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Transgenic *Eimeria tenella* Expressing Enhanced Yellow Fluorescent Protein Targeted to Different Cellular Compartments Stimulated Dichotomic Immune Responses in Chickens

Xiaoxi Huang,* Jun Zou,* Hanqian Xu,† Ye Ding,‡ Guangwen Yin,* Xianyong Liu,* and Xun Suo*

*Eimeria tenella*, one of the seven species of chicken coccidia, elicits protective immunity against challenge infection with both homologous and heterologous strains. We endeavor to use recombinant *E. tenella* as a vaccine vehicle for expressing and delivering pathogen Ags and investigate immune responses against these foreign Ags. In this study, two lines of transgenic *E. tenella* expressing a model Ag, enhanced yellow fluorescent protein (EYFP), targeted to the micronemes and to the cytoplasm of the recombinant parasites were constructed to study the impact of Ag compartmentalization on immunogenicity. The MTT assay, intracellular cytokine staining, and real-time PCR were performed to detect the EYFP-specific proliferation and effector functions of splenic lymphocytes of immunized chickens. ELISA was used to measure anti-EYFP IgG and IgA responses. The results showed that both lines of transgenic parasites stimulated EYFP-specific lymphocyte proliferation and IFN-γ expression in CD4 and CD8 T cells, whereas a higher level of Ag-specific lymphocyte proliferation was elicited by the transgenic line expressing micrornemetargeted EYFP. Furthermore, this line stimulated stronger IgA response than the one expressing cytoplasm-targeted EYFP after the second immunization. Our findings are encouraging for further investigation of the effect of Ag compartmentalization in transgenic *Eimeria* on immunogenicity and for the development of a eukaryotic vaccine vector using genetically modified Apicomplexa parasites. The Journal of Immunology, 2011, 187: 000–000.

**E. tenella** is an intracellular Apicomplexa parasite. Infection by *E. tenella* or other coccidia species occurs in almost all poultry farms and causes huge economic losses in the poultry industry (1). Fortunately, *Eimeria* infection can be effectively controlled by vaccination with attenuated *Eimeria* strains (2).

The life cycle of *Eimeria* comprises intracellular and extracellular stages, and the host’s immune responses against such parasites are complex and involve both cellular and humoral immune mechanisms. Cell-mediated immunity plays a major role in resistance to parasite infections, and the cellular immunity against *Eimeria* infection could be transferred by spleen cells and PBLs to naive recipients in chickens (3–6). Also, *Eimeria* infection induces high levels of IFN-γ (7), which effectively inhibits the intracellular development of *E. tenella* (8), and upregulates mRNA expression of IL-1β, which recruits inflammatory cells including macrophages, neutrophils, and lymphocytes to the site of infection (7). Humoral immunity is equally important in parasite infections. Chickens infected with *Eimeria* develop parasite-specific Abs in both the systemic circulation and mucosal secretions (3, 9). Studies showed that IgY transferred from immunized hen to embryos conferred effective protection of the offspring against an infectious dose of the parasite (10), and IgA inhibited sporozoite invasion and development in cultivated cells (11). The above findings and worldwide application of attenuated anticoccidial vaccines prompted us to develop *Eimeria* spp. as a vaccine vector for viral and bacterial pathogens.

Studies with recombinant intracellular pathogens showed that Ag compartmentalization could influence host immune responses. It was reported that the secreted, but not cytoplasmic, Ag from tachyzoites of recombinant *Toxoplasma gondii* induced Ag-specific CD8 T cell responses (12). In a study with *Listeria monocytogenes*, listeriolysin Ag, either secreted into the host cell cytoplasm or retained in bacterial cells, expressed in *L. monocytogenes*-primed CD8 T cell responses, but the specific CD8 T cells protected the host only against bacteria secreting the Ag (13). Another study with the same Ag expressed by *Salmonella* also showed that only the secretory Ag, but not the somatic Ag, provided protection against *L. monocytogenes* (14). These findings underline the importance of Ag compartmentation in the elicitation of immune protection of the host against pathogens.

Compared to bacteria and viruses, *Eimeria* have a complex life cycle and a unique feature of Ag compartmentalization in subcellular organelles and parasitophorous vacuoles. We studied the impact of Ag localization in *E. tenella* on the immune response of chickens to recombinant *Eimeria* infection. Two lines of transgenic *E. tenella* expressing the model Ag, enhanced yellow fluorescent protein...
(EYFP), in the microneme or cytoplasm elicited both cellular and humoral arms of host immunity against EYFP. The EYFP targeted into the microneme organelles stimulated higher Ag-specific lymphocyte proliferation and IgA production than EYFP localized in the cytoplasm. Our findings illustrate the importance of compartmentalization of Ags in the induction of *E. tenella*-specific immune responses and have important implications on the development of transgenic *E. tenella* as a vaccine vector.

**Materials and Methods**

**Chickens and parasites**

One-day-old Arbor Acre (AA) broiler chickens were purchased from Beijing Arbor Acres Poultry Breeding. They were housed in isolators and fed with a pathogen-free diet and water.

*E. tenella* (BJ strain) was maintained and propagated in cococcidia-free, 2–4- wk-old AA broilers. Oocysts were collected from feces of chickens 6–11 d postinfection and were isolated, purified, and sporulated as described previously (15).

**Plasmid construction**

Two plasmids, pSK-MsEA and pSK-TNED, expressing EYFP targeted to the microneme and parasite cytoplasm, respectively, were constructed (Fig. 1A). The pSK-MsEA plasmid was constructed from the pBluescript-SK plasmids (Invitrogen) by inserting the whole open reading frame of EYFP between the Ndel and NotI sites, the 3′ untranslated region of Actin (1.4 kb) from *E. tenella* between NotI and MfeI, and MClI promoter with its signal sequence of *E. tenella* between Apal and EcoRV. The Ndel and EcoRV sites in pBluescript-SK were removed during the construction process.

The pSK-TNED plasmid was constructed by inserting the whole nuclear protein of avian influenza virus between BglII and AvrII, replacing one open reading frame of EYFP in the plasmid TupYFP-YFP/sagCAT, a construct derived from the pBluescript-SK plasmid (a gift from Prof. Boris Striepen, Department of Cellular Biology, University of Georgia).

**Transfection and selection of transgenic parasites**

The plasmids were transfected to *E. tenella* sporozoites by restriction enzyme-mediated integration (16). The plasmid DNA (30 μg) was linearized with BamHI, and the enzyme was removed by phenol extraction. The linearized DNA and 100 U BamHI were added to a cocomix buffer supplemented with 2 mM ATP and 5 mM glutathione (17) containing 2× 10^5 sporozoites to a total volume of 800 μL. Electroporation was performed in a Gene Pulser Xcell Electroporation System (Bio-Rad, Hercules, CA) at 2000 V, 25 mF, and a pulse time of 0.3–0.4 ms. The sporozoites were allowed to recover for 20 min at room temperature and then inoculated to the ileocecal opening of 4-d-old chickens for in vivo stable transfection (18).

Oocysts in feces excrrected 6–11 d after inoculation were collected, sporulated, and then purified using a 10% sodium hypochlorite solution (15). The purified oocysts were filtered through a 40-μm nylon cell strainer (BD Falcon, Boston, MA) and then resuspended in PBS. The transfected oocysts expressing EYFP were sorted by the MoFlo Cell Sorter (DakoCytomation, Fort Collins, CO) on the single-cell mode. The fluorescent oocysts were propagated again in chickens by oral inoculation to obtain the next generation. After propagation and sorting for six generations, >90% excrrected oocysts were fluorescent. The oocysts were resuspended in 2.5% K2CO3 and stored at 4°C for immunization experiments.

**Indirect immunofluorescence assay**

The fluorescence distribution in stably transfected parasites was examined by indirect immunofluorescence assay. Sporozoites were fixed in 4% paraformaldehyde and stained with anti-chicken IFN-γ (Sigma–Aldrich) at 1:50 dilution in blocking buffer. After washing three times with blocking buffer and three times with double distilled water, the sections were stained with 2% uranyl acetate and lead citrate in double-distilled water. Control sections were incubated in the absence of primary Ab. The sections were imaged in a JEM-100S electron microscope (JEOL) at 80 kV (19).

**Histopathology and lesion scoring**

The cecal tissue was fixed, dehydrated with increasing concentrations of ethanol, embedded in paraffin, sectioned, and stained with H&E for histological evaluation. The lesion severity was scored according to the method of Johnson and Reid (21).

**ELISA**

Chicken IgG and IgA were detected by ELISA as previously described (22). The EYFP protein expressed in *Escherichia coli* and purified by an Ni-NTA Superflow cartridge (Qiagen) was used as the coating Ag to coat the 96-well microtiter plates (4 μg/mL). Abs bound to the coating Ag were detected with HRP-labeled goat anti-chicken IgG Ab (diluted in 1:2000; Proteintech Group USA) or goat anti-chicken IgA Ab (diluted in 1:1000; Bethyl Laboratories). The data were representative of three independent experiments. The baseline Ab titer was twice the mean readings for the serum or bile control samples.

**Spleenic lymphocytes proliferation**

Chickens inoculated with the transgenic or WT oocysts were sacrificed 7 d after the first or second immunization. Spleen was removed aseptically (23). Single-cell suspensions of splenocytes in RPMI 1640 cell-culture medium supplemented with 5% FCS were loaded onto a 96-well cell-culture plate (3×10^5 cells/well) and incubated in a humidified chamber. After propagation and sorting for six generations, >90% excrrected oocysts were fluorescent. The oocysts were resuspended in 2.5% K2CO3 and stored at 4°C for immunization experiments.

Three chickens of each group were sacrificed 7 d after each immunization inoculation. Lymphocyte suspensions from the spleen were incubated with 1×10^6 cells/ml were incubated in six-well cell culture plates. After incubation with the EYFP protein (5 μg/ml) for 6 h at 41°C in 5% CO2, the cells were treated with monensin (2 μg/ml) for 2 h. The cells were washed twice with PBS and stained with mouse anti-chicken CD4-FITC, mouse anti-chicken CD8-PE, and mouse anti-chicken CD3-SPRD (Southern Biotechnology Associates). After 15 min at 4°C, the cells were washed three times with PBS, the cells were fixed with 4% paraformaldehyde for 10 min on ice and then washed with PBS. The samples were incubated with 0.1% saponin for 10 min at room temperature, washed with PBS, and then stained with mouse anti-chicken IFN-γ–Pacific Blue for 20 min at 4°C. Mouse anti-chicken IFN-γ mAbs were kindly given by Dr. Lillehoj (Animal Parasitic Diseases Laboratory, Animal and Natural Resources Institute, U.S. Department of Agriculture) and conjugated to the Pacific Blue dye using a Pacific Blue mAb Labeling kit (Invitrogen). The fluorescence intensity was measured by the FACS Aria II (BD Biosciences) (24, 25). First, we gated for CD3* cells, then for CD8* or CD4* cells, and lastly for IFN-γ expression in CD8* or CD4* cells (26, 27, 28).
The data were recorded as stimulation ratio, which is the ratio of cells expressing IFN-γ stimulated by EYFP to cells expressing IFN-γ without EYFP stimulation.

**Real-time PCR**

Total RNA was extracted from lymphocytes stimulated with the EYFP protein as described above with the TRIzol reagent (Invitrogen). cDNA was synthesized using random primers and a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The primer pairs used for analysis of five specific genes (Table I) were designed with the PerlPrimer software according to Qiagen product instruction (http://www.qiagen.com), with β-actin as the reference gene (28, 29). Each pair of primers was confirmed by melting curve analysis. Quantitative real-time PCR was performed on the 7500 Real Time PCR System (Applied Biosystems) with a program of 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for 1 min following the instruction for the SYBR Green PCR master mix (Applied Biosystems). Each cDNA was amplified in a 20-μl reaction mixture containing 10 μl duplicate SYBR Green PCR master mix, cDNA

**FIGURE 1.** Plasmid construction and transgenic *E. tenella*. A, Plasmid pSK-MsEA containing the *E. tenella* MIC1 promoter and MIC1 signal sequence and pSK+TNED containing the *T. gondii* tubulin promoter without a signal sequence. B, Subcellular localization of EYFP expressed in transgenic *E. tenella* sporozoites and oocysts. EYFP expressed in EtTNED (*E. tenella* transfected with pSK+TNED) was observed in the cytoplasm and particularly nuclei of sporozoites; EYFP expressed in EtMsEA (*E. tenella* transfected with pSK-MsEA) was observed in the cavity of sporocytes and as spots scattered in the apical complex of sporozoites. Scale bars, 7.5 μm (top) and 10 μm (bottom). C, Colocalization of EYFP (yellow) with MIC2 protein (red) and nuclei stained by DAPI (blue) in sporozoites transfected with pSK-MsEA or pSK+TNED by indirect immunofluorescence assay. EYFP spots in the EtMsEA were observed in the micronemes with MIC2. Scale bars, 5 μm. D, Subcellular localization of EYFP in the EtMsEA sporozoites by immunoelectron microscopy. EYFP stained with gold (dense dark spots) were generally located in the micronemes. Scale bar, 200 nm.
corresponding to 1 μg reverse-transcribed RNA, and 250 mM (optimized concentration) forward and reverse primers. Samples from three chickens were analyzed for each group. Relative expression was calculated as the ratio of template copy numbers of a sample relative to the nonstimulated control after normalizing to their respective isotype control actin.

Statistical analysis

The data were expressed as the mean of three chickens per group ± SEM of at least three independent experiments and analyzed using Student t tests, with significance levels set at 0.05 and 0.01.

Results

Transgenic E. tenella were established to express EYFP targeted into the microneme and cytoplasm

To obtain transgenic E. tenella expressing the EYFP to targeted compartments, two plasmids, pSK-MsEA and pSK+TNED, were constructed (Fig. 1A). Plasmid pSK-MsEA contained the MIC1 promoter of E. tenella and its first 90 bp as the signal sequence to deliver the EYFP into the microneme organelles. Plasmid pSK+TNED contained the tubulin promoter from T. gondii, without any signal sequence, to deliver the EYFP into the parasite’s cytoplasm. Two stable transgenic lines of E. tenella were obtained by electroporation, in vivo propagation, and FACS.

Localization of the exogenous protein expressed in the stable transgenic parasites was determined by laser scanning confocal microscope and immunoelectron microscopy. As expected, EYFP expressed in the EtTNED parasites transfected with pSK+TNED was distributed evenly in unsporulated oocysts. After oocysts sporulation, EYFP was confined in the sporozoites and predominantly located in the nucleus. EYFP in the EtMsEA parasites transfected with pSK-MsEA was observed mainly in the cavity of the sporocyst with only faint fluorescence in sporozoites after sporulation (Fig. 1B). To further determine the definite localization of EYFP in the parasitic, immunofluorescence staining of sporozoites for MIC2 was performed. Immunostained MIC2 and partial EYFP were seen in the apical micronemes in the EtMsEA strain, suggesting colocalization of the two proteins (Fig. 1C). To confirm this result, immunoelectron microscopy was used to show the accurate localization of EYFP expressed in EtMsEA. As shown in Fig. 1D, EYFP was mostly localized in the micronemes of sporozoites of the EtMsEA parasite.

Transgenic E. tenella lines maintained immunogenicity with low pathogenicity

Our previous work showed that the reproductiveity of transgenic E. tenella expressing exogenous proteins was substantially decreased relative to the WT parasite (18). We examined the infectivity and immunogenicity of our transgenic E. tenella in chickens. Chickens inoculated with the transgenic E. tenella shed fewer oocysts than chickens infected with the WT parasites, especially after the boosting immunization. Very low numbers of oocysts were detected in the feces of all immunized groups after challenge with no significant difference among the EtMsEA, EtTNED, and WT groups. Chickens of the PBS group, however, shed much more oocysts than the chickens from the immunized groups (Fig. 2).

To determine the pathogenicity of transgenic lines, ceca were collected from chickens 5 d after each immunization dose and 7 d after the challenge and were histologically examined (Fig. 3). Trophozoites and second-generation schizonts were observed in the lamina propria and epithelial cells of the cecal gland of all chickens 5 d after primary (Fig. 3A) and secondary (Fig. 3B) immunization of all immunized groups. Cecal lesions including mucosal damage, inflammatory cell infiltration, and hemorrhage in the transgenic parasite groups were less severe than those in the WT group. Seven days after WT challenge (Fig. 3C), the PBS-
ex vivo. EYFP elicited significantly higher levels of IFN-γ expressed in CD8+ T cells from chickens immunized with the EtTNED parasites than cells from the WT group PPI. Similarly, CD8+ and CD4+ T cells from the EtTNED group expressed significantly high levels of IFN-γ PSI (Fig. 5A–D). To determine the IL-12 expression, real-time PCR (primers are shown in Table I) was used to analyze the mRNA transcription in lymphocytes stimulated by EYFP ex vivo at 7 d PPI. IL-12 mRNA level of the EtTNED group was higher than that of all other groups (Fig. 5E). The EtMsEA group had a similar IL-12 expression to the WT group.

*E. tenella* is an intracellular parasite. It is generally believed that Abs play a minor role in resistance to *E. tenella* infection. However, this parasite has a complicated life cycle, and studies showed that specific IgY and IgA could be induced by the parasite (31). To develop a novel vaccine vector, it is also important for us to understand Th2 responses stimulated by the exogenous Ag expressed by transgenic *E. tenella*. Therefore, we determined the expression of a Th2 cytokine, IL-4, which is an important effector cytokine of Th2 responses (30). Analysis of the IL-4 transcription revealed that there was no significant difference between the transgenic and control groups 7 d PPI (Fig. 5E).

To analyze the function of EYFP-specific T cells, the expression of perforin and granzyme A were analyzed. The results showed only the EtTNED upregulated the expression of perforin and granzyme A 7 d PPI, although the increase was not statistically significant because of the high variability (Fig. 5E). These results indicated that cytoplasmic Ags might stimulate stronger cytotoxic T cell function than secreted Ags.

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**FIGURE 3.** Cecal lesions of chickens 5 d after the first (A) and second (B) immunization with the transgenic (EtMsEA and EtTNED) or WT *E. tenella* or PBS and 7 d after challenge (C) with WT *E. tenella*. The sections were stained with H&E and examined at ×100 or ×400 magnification under a light microscope. Parasites (black arrows), lesions of lamina propria (black arrowheads), tissue hemorrhage (white arrows), and inflammatory cells infiltration (white arrowheads) were observed in the immunized groups after the first and second immunization doses and after challenge in the PBS group. In the WT group, tissue lesions were more severe and more parasites were observable than in the transgenic groups 5 d PPI and PSI. After challenge, there was barely any lesion in the two transgenic groups and WT group, but a large amount of oocysts were present in the cecal lumen, with severe tissue damage in the PBS group.

**FIGURE 4.** Splenic lymphocyte proliferation stimulated by EYFP in vitro. Splenocytes suspensions were prepared from the immunized chickens (three chickens/group, three cultures/chicken) 7 d PPI (A) or PSI (B) and stimulated with EYFP (model Ag), SA, BSA (BSA, irrelevant Ag), Con A (positive control), or culture medium (negative control). Cell proliferation was analyzed by the MTT colorimetric method and expressed as SI. The EYFP SIs of splenic lymphocytes from the transgenic groups (EtMsEA and EtTNED) were significantly higher than that of the WT group 7 d PPI, and the index of the EtMsEA group was significantly higher than that of the EtTNED group 7 d PSI. There was no difference for the SA SIs between the parasite-immunized groups. *p < 0.05, **p < 0.01.
Transgenic E. tenella stimulated EYFP-specific systemic humoral and mucosal immune responses

To examine whether the transgenic E. tenella stimulated humoral responses against the exogenous protein, serum samples were obtained from the chickens every 7 d PPI and every 5 d PSI. The anti-EYFP Ab was detected by ELISA. As shown in Fig. 6A, the transgenic E. tenella induced significantly higher specific Ab against EYFP than WT oocysts. The two lines of transgenic parasites elicited similar total IgG responses in chickens against EYFP. These results indicated that the transgenic parasites elicited specific humoral responses, and the localization of the heterogenous protein did not influence the humoral immune response.

The intestinal mucosa provides an important immunologic barrier to E. tenella, which complete their life cycles within the epithelial cells of the cecum. Sporozoites are also found in macrophages or intraepithelial lymphocytes, through which the parasite is translocated into the lamina propria and gains access to the crypt epithelial cells (7). To evaluate whether the heterogenous proteins expressed in E. tenella elicit mucosal immune responses, bile was collected from immunized AA broilers at 7 d intervals PPI and PSI, and anti-EYFP IgA was detected by ELISA. We found that the titer of anti-EYFP IgA induced by the EtMsEA oocysts was much higher than that by the EtTNED oocysts and peaked at the 21st day PPI (Fig. 6B). The results suggested that the EYFP expressed by the transgenic parasites elicited IgA responses, and the EYFP located in the microneme stimulated stronger responses than the protein located in the parasite cytoplasm.

Discussion

In the current study, we successfully obtained two lines of transgenic E. tenella expressing EYFP as a model Ag targeted to different compartments and assessed the effect of compartmentalization of a heterologous Ag on the induction of cellular and humoral immune responses in chickens. The EYFP localization was confirmed by confocal fluorescence microscopy and immunoelectron microscopy. As expected, EYFP in the EtMsEA transgenic line transfected with the plasmid containing the MIC1 promoter and the signal peptide of E. tenella colocalized with microneme organelles, and a large amount of EYFP expressed in the EtMsEA parasites was secreted to the cavity of sporocysts. The EtTNED line expressed EYFP to the nuclei and cytoplasm.

Studies with intracellular parasites, such as T. gondii (12), Leishmania major (32), and Trypanosoma cruzi (33), expressing a model Ag targeted to subcellular compartments showed that only secretory Ags, not cytosolic or transmembrane proteins, were found to be presented to and primed CD8 T cells. We assessed the cell-mediated immunity by Ag-specific splenic lymphocyte proliferation ex vivo and Ag-stimulated IFN-γ production by CD8 and CD4 T cells isolated from the immunized chickens. Our finding is different from observations in studies with other intracellular parasites expressing model Ags. The secreted EYFP localized in micronemes induced significantly greater specific T cell proliferation, but lower specific IFN-γ expression and cytotoxic T cell function than cytoplasmic EYFP.

The secreted Ag also induced greater mucosal immunity than the cytoplasmic Ag. Sustained high levels of IgA in bile were detected in chickens after a single inoculation of the EtMsEA oocysts expressing EYFP to the secretory organelle, micronemes.

with high expression of perforin and granzyme A. IL-12 expression of the EtMsEA group was not different from that of the WT group. *p < 0.05, **p < 0.01.
In comparison, the parasites transfected with EtTNED expressing EYFP to the cytoplasm induced relatively low levels of EYFP-specific IgG only after two inoculations of the EtTNED oocysts. The stronger mucosal immunity elicited by the secreted Ag could be explained by the direct, repeated exposures of the host cells to the expressed protein after oral inoculation. Firstly, after oral inoculation of the sporulated oocysts, a large amount of the expressed EYFP in the cavity of sporocysts is released into the lumen of the duodenum and internalized and processed by intestinal epithelial lymphocytes, membranous cells, or APCs in the Peyer’s patch. Secondly, the Ag targeted to the microneuron is secreted during invasion of the host cells, and the secreted Ag is again presented to and processed by the host immune system. Thirdly, multiple cycles of cell invasion by T. gondii merozoites in the host intestine further boost the immunogenicity of the secretory Ag. Because a parasitophorous vacuole is formed around E. tenella in host cells, preventing most parasitic proteins from entering the host cell cytosol, the cytoplasmic Ag may be processed by the host immune system only after the parasites are killed by the host and the protein released from the parasite. Our findings indicate that transgenic Eimeria with exogenous Ags expressed in the secretory organelles are likely to induce strong intestinal mucosal immunity and thus could be used as an effective vaccine vector for gastrointestinal tract pathogens. Although the apical complex and unique invasion process of apicomplexan parasites increased complexity in the stimulation of host immune responses against the parasites, these characteristics of Eimeria parasites provide us with a tool to design live vaccine vectors expressing Ags in targeted compartments to stimulate specific host immunity pathways.

MHC class II (MHC-II) presentation has been shown to be restricted to secreted proteins in T. gondii with a tool to design live vaccine vectors expressing Ags in targeted compartments to stimulate specific host immunity pathways. Previous studies on parasite Ags presented by MHC-II molecules investigated mainly the process after parasite invasion or death (35, 36). The impact of Ag localization on immunogenicity has been studied with protozoan parasites. For example, secreted Ags and Ags expressed on the plasma membrane of Leishmania were presented by MHC-II molecules to CD4 + T cells (36, 37). Little information on immune responses stimulated by Ags secreted into the cavity of sporocysts has been published. Our studies revealed an additional Ag localization where the Ags might be presented by MHC-II molecules, provoking strong mucosal immunity.

Both E. tenella and T. gondii are Apicomplexan parasites. Previous studies showed that secreted, but not cytoplasmic, Ags of T. gondii primed IFN-γ, a cytotoxic effector cytokine, expressed by CD8 + T cells in mice (12). We observed that both cytoplasmic and secretory Ags expressed by E. tenella stimulated IFN-γ expression by CD8 + and CD4 + T cells of chickens, and the cytoplasmic Ag elicited higher specific IFN-γ expression in CD8 + and CD4 + T cells than the secretory Ag. The difference between T. gondii and E. tenella might be related to the parasite’s distinctive life stages in the host. T. gondii does not undergo the sexual stage in mice, whereas E. tenella go through both asexual and sexual phases and complete the life cycle in chickens. The sexual stage may be important for cytoplasmic Ags in the stimulation of host immune responses. There is evidence that proteins in gametocytes of E. maxima induced protective immunity against experimental E. maxima infection and reduced fecal oocyst shedding (38, 39).

In conclusion, our results demonstrated that the heterologous Ag expressed by transgenic E. tenella elicited cellular, humoral, and mucosal immunity. The transgenic E. tenella maintained the immunogenicity, yet with low pathogenicity. Meanwhile, the localization of Ags in E. tenella is an important determinant of host immune response pathways. The oocyst and sporocyst wall may serve as a capsule to deliver the Ag directly into the lumen of

Table 1. Primers for real-time PCR

<table>
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<th>Primers</th>
<th>Product Size (bp)</th>
<th>Efficiency (%)</th>
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<td>104</td>
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<tr>
<td>perforin</td>
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<td>105</td>
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</table>

FIGURE 6. The Ab response to EYFP expressed by transgenic E. tenella after primary (day 1) and secondary (day 22, indicated by an arrow) immunization. A. Total serum IgG against EYFP determined by ELISA. The EYFP-specific IgG elicited by the two transgenic parasites was significantly higher than the IgG in the WT group, with no difference between the transgenic groups. B. EYFP-specific IgA in bile detected by ELISA. EYFP-specific IgA titer of the EtMeSA group was significantly higher than that of the other groups and peaked on day 21 PPI. Although there is hardly any response in the EtTNED group after the first immunization, high IgA titer was detected on day 7 PSI. The data were representative of three independent experiments.
intestines by protecting the antigenic protein from degradation under acidic conditions in the stomach. Our findings suggest that *E. tenella* can be developed as an effective vaccine vector to deliver heterologous Ags against viral or bacterial pathogens.

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**Disclosures**

The authors have no financial conflicts of interest.

**References**


