Original Paper

Pilot Study on Interferon-γ-producing T Cell Subsets after the Protective Vaccination with Radiation-attenuated Cercaria of *Schistosoma japonicum* in the Miniature Pig Model

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Abstract: CLAWN miniature pig has been shown to serve as a suitable host for the experimental infection of *Schistosoma japonicum*. In this study, we found that radiation-attenuated cercaria (RAC) vaccine gave CLAWN miniature pigs protective immunity against subsequent challenge infection with *S. japonicum* cercaria. To characterize the protective immune response of the pig model vaccinated by attenuated cercaria, flow cytometric analysis of the reactive T cell subsets was performed. The intracellular interferon (IFN)-γ and the cell surface markers revealed the peripheral blood CD3+ T-lymphocytes produced significant amounts of IFN-γ during the immunization period and after the challenge infection. CD4+ αβ-T cells as well as CD4+/CD8α^{mid} double positive and/or CD8α^{high} αβ-T cells were the major IFN-γ-producing CD3+ T cells. On the contrary, γδ T cells did not produce intracellular IFN-γ. Our results suggested that RAC-vaccinated miniature pigs showed effective protective immunity through the activation of αβ T cells bearing antigen specific T-cell receptors but not through the activation of γδ T cells.

Key words: αβ T cell receptor, γδ T cells, *Schistosoma japonicum*, miniature pig, radiation attenuated cercaria, Vaccine, IFN-γ

INTRODUCTION

Schistosomiasis is one of the neglected tropical diseases [1]. An estimated 200 million people suffer from schistosomiasis and 600 million people are at risk of contracting this disease. Recent re-evaluation of the disease burden has revealed that schistosomiasis exerts a far greater impact than was originally thought [2, 3]. Among the several schistosoma species identified to date, *Schistosoma japonicum* is known to cause endemic diseases in Asia, especially in China and the Philippines [4–6]. Despite the implementation of an extensive control program over the past 20 years, infection remains high in these endemic areas [5, 7, 8]. In contrast to other schistosoma species including *S. mansoni* and *S. haematobium*, *S. japonicum* is mainly harbored by animal species such as water buffalo, cattle and pigs [9]. Such a variety of hosts makes the control of *S. japonicum* difficult. Current control programs re-

ly mainly on the annual administration of Praziquantel (PZQ) to residents of the endemic areas [10–12]. PZQ is very effective at treating the disease but it does not protect against re-infection [13]. Subsequent *S. japonicum* infections are thought to contribute to both the transmission and persistence of schistosoma infection in the prevalent areas.

As the areas that require coverage by the annual PZQ treatment are very large [13], additional therapies are urgently required. The development of effective vaccines against *S. japonicum* infection is currently underway [10]. Two major prerequisites need to be met in developing the vaccine. First, the animal model should be able to tolerate *S. japonicum* infection over a long period and should be chosen from a natural reservoir host. In this context, we previously reported that the CLAWN miniature pig was a unique and appropriate experimental model of *S. japonicum* infection [14]. In addition, CLAWN miniature pigs are easy to handle due to their small size in comparison to

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domestic pigs. *S. japonicum* can infect and establish infection in this species of pig. The evaluation of long-term infection is also feasible in this pig within animal facilities. The second prerequisite to consider during vaccine study in animal models is that the animal should show an immune response against the parasite and subsequently be immunized against such parasites or parasite antigens [10]. Radiation-attenuated cercaria (RAC) may serve as a positive control, indicating that the host may potentially recognize the parasite and develop protective immunity. During *S. mansoni* infection in mice, RAC inoculation confers host protective immunity against subsequent infection [2]. However, unlike *S. mansoni* infection in mice, effective, protective immunity against subsequent infection with *S. japonicum* in mice conferred by RAC was minimal [15, 16]. In our previous study, we reported that the CLAWN miniature pig showed protective immunity following RAC inoculation [17]. In order to assess the ability of the CLAWN miniature pig to mount an immune response that leads to immunization, we evaluated the effects of RAC inoculation on subsequent S. japonicum cercaria infection and analyzed the immune response elicited by RAC inoculation.

METHODS

Animals

Four CLAWN miniature pigs were purchased from the Japan Clawn Farm Institute (Kagoshima, Japan). The body weight of the animals used in this study varied from 2.5–3.0 kg and the pigs were 5–6 weeks of age. The pigs were separated into two groups based on their swine leukocyte antigen (SLA), fed standard nutrient chow based on their body weight and given water ad libitum. The experimental protocol was pre-approved by the Animal Ethics Committee of Nagasaki University (No. 071207-1).

Parasite, parasitological technique and blood collection

One group of miniature pigs was pericutaneously inoculated twice with 400 RAC (total 20 krad, 33 Gy/min, using 6012 Co irradiator: Pony Industry Co., Ltd., Osaka, Japan) with a 3-week interval between inoculations. As a control, the other group was injected with PBS at the same time as the inoculated group. Four weeks after the irradiated cercaria inoculation, the miniature pigs were further challenged with 200 cercariae. The cercariae were shed from Oncomelania hupensis snails infected with a Chinese strain of *S. japonicum* and maintained at the Jiangsu Provincial Institute of Parasitic Disease Control (Wuxi, Jiangsu Province, China). *S. japonicum* adult worms were recovered from the liver and mesenteric veins using the

perfusion method [14]. A portion of the left hepatic lobe (1–2 cubic cm) was digested in 3% KOH at 37°C for 24 h after recording the weight. The egg number counted in one tenth of the digested fluid was evaluated to determine the total number of eggs per gram of tissue.

Analysis of peripheral blood lymphocytes

Blood samples were collected from the auricular vein every 2 weeks. Whole peripheral blood was then lysed with ACK lysis buffer and stained with monoclonal antibodies. The antibodies used were as follows: anti-swine FITC-CD3 antibody (clone: BB23-8E68C8), Biotin-CD16 (FcG7), PE-CD4 (74-12-4), FITC-CD8 (76-2-11) and APC-gamma-delta T cell receptor (γδ TCR) (clone: MAC320). All antibodies were purchased from Becton Dickinson (Tokyo, Japan). To analyze the source of IFN-γ, peripheral blood lymphocytes (PBL) were cultured in RPMI-1640 medium supplemented with 10% FBS and 50 mM 2-mercaptoethanol. The cells were cultured at a density of 3×10^6/ml in 48-well flat bottom culture plates (Corning, Inc., NY, USA) for 3 days with schistosoma adult worm antigen (SWA, 50 μg/ml). The cells were then cultured for 4 h with brefeldin A (10 μg/ml), PMA (10 ng/ml, Sigma-Aldrich, Tokyo Japan) and ionomycin (1 μg/ml, Sigma-Aldrich), harvested and stained with the fluorochrome-conjugated monoclonal antibodies (mAb) listed above for the analysis of cell surface markers. For the intracellular cytokine staining, following incubation with antibody, the cells were permeabilized using the Cytofix/Cytoperm Fixation Permeabilization Kit (Becton Dickinson) and stained with PE-(phycoerythrin) conjugated anti IFN-γ mAb (clone: P2G10, Becton Dickinson). The stained samples were then applied to a FACS calibur (Becton Dickinson) and analyzed using the CellQuest program (Becton Dickinson) and FlowJo (Tree Star, OR, USA).

Cytokine measurement

In a separate experiment, cells were stimulated with PHA (phytohemagglutinin) and cultured for 2 (IFN-γ) to 4 (IL-4, IL-10) days. The IL-4, IL-10, IFN-γ concentrations in the culture supernatant were measured using ELISA sandwich assay according to the manufacturer's instructions (R&D Systems, MN, USA).

RESULTS

RAC inoculation confers protective immunity to subsequent *S. japonicum* infection in CLAWN miniature pigs

In this study, CLAWN miniature pigs were immu-

nized twice with RAC at 3-week intervals. Four weeks after the second immunization, CLAWN miniature pigs were inoculated with *S. japonicum* cercaria. The efficacy of immunization was examined by calculating the number of worms recovered from CLAWN miniature pigs 6 weeks after infection (Table 1). In the immunized miniature pigs, the worm reduction rate was found to be greater than 80%. We also noticed a marginal number of deposited eggs in the liver in both vaccinated and control pigs. There was no

difference between the two groups. The paucity of eggs in the liver was observed previously, but no clear reason was disclosed. This result confirmed the previous findings that RAC inoculation effectively conferred protective immunity against *S. japonicum* infection in miniature pigs [17].

Analysis of the immune response
Cellular composition
In order to evaluate the immunity elicited by RAC

Table 1. Effect of RAC inoculation against subsequent *S. japonicum* challenge infection

	Miniature pigs	Eggs count in the liver (eggs/gram liver)	Worm number			Pair	[a]Reduction rate
			Total	Male	Female		
RAC	BQ47	6	26	18	8	0	89.40%
	BQ48	4	15	10	5	1	81.62%
Cont.	BQ57	7	146	123	23	0	—
	BQ59	8	137	107	30	0	—

[a] Reduction rate: Worm reduction rate (%) = [numbers of worms in the control group - numbers of worms in the vaccinated group]/ Numbers of worms in the control group × 100.
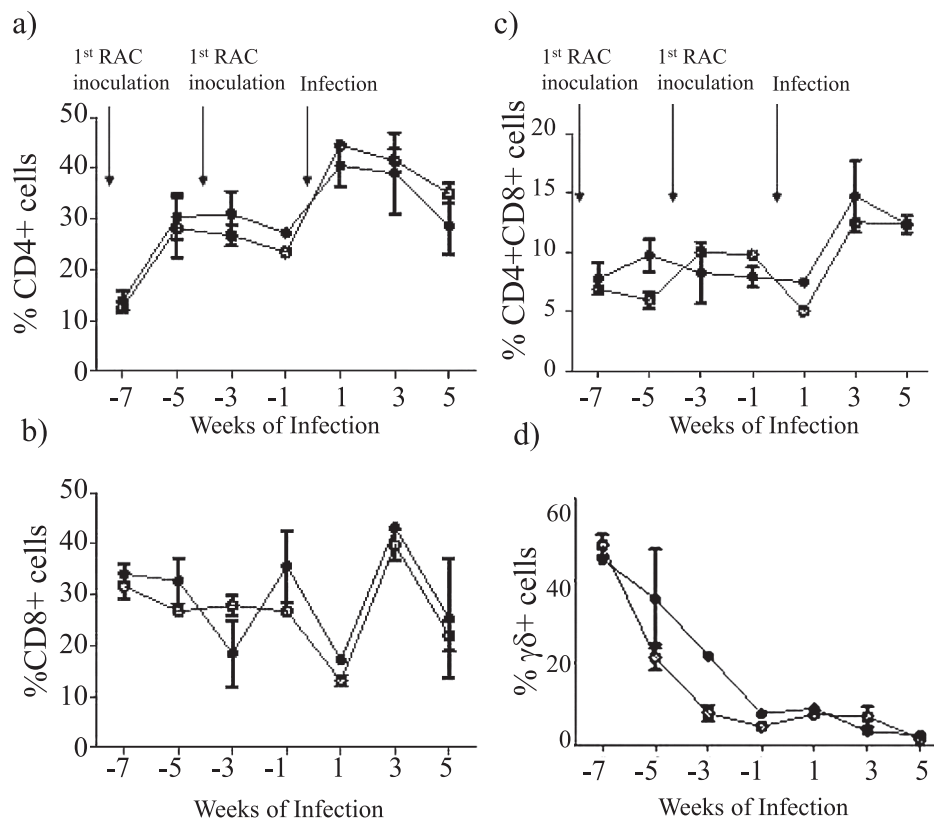

Fig. 1. Chronological changes of peripheral blood lymphocyte (PBL) composition in RAC-immunized miniature pigs
Four miniature pigs were separated into two groups based on their SLA. One group of pigs was immunized by two inoculations of 400 of RAC at 3-week intervals. Four weeks later, the pigs received a second inoculation with 200 *S. japonicum* cercaria. Blood was then collected every 2 weeks and PBL was analyzed using a FACS caliber. The percentage of each lymphocyte population in the lymphocyte-gated cells is presented as mean ± SEM. Open circles; control group, closed circles; immunized group. (a) CD4+, (b) CD8+, (c) CD4+/CD8α^{mid} and (d) γδ TCR+ lymphocytes.

inoculation, the composition of peripheral blood mononuclear cells (PBMC) was sequentially analyzed (Fig. 1). We did not identify any significant differences between the CD3+ cell (T cells) and CD21+ cell (B cells) ratio between the immunized and control miniature pigs (data not shown). The ratio of T cell populations—for example, the CD4+ cells, CD8α^{high} cells and CD4+/CD8α^{mid} cells—in the immunized pigs also showed no difference from that in the control pigs (Fig. 1a–c). CD4+ T cells were increased 1 week after infection. In contrast, the CD8α^{high} T cells were decreased at 1 week after infection in both groups of miniature pigs and then increased 3 weeks after infection (Fig. 1a, b). CD4+/CD8α^{mid} cells were increased at 3 weeks after infection (Fig. 1c). One of the features of the pig PBMC is that it contains a large proportion of T cells that express γδ TCR [18, 19]. In this study, almost 50% of lymphocytes comprised T cells that expressed γδ TCR prior to immunization. However, during the course of immunization and infection, the percentage of this population of cells in both the immunized and control pigs showed a remarkable decrease (Fig. 1d). This finding suggests that γδ TCR+ T cells play a minor role in the protective immunity elicited by RAC inoculation. Granulocytes, especially eosinophils, have been shown to play an important role in the development of protective immunity in schistosoma infections [20, 21]. In our study, the eosinophil number was not increased following immunization, but increased 6 weeks following infection in both groups (data not shown).

Cytokine production

We also evaluated cytokine response using ELISA (Fig. 2). In order to achieve this, peripheral blood was collected and the PBMC were isolated. PHA was added to PBMC for 2 days to stimulate IL-4 levels and 4 days to stimulate IFN-γ and IL-10. Cytokines in the culture supernatant were then measured by ELISA. The RAC-immunized miniature pigs produced more IFN-γ and IL-4 than the control miniature pigs during the immunization period, although the differences were not statistically significant due to the small number of pigs. IFN-γ production was found to peak 3 weeks after infection. The amount of IFN-γ was greater than that of IL-4 produced in the RAC-immunized miniature pig PBMC. Unlike IFN-γ production, IL-4 production peaked at 1 week after infection. Throughout the course of immunization and infection, the
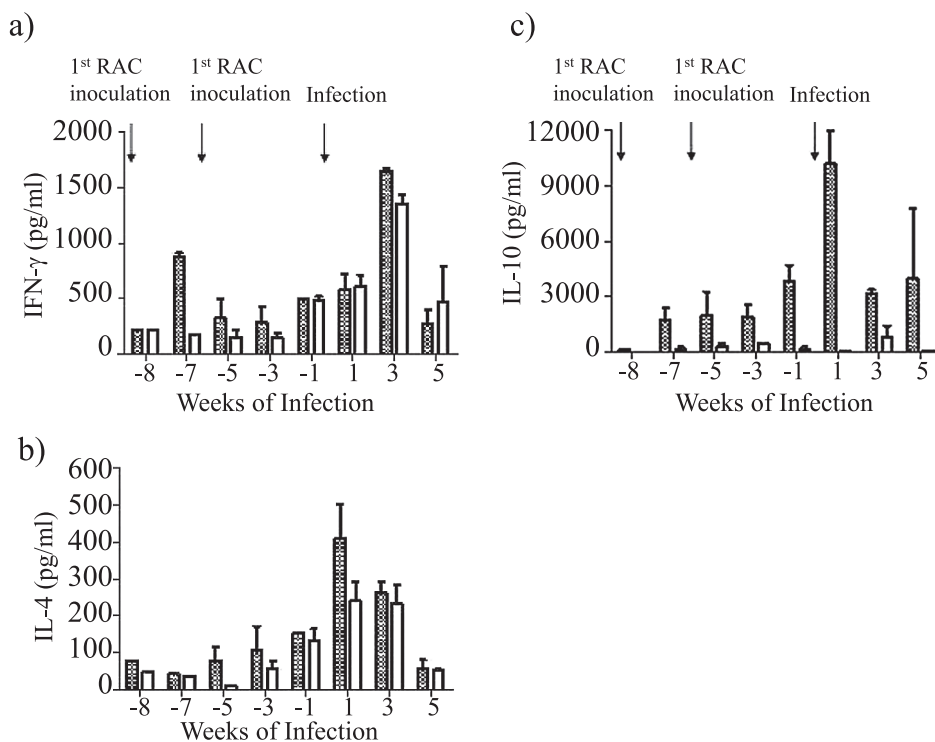

Fig. 2. Cytokine production by PBL in RAC-immunized miniature pigs
PBMC were cultured with PHA for 4 days and cytokine levels measured using ELISA. (a) IFN-γ, (b) IL-4 and (c) IL-10 levels. Open columns show the data from the control group. Shaded columns show the data from the immunized group. Data are presented as the mean ± SEM.
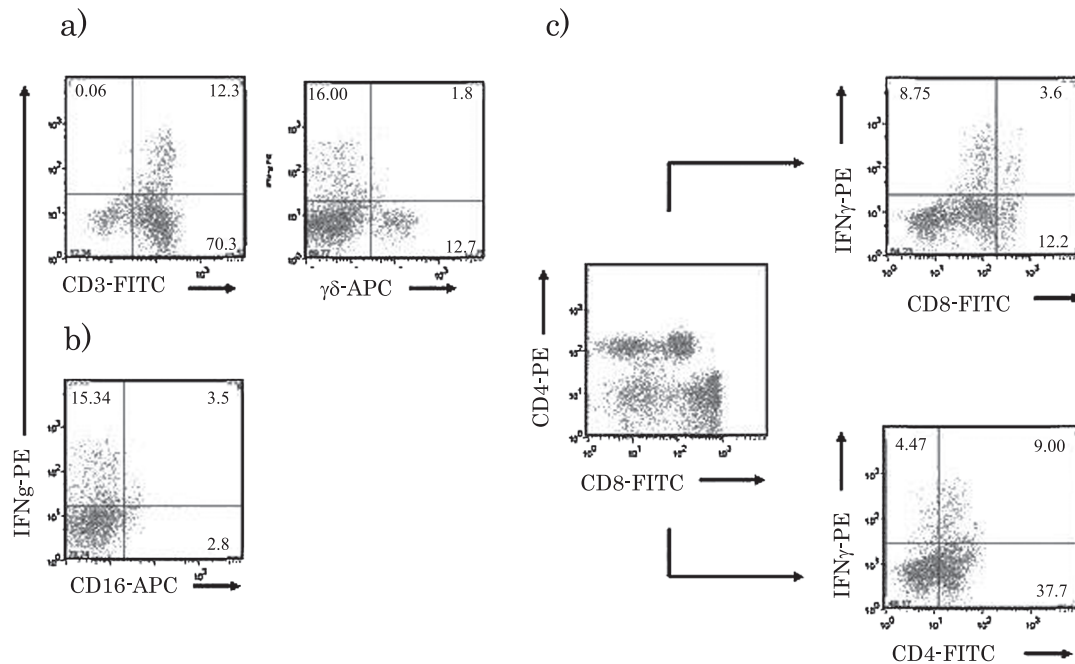
a)

b)

c)

Fig. 3.   Flow cytometric analysis of the cellular source of IFN-γ in RAC-immunized miniature pig PBL
Peripheral blood of the immunized pigs was collected at the time of scarification. PBMC were then stimulated with SWA for 3 days. The samples were cultured with PMA, ionomycin and breferdin A for 4 h. Cells were stained with CD3, CD16, γδ TCR, CD4, CD8α and IFN-γ antibodies. Lymphocyte-gated cells were analyzed. IFN-γ expression was examined in (a) CD3, γδ TCR+ (b) CD16+ (c) CD4+ and/or CD8α cells.

immunized miniature pig PBMC produced significantly higher levels of IL-10 than the control miniature pigs.

Cellular source of IFN-γ

Our observation of cytokine response suggested that RAC immunization in miniature pigs elicits an IFN-γ-mediated immune response, similar to the findings reported for *S. mansoni* infection in mice [22, 23]. Therefore, we examined the cellular source of IFN-γ, as few papers have reported the source of IFN-γ during *S. japonicum* infection in pigs. In order to examine the cellular source of IFN-γ, immunized miniature pig PBMC were cultured in the presence of SWA and then stimulated with PMA and ionomycin for 4 h. The PBMC were then stained for intracellular IFN-γ and analyzed using flow cytometry (Fig. 3). Based on forward and side scatter, IFN-γ-positive cells were lymphocytes (data not shown). Among the lymphocytes observed, almost all of the IFN-γ-positive cells were also positive for CD3 and negative for γδ TCR (Fig. 3a). Thus, we suggest that the conventional T cells expressing αβ TCR are the most likely major source of IFN-γ in the immunized miniature pigs. We also examined the natural killer (NK) cells, which are strong producers of IFN-γ [24], using CD16 as a marker of NK cells among other lymphocytes. CD16 is a low affinity receptor for IgG and

is expressed on monocytes and a population of NK cells [25]. Additional specific markers of porcine NK cells have yet to be clearly established [18].

We found a number of CD16+ lymphocytes, initially thought to be NK cells, that were positive for IFN-γ (Fig. 3b). However, this population only represented 2.3% of the total lymphocytes. In contrast, 15.9% of the lymphocyte population was positive for IFN-γ, but not for CD16. This result suggests that only a small proportion of NK cells was able to produce IFN-γ. The lymphocyte subpopulations were further examined to determine their CD4 and CD8α$^{\text{mid}}$ expression (Fig. 3c). We found that the CD8α$^{\text{mid}}$ and CD8α$^{\text{high}}$ cells were positive for IFN-γ, and that a portion of the CD4+ cells were also positive for IFN-γ. In the pig lymphocyte population, it has also been shown that the CD8α$^{\text{mid}}$ cells express CD4 and that these cells were considered to form a part of the CD4+ T cell group [26]. Therefore, both CD8+ and CD4+ T cells represented the main producers of IFN-γ.

DISCUSSION

This study revealed that CLAWN miniature pigs could be successfully immunized using RAC inoculation and that the specifically generated αβ but not γδ T cells

produced high levels of IFN-γ in response to antigens. IFN-γ was found to be mainly produced by the CD4+/CD8α^{mid} T cells and CD8+ T cells. As noted in previous reports including ours [14–16], in both groups of pigs, CD4+ T cell number was increased 1 week after infection. At the same time, the CD8+ T cell number was decreased. In addition, RAC immunization itself reduced the CD8+ T cell number after the second immunization. Thus, RAC immunization appeared to increase the CD4+/CD8α^{mid} T cell ratio. γδ TCR+ T cells comprise one of the major components of the porcine PBMC. During infancy, these cells comprise 50% of the lymphocyte population [18, 19]. As the pigs age, this ratio gradually decreases [4, 18]. Since no remarkable differences were observed between the immunized and control groups, the observed decrease in γδ TCR+ T cells may be attributable to the physiological changes caused by growth.

We also examined cytokine production using ELISA. In the immunized pig group, we found a higher level of IFN-γ production than in the control group. This increased IFN-γ production was even observed during the immunization period of -7 to -3 weeks of infection. In addition, the immunized group produced more IL-4, and RAC immunization activated production of both Th1 and Th2 cytokines in the lymphocytes. Since IFN-γ production predominated over IL-4 production, RAC immunization induced IFN-γ-based protective immunity, a finding also reported in a mouse model of *S. mansoni* infection [22, 23]. IFN-γ production peaked at 3 weeks after infection, a result that was consistent with the increase in CD8+ T cell and CD4+/CD8α^{mid} T cell numbers in both groups. This observation was further supported by the results presented in Fig. 3 showing that IFN-γ was mainly produced by these two types of T cells during the infection period. We suggest that CD8 T and CD4+/CD8α^{mid} T cells, as well as the corresponding IFN-γ production, increased during this period because these lymphocytes might accumulate in the lung 1–2 weeks after infection. This accumulation is thought to have been caused by the migration of schistosomula into the lung tissue [27]. After the exit of schistosomula from the lung, the accumulated lymphocytes are probably dispersed into the peripheral blood before migrating further. We also found that IL-4 production reached a peak at 1 week after infection, earlier than IFN-γ production. CD4+ cell number was also increased during this period, a finding suggesting that IL-4 was produced in CD4+ T cells during the early stages of infection. IL-10 was also found to be significantly produced in the immunized, but not control, group. Given that IL-10 is thought to function as a regulatory cytokine that reduces the inflammatory response, the synthesized IL-10 might be produced in re-

sponse to inflammation induced by immunization. In order to determine the cellular source of IFN-γ, we undertook intracellular cytokine staining. Since activation by schistosomal antigen alone was insufficient to detect intracellular cytokine signals, we also used PMA and ionomycin stimulation. As expected, CD3+ cells were shown to be the main source of IFN-γ synthesis. We found that approximately half of the CD16+ cells demonstrated IFN-γ production (2.3% of lymphocyte population); however, this level was less than that of the CD3+ population. According to Gerner W et al. [18], NK cells of porcine present CD3-negative and CD8a-positive. But, CD3-negative cells produced very little IFN-γ. Therefore, CD16-positive cells producing IFN-γ may be NKT cells (Fig. 3). Furthermore, lymphocytes expressing γδ TCR were not positive for IFN-γ. This finding is consistent with the result in Fig. 1 demonstrating that this type of T cell was decreased over time. Thus, we suggest that γδ TCR+ T cells play a minor role in RAC-induced IFN-γ production.

In this study, we used CD16 as a marker of NK cells among the lymphocyte population. Although a CD16-population existed [25], our observations showed that almost all of the IFN-γ producing cells were positive for CD3. Therefore, NK cells appeared to play a minor role in IFN-γ synthesis.

Among the IFN-γ+ cells, which are known to mainly comprise T cells expressing the αβ chain, CD8α^{high} and CD8α^{mid} cells were positive for IFN-γ. As shown in Fig. 3 (c), CD8α^{mid} cells also expressed CD4. Thus, CD8α^{mid} cells positive for CD4 and CD8α^{high} cells are the source of RAC-induced IFN-γ production. Considering the fact that the percentage of IFN-γ+/CD4+/CD8α^{mid} cells was almost twice that of IFN-γ+/CD8α^{high} cells, CD4+/CD8α^{mid} cells may be the main producer of IFN-γ in the miniature pig.

The fact that CD8α^{high} cells also produce IFN-γ may be attributable to the PMA and ionomycin used to activate PBL. PBMC were cultured with SWA for 3 days and then stimulated with PMA and ionomycin to preferentially simulate the schistosoma-specific cells. We expected that 3 days of culture with SWA would be sufficient to expand the specific cells. However, it may be possible that the PMA and ionomycin non-specifically simulated the cells. In our previous study, we reported that two proteins synthesized by eight candidate genes showed strong reactivity to the serum of RAC-immunized miniature pigs [17]. Given that RAC induced IFN-γ-based immunity, it is expected that these two vaccine candidates may induce Th1 immunity against infection. Despite numerous reports emphasizing the role of IFN-γ in RAC immunization, Th2 immunity has been shown to be important for protective immunity [28, 29]. As shown in Fig. 2b, the RAC-

immunized group demonstrated a higher IL-4 production. In a human study, Th2 immunity was shown to be related to resistance against re-infection with *S. mansoni* and *S. japonicum* [30, 31]. Thus, it may also be important to study the immunity governed by Th2 and elicited by immunization with adjuvant of cholera toxin or alum. In this study, RAC-induced IFN-γ-based immunity and IFN-γ were mainly produced by CD4+/CD8α ^{mid} and CD8α ^{high} cells.

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