1.41 $\pm$ 0.08 ^a	0 ^a	0 ^a	0 ^a
Unimmunized, Infected	371 $\pm$ 25 ^b	2.158 $\pm$ 0.12 ^b	15,739 $\pm$ 0.68 ^b	2.7 $\pm$ 0.6 ^b	16 $\pm$ 5.6 ^b
Immunized with CD80 ⁺ exosomes, Infected	649 $\pm$ 18 ^a	1.45 $\pm$ 0.09 ^a	302 $\pm$ 0.71 ^c	0.9 $\pm$ 0.6 ^c	0 ^a
Immunized with CD80 ⁻ exosomes, Infected	523 $\pm$ 20 ^c	1.63 $\pm$ 0.09 ^c	7,890 $\pm$ 0.87 ^d	1.5 $\pm$ 0.6 ^d	0 ^a

Each value represents the mean $\pm$ SD of seven cages per each treatment group (8 birds/cage). Within each group, values with different superscripts are significantly different ($p < 0.05$) according to the Duncan’s multiple range test. Lesion score ranges from 1 (none) to 4 (high) according to Jonson and Reid (1970).

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5