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***Giardia duodenalis*: Dendritic cell defects in IL-6 deficient mice contribute to susceptibility to intestinal infection**

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Abstract

Interleukin (IL)-6 is important in numerous infections. IL-6 can promote T cell survival and differentiation toward Th17 cells, as well as B cell proliferation and differentiation to plasma cells. *Giardia duodenalis* is a protozoan parasite that replicates in the lumen of the small intestine in humans and many other mammals resulting in diarrhea, cramps and developmental delays in children. IL-6 is required for control of this infection, but it is unclear what its role is or which cells are required to produce this cytokine to generate efficient immunity. We have analyzed infections in a series of chimeric mice in which specific cell types lacked the ability to produce IL-6 in order to determine which sources of IL-6 played an important role in controlling this infection. Analysis of bone marrow chimeras indicate that radiation-sensitive, bone-marrow derived cells must produce IL-6. T cell chimeras show that T cell production of IL-6 is not required. Finally, by transferring dendritic cells from wild-type mice into IL-6 deficient recipients, we show that dendritic cell defects are responsible for the inability of IL-6 deficient mice to respond to *Giardia* challenge.

Keywords

MUCOSAL IMMUNITY; DENDRITIC CELLS; INTERLEUKIN-6; *Giardia*

1. Introduction

Infection with *Giardia duodenalis* (syn.: *G. lamblia*, *G. intestinalis*) is the most common parasitic cause of diarrhea in humans in the developed world. It is also a significant contributor to diarrheal disease in the developing world where >90% of children have their first *Giardia* infection before the age of three (Adam, 2001). Infections can cause, diarrhea, cramps, nausea and nutrient malabsorption and chronic and repeated infections in children leads to physical and cognitive developmental defects (reviewed in Cotton et al., 2011; Faubert, 2000). While rapid reinfection following drug treatment has been noted in children in the developing world (Gilman et al., 1988), studies of *Giardia* outbreaks in the developed world suggest that adults previously infected with this parasite can develop resistance to subsequent infections (Isaac-Renton et al., 1994; Istre et al., 1984), suggesting that vaccine development should be possible. However, no vaccine is currently available for human use.

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Previous work using mouse models to determine the role of various cytokines in immunity to *Giardia* has shown that IL-6 plays a critical role in control of primary infections with this parasite (Bienz et al., 2003; Zhou et al., 2003). Mice lacking IL-6 had higher parasite burdens than wild-type controls and the infections required ~8 weeks to be eliminated compared to 1–2 weeks in control mice. Both wild-type and control mice produced strong IgA responses against the parasite following infection. While levels of IL-4 mRNA in the mesenteric lymph nodes were significantly increased following infections in IL-6 deficient mice, IFN mRNA levels were significantly depressed compared to infected wild-type mice. The role of IL-6 in *Giardia* immunity, however, has remained enigmatic. We and others have identified many possible sources for IL-6 including mast cells, intestinal epithelial cells and T cells (Li et al., 2004; Matowicka-Karna et al., 2009; von Allmen et al., 2006 and unpublished data).

IL-6 has been shown to have numerous roles in immune responses, including enhancing production of antibodies (especially IgA, Muraguchi et al., 1988), directing T cell development away from Treg toward Th17 cells (Kimura et al., 2007; Weaver et al., 2006) and in the development of dendritic cells (Bleier et al., 2004; Park et al., 2004). IL-6 deficient DCs have been shown to be functionally different from IL-6 competent DCs, both in their maturation state and their ability to activate T-cells. (Bleier et al., 2004; Park et al., 2004). In this study we have used several different forms of chimeric mice in order to better understand the role of IL-6 in giardiasis. Our results suggest that the importance of IL-6 in this system is related to dendritic cell function.

2. Materials and Methods

2.1 Mice

C57BL/6, IL-6 deficient, and Tcr β deficient female mice were all obtained from Jackson Laboratories (Bar Harbor, ME) and housed either at the NIH or Georgetown University animal facilities. All mouse experiments were approved by the NIH or Georgetown University Animal Care and Use Committees.

2.2 Parasites and extracts

The GS(M)/H7 clone of *Giardia lamblia* (Aggarwal and Nash, 1988) was propagated in vitro in Keister's modified TYI-S-33 medium (Keister, 1983). Parasites were harvested by chilling on ice, collected by centrifugation and washed three times with endotoxin-free phosphate buffered saline (PBS) (Invitrogen corporation, Grand Island, NY). Extract was made by three freeze-thaw cycles, and stored in aliquots at -70°C . Total protein concentration was measured by converting absorbance, with $A_{280} = 1$, defined as 1 mg/ml.

2.3 Reagents

Recombinant murine GM-CSF was purchased from PeproTech, Rocky Hill, NJ. Allophycocyanin (APC)-conjugated antibody against murine CD11c was obtained from BD-Pharmingen (San Diego, CA).

2.4 Chimeric mice

2.4.1 Bone marrow transfers—Eight week old female mice were given 950R whole body irradiation from a cesium-137 source. The next day, these mice were given 10 million bone marrow cells prepared from the femurs and tibias of female mice by i.v. injection. Mice were maintained on sterilized food and water supplemented with 1.4 mg/ml neomycin to reduce the chances of infection. *Giardia* infections were performed 8 weeks following bone marrow injection.

2.4.2 Spleen cell transfers—Spleens were removed from female mice and single cell suspensions were prepared. Recipients received 10 million cells by i.v. injection one day before infection with *Giardia*.

2.4.3 Dendritic cell transfers—DCs were generated from mouse bone marrow following a previously described protocol (Lutz et al., 1999). Briefly, bone marrow from the femur and tibia of mice were harvested in DC culture media (RPMI 1640, 10% fetal calf serum (FCS), 50 M 2-mercaptoethanol, 100 U/mL penicillin, 100 g/mL streptomycin and 20 ng/mL GM-CSF), and grown in 100 mm petri dishes. Four million cells were seeded per dish in a total initial volume of 10 mL. Cultures were maintained in CO₂ 5% atmosphere incubators at 37 °C. Fresh media with 20 ng/ml GM-CSF was supplemented on day 3, while half of the culture media was replaced with fresh media and GM-CSF on days 6 and 8. Non-adherent cells were harvested on day 10 of culture and used as DCs. Cells were typically 80–90% CD11c⁺ by flow cytometry (data not shown). Cells were washed twice with sterile PBS before resuspending in fresh media at a concentration of 5×10^6 cells/ml. 100 l of this preparation (5×10^5 cells/ml), or media alone was administered intraperitoneally or intravenously to each mouse.

2.5 Infections

Mice were infected by gavage with 0.5–1.0 million trophozoites in 0.1 ml sterile PBS. Infected mice were euthanized and ~10 cm of their proximal small intestines were removed and placed in 2 ml of PBS on ice. The small intestines were minced, and after 30 minutes on ice, parasites were either counted with a hemocytometer or examined microscopically for the presence or absence of trophozoites. At least four separate grids on the hemocytometer were counted for each mouse, and mice with no detectable parasites were considered to have cleared the infections. Since a single parasite on one grid corresponds to 10^4 trophozoites/ml, the limit of detection was 5×10^3 trophozoites/mouse.

2.6 Statistics

A comparison of the numbers of parasites in mice between the groups was done by one tailed Mann-Whitney test using GraphPad Prism version 3.00 for Windows, GraphPad Software, San Diego California USA.

3. Results and Discussion

3.1 Bone marrow derived cells are an important source of IL-6

IL-6 can be produced by numerous cell types in the body, including those derived from bone-marrow as well as stromal cells. Our initial characterization of the source of IL-6 was therefore to produce reciprocal bone marrow chimeras using IL-6 deficient and wild-type mice. As seen in Table 1, all chimeras produced using wild-type bone-marrow had no detectable parasites at different time points after challenge with *Giardia*. In contrast, mice produced using IL-6 deficient bone marrow exhibited high numbers of parasites in the small intestine after *Giardia* challenge, even four weeks post-infection. Thus, IL-6 production by bone-marrow derived cells was more important than IL-6 produced by radiation-resistant stromal cells in control of *Giardia* infection.

3.2 T cell production of IL-6 is not necessary

Since CD4⁺ T cells had been shown to be essential for control of *Giardia* (Heyworth et al., 1987; Singer and Nash, 2000), we next examined whether these cells were a necessary source of IL-6. In this case, chimeric mice were produced by adoptive transfer of spleen cells from wild-type or IL-6 deficient donors into T cell deficient recipients. Table 2 shows

that mice receiving T cells from either wild-type mice or IL-6 deficient mice were able to clear *Giardia* infections. In contrast, T cell deficient recipients that received no spleen cells, or spleen cells from T cell deficient donors could not control the infection. This indicates that T cell production of IL-6 is not necessary for control of infection and suggests that another cell type is likely to be important.

3.3 *Giardia*-pulsed DCs confer protection against *Giardia* infection

We hypothesized that DCs might be an important source of IL-6 since this cytokine has been shown to influence T cell subset development (Weaver et al., 2006) and since IL-4 and IFN production are skewed in IL-6 deficient mice infected with *Giardia* (Bienz et al., 2003). We therefore sought to establish a method for testing the role of DCs. We chose a DC vaccination strategy, similar to what has been used for cancer models as well as some infectious diseases (Draube et al., 2011; Remer et al., 2007; Su et al., 1998). To determine if adoptive transfer of DCs can confer protection against *Giardia* infection, we administered DCs pulsed with 1.0mg/ml of *Giardia* extract (5×10^5 cells/mouse in 100 l media), an equal volume of media, an equal number of unpulsed DCs or an equivalent amount of *Giardia* extract into the peritoneal cavity of mice. Fourteen days later, we orally infected each mouse with 10^6 live parasites by gavage, and then euthanized them 5 days after the infection. Parasite numbers in the small intestine were counted showing that on average there was a 70% reduction ($p < 0.05$) in the number of parasites in the group receiving antigen-pulsed DCs compared to each of other groups (Fig. 1). This suggested that the DCs loaded with *Giardia* extract were able to induce a protective immune response against the parasite. In an attempt to optimize the protective effect of the DC transfer, we compared intravenous and intraperitoneal injections. There was no significant difference in parasite numbers between the two routes (data not shown) and so i.p. injections were used for future studies.

3.4. Transfer of *Giardia*-pulsed IL-6 competent DCs into IL-6 deficient mice corrects the defect in *Giardia* clearance in IL-6 deficient mice

Considering that IL-6 deficient mice control *Giardia* infection poorly and have altered intestinal cytokine responses (Bienz et al., 2003; Zhou et al., 2003), we hypothesized that IL-6 competent DCs pulsed with *Giardia* extract would induce protection in IL-6 deficient recipients. To test this hypothesis, we administered *Giardia*-pulsed, IL-6 competent DCs, an equal volume of media or an equal number of unpulsed, IL-6 competent DCs into the peritoneal cavity of IL-6 deficient mice. Fourteen days later, we orally infected each mouse with 10^6 live parasites by gavage, and then euthanized them 12 days after the infection, when low parasite numbers are expected in untreated wild type mice but high numbers are typically seen in IL-6 deficient mice. There was an average 91% reduction in the number of parasites in the group receiving antigen pulsed DCs compared to the group receiving media only, and an 80% reduction compared to the group receiving DCs alone (Fig. 2A and B). Only the difference between the group receiving *Giardia* pulsed DCs and the group receiving media was consistently significant between experiments. These data indicate that defects in DC function in IL-6 deficient mice are responsible for the failure to readily control this infection.

3.5 Conclusions

Our data clearly identify a bone-marrow derived, non-T cell population as the important source of IL-6 during *Giardia* infections in mice. Furthermore, they show that adoptive transfer of DCs pulsed with *Giardia* antigen can induce protection against subsequent *Giardia* challenge. Moreover, IL-6 deficient mice receiving *Giardia*-pulsed IL-6 competent DCs can produce a more effective anti-*Giardia* response than control IL-6 deficient mice. These results suggest that IL-6 competent DCs can correct the *Giardia* clearance defect of IL-6 deficient mice, and that IL-6 from macrophages, mast cells, T-cells and intestinal

epithelial cells are not required for *Giardia* control. As described previously, DCs from IL-6 deficient mice have a functional defect (Bleier et al., 2004; Park et al., 2004). Whether the lack of protection against *Giardia* in IL-6 deficient mice is due to abnormal DC development and function in an IL-6 free environment, or is due to their inability to secrete IL-6 remains unanswered. The induction of anti-*Giardia* protection by IL-6 competent DCs to IL-6 deficient mice shown in our results could be due to a correction of either or both problems. Our previous work indicates that *Giardia* induces production of only small amounts of IL-6 from bone marrow-derived DCs (Kamda and Singer, 2009).

IL-6 is one of the cytokines crucial in determining differential T-cell development, as in the distinction between Treg versus Th17 cells (Weaver et al., 2006). It has been suggested that an early IL-6 production by DCs has a specific role in T-cell differentiation compared to late IL-6 production (Jones, 2005). Given that DCs are professional APCs, the presence of IL-6 during the initial contact between DCs and T-cells could be a key determinant of the type of response that ensues. IL-6 is one of the few DC cytokines induced by *Giardia* (Kamda and Singer, 2009), and a deficiency in IL-6 leads to poor anti-*Giardia* immunity. Taken together, DC specific IL-6 could be a key regulator of anti-*Giardia* immune responses.

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Highlights

- IL-6 from bone-marrow derived non-T cells is required for immunity to *Giardia*
- Transfer of dendritic cells with *Giardia* antigen induces protective immunity
- Wild-type dendritic cells plus antigen can protect IL-6 deficient mice

Figure 1.

Parasite numbers from small intestine of mice receiving *Giardia*-pulsed DCs and control mice. 5×10^5 *Giardia* -pulsed DCs in 100 l media (diamonds) were injected in the peritoneal cavity of mice. Control mice received either 100 l of media (No vaccine - squares), 5×10^5 unpulsed DCs only (circles), or *Giardia* extract without cells (triangles) with 1 mg/ml total protein by the same route. All mice were infected orally with 10^6 live *Giardia* trophozoites by gavage 14 days later, then sacrificed 5 days after infection and parasites counted as in methods. Each point represents an individual mouse, and data are representative of three independent experiments. A one tailed Mann-Whitney test analysis was used to compare parasite numbers between the various groups. * $p < 0.05$.

Figure 2.

Parasite numbers from small intestine of IL-6 deficient and control mice after DC adoptive transfer. 5×10^5 *Giardia*-pulsed DCs from IL-6 competent mice, in 100 l media (diamonds) were injected in the peritoneal cavity of IL-6 deficient mice. Control mice received 100 l media (filled squares), or 5×10^5 unpulsed DCs in 100 l media (circles), by the same route. In one of the two experiments (B), a group of wild-type mice (open squares) received no treatment before infection. Two weeks later, all mice were infected orally with 10^6 live *Giardia* trophozoites by gavage, then sacrificed 12 days after infection, and parasites counted as described in the methods. Each point represents an individual mouse. A one-tailed Mann-Whitney test analysis was used to compare parasite numbers between the various groups. * $p < 0.05$.

Table 1*Giardia* infections in bone marrow chimeric mice^a

Bone Marrow Donor	Irradiated Recipient	Day Analyzed ^b	# Infected ^c
Wild-type	Wild-type	7	0/3
		14	0/3
		28	0/3
Wild-type	IL-6 -/-	7	0/3
		14	0/3
		28	0/3
IL-6 -/-	Wild-type	7	2/3
		14	1/3
		28	2/3
IL-6 -/-	IL-6 -/-	7	1/3
		14	3/3
		28	2/3

^a Chimeric mice were prepared as described in materials and methods.^b Relative to the day of infection.^c Number of mice with parasites detected in the small intestine compared to the total number of mice analyzed.

Table 2

Giardia infections following adoptive transfer of splenocytes into T cell deficient recipients.

Splenocyte Donor	Recipient	# Infected ^a
None	Wild-type	0/4
Wild-type	Tcrβ ^{-/-}	0/4 ^b
IL-6 ^{-/-}	Tcrβ ^{-/-}	0/4 ^b
Tcrβ ^{-/-}	Tcrβ ^{-/-}	4/4
None	Tcrβ ^{-/-}	4/4

^a Mice were euthanized 7 days after infection and duodenum and jejunum scored for trophozoites

^b p<0.05 compared with Tcrβ^{-/-} mice receiving no splenocytes.