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Evaluation of oral Lanzhou lamb rotavirus vaccine via passive transfusion with CD4+/CD8+ T lymphocytes

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ABSTRACT

Lanzhou Lamb derived Rotavirus (RV) Vaccine (namely LLR) for children is only used in China. Since there were no reports on evaluation of LLR, even the data of phase IV clinical trial, we proceed the evaluation of LLR through focusing on T-cell to investigate whether LLR could induce the potential function involving in protection as a vaccine. Four groups of nude mice were transfused with CD4+/CD8+ T-cells isolated from LLR-immunized (primed) and LLR-unimmunized (naïve) mice via intraperitonea (i.p.) respectively. Consequently, the adoption mice were challenged with mice-origin wild rotavirus EDIM (Epizootic Diarrhea of Infant Mice) by intragastric administration. Series of fecal/serum samples were collected and viral shedding, then serum IgA/IgG and secreted IgA were assayed. Compared to the mice transfused with T lymphocytes from naïve mice, the nude mice transfused with CD4+ T lymphocytes from primed mice induce fecal and serum IgA increasing more rapidly, and have a shorter duration of virus shedding too. Whereas, no significant difference in virus clearance was found between the mice transfused with CD8+ T lymphocytes isolated from primed and naïve mice. Therefore, we cleared the distinct roles of transfused CD4+/CD8+ T lymphocytes for rotavirus clearance in nude mice, that the viral clearance conducted by CD4+ T lymphocytes. Meanwhile, it has ability to help induction of LLR specific immunogenicity. Comparing with the transfusion of cell from primed and naïve mice, LLR can induce CD4+ T lymphocytes memory which is a potential index to reflect the immunogenicity and protection, while CD8+ T lymphocytes remove rotavirus by CTL with little memory ability.

1. Introduction

Rotavirus (RV) infection has been highly regarded since 1973 when the association between rotavirus infection and acute infant diarrhea was recognized (Bishop, 2009). So far, rotavirus is also one of the main etiological agents of severe dehydrating diarrhea in infants less than 5 years old worldwide. The World Health Organization (WHO) has coordinated the Global Rotavirus Surveillance Network since 2008 (Agócs et al., 2014). Although there is no specific treatment, attenuated live rotavirus vaccines have been verified to be effective in preventing rotavirus-caused diarrhea, especially the severe cases. There are five vaccines against rotavirus infection in the market till now. Two commercial vaccines, RotaTeq and RotaRix, have been used to prevent rotavirus infection in dozens of countries and regions (Kollaritsch et al., 2015; Desselberger et al., 2009), furthermore, two new rotavirus vaccines, Rotavac and Rotavin, was licensed in India (Anon, 2016) and Vietnam (Dang et al., 2012) respectively. However, previous studies indicated that RotaTeq and RotaRix are less effective in developing countries than in developed ones (Jiang et al., 2010), impeding their introduction in other countries including China. In fact, the LLR vaccine (Lanzhou Lamb derived Human Rotavirus Vaccine) has been authorized to use in China since 2000 and it is the only vaccine approved as rotavirus vaccine in China till now (Soares-Weiser et al., 2012). Besides, there are also several other rotavirus vaccines completed or undergoing phase III clinical trials in China. From 2006 to September 2014, more than 54 million doses of LLR vaccines have been lot released. However, there are still several confusions about the using of LLR, since there are limited published reports referred to preclinical or clinical effectiveness of LLR

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vaccine against infant gastroenteritis (Fu et al., 2007, 2012, 2010; He et al., 2013).

In this article we focus on the evaluation of effectiveness of LLR vaccine in animal models, instead of in human beings, and intend to look for a way for evaluation of the effectiveness of LLR to understand other novel vaccines well before implantation in children. To be noted, the immunogenicity is the principal consideration in the evaluation of vaccine's effectiveness. Although the immunogenicity studies of RotaTeq and RotaRix have been already conducted in animals and clinical trials, no related report has been found about the immunogenicity of LLR. Concluding from the previous studies IgA and IgG are normal indicators as evaluating the immunogenicity of rotavirus vaccines (Blutt et al., 2012; Westerman et al., 2005). Moreover, the exact correlation of protection remained indeterminate (Franco et al., 2006; Offit, 1994; Blutt et al., 2008), even the levels of RV-specific serum IgA and fecal sIgA seem to correlate with protection better (Angel et al., 2012; Patel et al., 2013). The cell-mediated immunity, as well as the humoral and mucosal immunity, and the earliest initiated innate immune response (Holloway and Coulson, 2013) against rotavirus were also proven to be significant in animal and human beings (Franco and Greenberg, 1995; Jaimes et al., 2002, 2005; McNeal et al., 1997). It has already been showed that there is a RV-specific cytotoxic T cell response in host, such as the passive transfer of immune cytotoxic T lymphocytes (CTLs), which, according to previous studies, can both protect against acute rotavirus induced diarrhea in suckling mice (Offit and Dudzik, 1990) and clear the chronic rotavirus infection from adult SCID (severe combined immunodeficient) mice (Blutt et al., 2012), but the exact role of T lymphocytes in preventing primary infection or re-infection is still unclear.

Therefore, in order to explore the immunological response of LLR, especially in which kind of T lymphocytes and how the T lymphocytes play a role against challenge after primed by LLR vaccine, we transfused the T lymphocytes isolated from primed mice and naïve mice into the nude mice. After challenged by mice-origin wild rotavirus EDIM (Epizootic Diarrhea of Infant Mice), we evaluated the immunological response in the duration of virus infection and clearance. The results showed there are different routes employed by $CD4^+$ and $CD8^+$ T lymphocytes to evaluate the rotavirus vaccine. That would be a new surrogate instead of unsatisfied serum antibody for preclinical or clinical trial. This suggests that we should pay more attention to passive immunology model and cell-mediated immune response induced by rotavirus vaccines in the process of production and evaluation of rotavirus vaccines.

2. Materials and methods

2.1. Viruses, cells and animals

LLR has been licensed in China since 2000. The titer of LLR vaccine used in this study was 6.2 $lgCCID_{50}$/ml. The rotavirus wild strain EDIM (G16 genotype) was cultured in MA104 cell, using RPMI-1640 medium in 37 °C, 5% CO_2 incubator. The two-week old female mice and nude mice (BALB/c) were provided by and breed in the Center for Experiment Animal, NIFDC (National Institutes for Food and Drug Control), and the animal experiment was approved by The Animal Care & Welfare Committee of NIFDC, China.

2.2. LLR immunization and isolation of $CD4^+$ and $CD8^+$ T lymphocytes

BALB/c mice were divided into two groups (72 mice each group) and intragastrically administrated with 0.3 ml LLR vaccine (group 1) or normal saline (group 2) once a week and three times totally. Four days after the third immunization, the spleens were isolated for separating out T lymphocytes using cell sorter with Dynabeads FlowComp Mouse $CD4^+$/$CD8^+$ kit (Invitrogen). Briefly, 10 μl $CD4^+$ FITC, $CD8^+$ PE, $CD3^+$ PerCP antibodies and 50 μl EDTA-anticoagulated blood from group 1 and group 2 mice were added into the bottom of BD TruCount Tubes. The mixture was vortexed and reacted in dark for 15 min. After adding 450 μl red blood cell lysis buffer and reaction for another 15 min, the proportion and absolute number of $CD4^+$ and $CD8^+$ T lymphocytes were analyzed and calculated by using flow cytometry and CellQuest software. After calculated by using Typan Blue staining, the live $CD4^+$ and $CD8^+$ T cells were intraperitoneally transfused into nude mice as soon as possible.

Before transfusion, the IFN-γ-secreting $CD4^+$ and $CD8^+$ T lymphocytes were analyzed using ELISPOT according to the manufacturer's instruction for evaluating the cell mediated immunity. Briefly, the immune-spot plate was coated with purified IFN-γ antibody overnight, followed by blocking with blocking buffer containing 10% fetal bovine serum. Rotavirus vaccine LLR was used as stimulator with 5.0 $lgCCID_{50}$ per well in 100 μl volume. Subsequently, the isolated $CD4^+$ and $CD8^+$ T lymphocytes were added with 10^5 per well. After incubated in 37 °C, 5% CO_2 incubator for 20 h, HRP-labeled anti-IFN-γ antibody and AEC substrate was used for detection under CTL ELISPOT reader.

2.3. Passive transfusion of isolated T lymphocytes to nude mice and challenge with EDIM

The nude mice were divided into four groups (18 nude mice each group), which were passively transfused with $CD4^+$ T lymphocytes isolated from primed (group 3) or naïve (group 4) mice, and $CD8^+$ T lymphocytes isolated from primed (group 5) or naïve (group 6) mice, respectively. The amounts of transfused $CD4^+$ and $CD8^+$ T lymphocytes were 1.5×10^6 and 0.75×10^6 cells/200 μl respectively to obtain the equivalent count of T lymphocytes in peripheral blood in nude mice as in normal mice. Simultaneously, the transfused nude mice were challenged with 0.3 ml EDIM (1.2×10^7 $CCID_{50}$/ml) by gavage. Post-challenged, the status of these mice were observed and evaluated to determine whether and how the transfused $CD4^+$ and $CD8^+$ T lymphocytes played a role in virus clearance, estimated by the indexes including body weight, rotavirus and RV-IgA in fecal, sera RV-IgA and RV-IgG, intestinal pathogenecity and viral distribution in small intestine. For this purpose, they were weighed each day from day 0 to day 15 and sampled the fresh stool from day 0 to day 8 for IgA and viral shedding assay. The blood samples were collected through venous sinus of eye orbit from four randomized picked out mice every two days for IgA and IgG assay.

2.4. ELISA to measure rotavirus, to measure anti-RV IgA, IgG in serum and sIgA in feces

Rotavirus in feces was measured by using ProSpecT Rotavirus Microplate Assay (OXOID) according to manufacturer's instruction. The ELISA to measure rotavirus-specific IgA, IgG and sIgA has been used in home. Briefly, plates were coated with diluted EDIM virus overnight at 4 °C, followed by blocked at 37 °C for 2 h and dried overnight at room temperature. Then the serum or treated feces were incubated in the coated plates with an initial dilution of 1/10. The amount of antibody was reflected by the values of OD following incubation with a HRP-conjugated goat anti-mouse IgA and IgG antibody. Sample was considered positive when the ratio of sample OD/negative OD ≥ 2.1.

Table 1
The purity assay of the isolated $CD4^+$/$CD8^+$ T cells.

	Purity (%, average/stdev)	
	Group 1	Group 2
$CD4^+$ T cells	92.93/4.66	92.63/4.59
$CD8^+$ T cells	92.51/3.26	91.83/2.37

Table 2
The activity assay of the isolated $CD4^+$/$CD8^+$ T cells.

	Group 1	Group 2	
$CD4^+$ T cells	Total cells No. (average/stdev)	111.2/7.2	115.2/10.0
	Dead cells No. (average/stdev)	5.6/1.7	5.0/1.6
	Activity	96.96%	95.66%
$CD8^+$ T cells	Live cells No. (average/stdev)	58.6/4.7	59.2/7.2
	Dead cells No. (average/stdev)	5.4/1.8	5.4/2.7
	Activity	90.78%	90.88%

3. Results

3.1. Isolation and transfusion of $CD4^+$/$CD8^+$ T lymphocytes

In order to mimic the natural status of $CD4^+$/$CD8^+$ T lymphocytes circulating in peripheral blood of normal mice, the accounts of transfused $CD4^+$/$CD8^+$ T lymphocytes should keep consistence with that in normal mice. To achieve this goal, the purity (Table 1) and activity (Table 2) of isolated $CD4^+$/$CD8^+$ T lymphocytes were assayed. Subsequently, a serial concentration of T lymphocytes was transfused ($CD4^+$ T lymphocytes: 4.5×10^6, 1.5×10^6 and 0.5×10^6 per 200 μl; $CD8^+$ T lymphocytes: 2.25×10^6, 0.75×10^6 and 0.25×10^6 per 200 μl). The results showed that, when $CD4^+$ T lymphocytes and $CD8^+$ T lymphocytes were transfused with 1.5×10^6 and 0.75×10^6 cells/200 μl respectively, the count of T lymphocytes in peripheral blood of nude mice was equivalent to that in normal mice with the same age. The ELISPOT assay showed that there is a minor difference in IFN-γ-secreting T lymphocytes between LLR immunized mice and unimmunized mice, that is, 178.8 vs 110.8 IFN-γ-secreting $CD4^+$ T lymphocytes, and 154.8 vs 119.2 IFN-γ-secreting $CD8^+$ T lymphocytes per 100,000 cells as shown in Fig. 1.

3.2. Passively transfused $CD4^+$/$CD8^+$ T lymphocytes shortened viral shedding

In the group transfused with 1.5×10^6 primed $CD4^+$ T lymphocytes, the mice were challenged with EDIM strain and the viral shedding reached to peak in day 1 post challenged, followed by a decline to negative in day 4. The viral shedding in the group transfused with 1.5×10^6 naïve $CD4^+$ T lymphocytes also reached to peak in day 1, but declined to negative in day 7, which was 3 days later than in primed group (Fig. 2A). In the groups transfused with 0.75×10^6 primed $CD8^+$ T lymphocytes, the viral shedding reached to peak in day 1 samely as in $CD4^+$ group, followed by a decline to negative in day 5, which was 1 day later than that in the group of 1.5×10^6 primed $CD4^+$ T lymphocytes, but 1 day earlier than that in the group of 0.75×10^6 naïve $CD8^+$ T lymphocytes (Fig. 2B). Whatever in groups transfused with primed or naïve $CD4^+$ or $CD8^+$ T lymphocytes, viral shedding reached to peak in day 1 simultaneously. However, the viral shedding would be earlier terminated by transfused with T lymphocytes isolated from primed mice than that from naïve mice.

3.3. Passively transfused $CD4^+$ T lymphocytes enhanced sIgA production in feces but not $CD8^+$ T lymphocytes

In the groups transfused with 1.5×10^6 primed $CD4^+$ T lymphocytes, sIgA increased rapidly post challenged with EDIM and reached the peak and maintained in day 3 and day 4, followed by a decrease began from day 5 till to negative in day 8. Whereas, sIgA in control group slowly increased from day 3, reached the peak in day 5–6 and maintained for 1–2 days, followed by a decline in day 7 (Fig. 3A). However, in the group transfused with 0.75×10^6 primed $CD8^+$ T lymphocytes, sIgA increased slowly from day 5, reached the peak and remained the level from day 6 to day 8, which showed no difference with that in the control group (Fig. 3B).

3.4. Passively transfused $CD4^+$ T lymphocytes enhanced serum IgA and IgG production but not $CD8^+$ T lymphocytes

The results (Fig. 4A) showed that serum IgA began to rise in day 3, reached the peak in day 7 followed by a decline from day 9 in group transfused with 1.5×10^6 primed $CD4^+$ T lymphocytes. However, in the control group serum IgA began to rise in day 5, which was 2 days later compared to the former group, and the test value showed that there was a significant difference in the peak value between the two groups. As sIgA, serum IgA in the group transfused with 0.75×10^6 primed $CD8^+$ T lymphocytes showed no obvious difference with the control group. In the two groups, after challenged, serum IgA began to rise in day 7, reached the peak in day 11 and maintained (Fig. 4B).

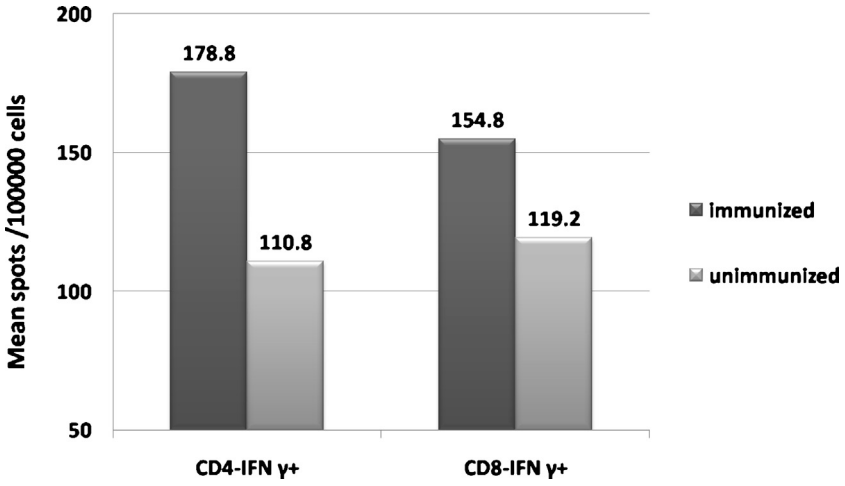

Fig. 1. IFN-γ-secreting $CD4^+$ and $CD8^+$ T cells isolated from LLR immunized and unimmunized mice.

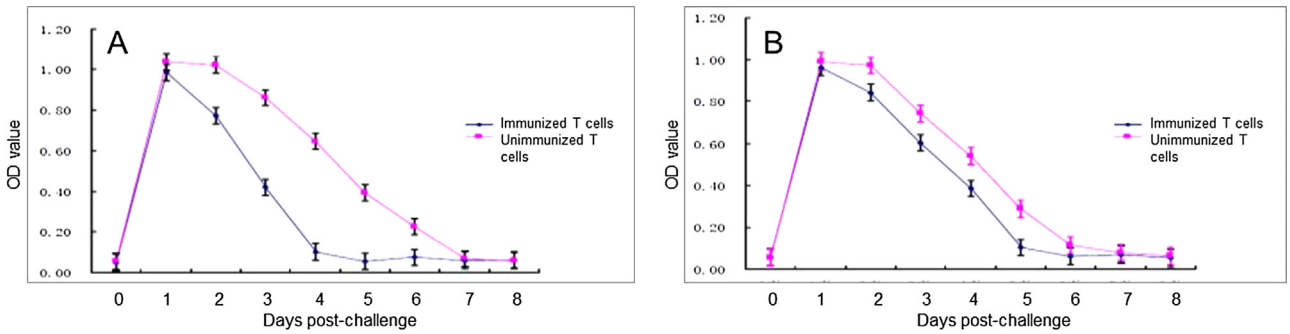

Fig. 2. Viral shedding in nude mice after transfused with $CD4^+$ (A) and $CD8^+$ (B) T cells isolated from LLR-immunized and unimmunized nude mice.

RV-IgG in the group transfused with 1.5×10^6 primed $CD4^+$ T cells increased in day 3 post-challenged and reached the peak in day 7, which was two-fold higher than that in day 0. However, RV-IgG in control group increased in day 7 and reached the peak in day 11, which was 4 days delayed compared to that in the former group (Fig. 5A). Similar to sIgA and serum IgA, serum IgG in the group transfused with 0.75×10^6 primed $CD8^+$ T lymphocytes also showed no difference with the control group, with a slowly increasing began on day 5, reached to the peak in day 11, followed by a decline (Fig. 5B).

4. Discussion

As mentioned in Section 1, the humoral immunity against rotavirus, especially antibody was an important part of immunogenicity, while partially proven to be significant in animal and human beings (Franco and Greenberg, 1995; Jaimes et al., 2002, 2005; McNeal et al., 1997). However, the roles of T lymphocytes and passive immunology model were limitedly noticed in the previously published studies referred the evaluation of rotavirus vaccines in clinical trials.

In this study, we evaluated the cell-mediated immunity induced by LLR vaccine using two-week old nude mice as animal model. Back to 1990, Offit and Dudzik performed the passive transfusion of rotavirus-specific cytotoxic T cells into suckling mice to observe its role in protecting against gastroenteritis (Offit and Dudzik, 1990). However, suckling mice were not sufficient to present the function of T lymphocytes, owing to the existence of autologous T lymphocytes and potential maternal antibody. When the competent immunogenicity was established, the optimal age for challenge by EDIM would be passed, whereas economic nude mice were introduced to avoid this influence. The nude mice were transfused with T lymphocytes via i.p. route as Offit reported (Offit and Dudzik, 1990) instead of intravenous injection through tail veins, for two-week old nude mice were so tiny that intravenous injection through tail veins was impracticable. As expected in this study, both the transfused $CD4^+$ and $CD8^+$ T lymphocytes can be detected in peripheral blood through intraperitoneal route trasfusion.

Besides the route of transfusion, another issue should be noticed, that is the precise count of transfused T lymphocytes. Human RV-specific $CD4^+$ T lymphocytes circulating in blood can be transmigrated to the small intestine that attributed to their expression of intestinal homing receptor $\alpha4\beta7$ (Rojas et al., 2003; Weitkamp et al., 2005; Parra et al., 2014a), while adoptively transferred $CD8^+$ T cells leave the circulation and enter the intestinal tract after binding to specific receptors (vascular addressins) located on specialized capillary endothelial cells (Berg et al., 1989). However, the efficacy of uptake depended on the expression of such receptors in small intestine of nude mice. Therefore, the precise count of transfused $CD4^+$/$CD8^+$ T lymphocytes is crucial for evaluating virus clearance. In order to obtain the equivalent count of T lymphocytes in peripheral blood in nude mice as same as in normal mice, a serial concentration of T lymphocytes was transfused ($CD4^+$ T lymphocytes: 4.5×10^6, 1.5×10^6 and 0.5×10^6 per 200 μl; $CD8^+$ T lymphocytes: 2.25×10^6, 0.75×10^6 and 0.25×10^6 per 200 μl). Finally, the results showed that our goal could be achieved when $CD4^+$ T lymphocytes and $CD8^+$ T lymphocytes were transfused with 1.5×10^6 and 0.75×10^6 cells/200 μl respectively.

Paradox existed in the function of T lymphocytes against rotavirus infection. Nude mice have been reported to recover from murine rotavirus-caused gastroenteritis in the absence of a significant antibody or a functional T-cell response (Eiden et al., 1986). Recent studies also showed that circulating RV-T lymphocytes seem to have a relatively poor functional profile compared to tetanus toxoid and influenza (Parra et al., 2014b). RV-specific cytotoxic T cells, contrast to these reports, have been proven to passively protect against gastroenteritis in suckling mice (Offit and Dudzik, 1990). Therefore, to re-explore the role of RV-specific T cells, induced by rotavirus vaccine LLR in rotavirus clearance, two-week old nude mice were passively transfused with $CD4^+$ or $CD8^+$ T cells separately that isolated from primed mice.

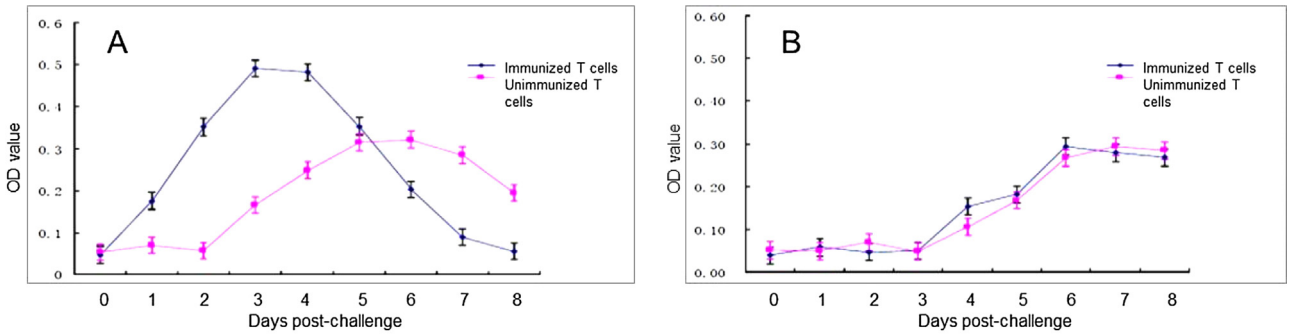

Fig. 3. Feces IgA changes in nude mice after transfused with $CD4^+$ (A) and $CD8^+$ (B) T cells isolated from LLR-immunized and unimmunized nude mice.

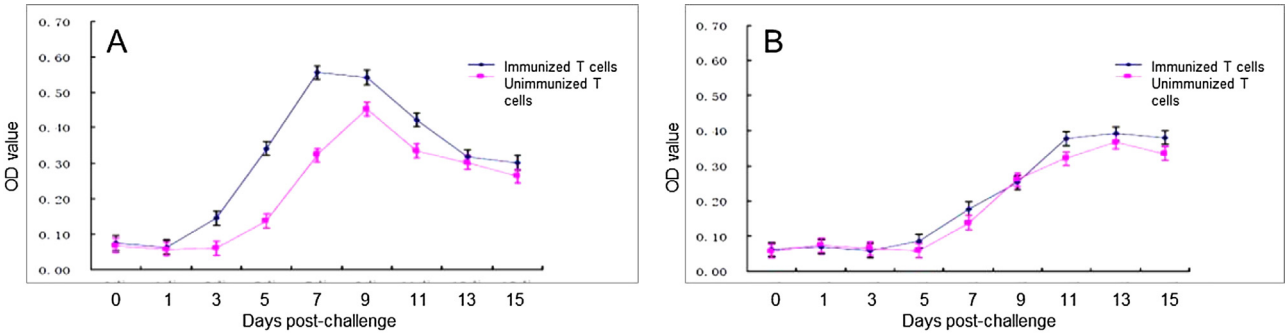

Fig. 4. Serum IgA changes in nude mice after transfused with $CD4^+$ (A) and $CD8^+$ (B) T cells isolated from LLR-immunized and unimmunized nude mice.

Compared to control group, the mice passively transfused $CD4^+$ T lymphocytes from primed mice expedited and enhanced the production of serum IgA, IgG and sIgA, and shortened the duration of viral shedding. The results demonstrated that, as expected, rotavirus specific $CD4^+$ T lymphocytes can be induced in mice through immunization with LLR vaccine, and the passively intraperitoneally transfusion of LLR-primed $CD4^+$ T lymphocytes transfer the immunological memory to nude mice for virus clearance. Previous studies proved that the memory $CD4^+$ T lymphocytes induced an earlier amplification and proliferation of B cells than naïve $CD4^+$ T lymphocytes (MacLeod et al., 2011). The rapid action of antibodies in our study was also motivated by the memory $CD4^+$ T lymphocytes specific to LLR vaccine.

Interestingly, unlike $CD4^+$ T lymphocytes, there was no much difference between primed and naïve $CD8^+$ T lymphocytes in virus clearance and antibody production in nude mice. In the previous studies, $CD4^+$ T lymphocytes alone were able to protect rotavirus infection, whereas it cannot be achieved with only $CD8^+$ T lymphocytes (VanCott et al., 2001). Moreover, in the infection models of poxvirus and listeria monocytogenes, the initiation of the memory function of $CD8^+$ T lymphocytes partly depended on $CD4^+$ T lymphocytes (Novy et al., 2011). In our study, $CD8^+$ T cell and $CD4^+$ T lymphocytes were passively transfused to nude mice separately. Consequently, the immunological memory of $CD8^+$ T lymphocytes cannot be activated without $CD4^+$ T lymphocytes. Therefore, the coordination of $CD4^+$ and $CD8^+$ T lymphocytes in rotavirus infections should be studied further.

Our findings demonstrated that, available nude mice were successfully used in evaluation of rotavirus vaccine, especially enhance cell-mediated immunity induced by rotavirus vaccine. In addition, as a lamb originated human rotavirus vaccine, LLR induced $CD4^+$ T cells response had the ability of immunological memory, accelerated virus clearance and rapid production of IgA, and shortened the viral shedding in nude mice compared to control which plays a predominant role in viral clearance. Disparately, LLR-induced weak $CD8^+$ T lymphocytes response, without the help of $CD4^+$ T lymphocytes, cleared virus infection through CTL instead of immunological memory, which was the same as in control group.

Another issue is that the percentage of antigen specific T lymphocytes may need to be determined in the transfused T lymphocytes, which could be the major contributor to the immunity. Previous studies showed that higher frequencies of RV specific IFN-γ-secreting $CD8^+$ and $CD4^+$ T lymphocytes circulate in symptomatically infected adults and RV-exposed laboratory workers, compared with healthy volunteers, but low level of IFN-γ-secreting $CD8^+$ and $CD4^+$ T lymphocytes in children with RV-induced diarrhea (Jaimes et al., 2002; Rojas et al., 2003; Mesa et al., 2010). Several studies also found that circulating RV-specific T lymphocytes have a poor functional profile or rotavirus is a relatively poor inducer of circulating memory T lymphocytes that secrete IFN-γ (Jaimes et al., 2002; Parra et al., 2014b). In this study, T lymphocytes isolated from spleen of mice was used for passive transfusion, so we also evaluated the roles of IFN-γ-secreting $CD4^+$ and $CD8^+$ T lymphocytes isolated from spleen of mice in cell-mediated immunity against rotavirus re-infection. The ELISPOT assay showed that there is a minor difference in IFN-γ-secreting T lymphocytes between LLR primed and naïve mice. However, the difference of IFN-γ-secreting $CD4^+$ T lymphocytes between LLR primed and naïve mice (178.8 vs 110.8 per 100,000 cells) was more remarkable than the difference of IFN-γ-secreting $CD8^+$ T lymphocytes between LLR primed mice and naïve mice (154.8 vs 119.2 per 100,000 cells). The results verified the above findings once again indirectly, that IFN-γ-secreting $CD4^+$ T lymphocytes played a more important role in cell-mediated immunity against rotavirus infection than IFN-γ-secreting $CD8^+$ T lymphocytes.

In summary, the cell-mediated immunity against rotavirus was proven to be significant in mice once more, especially the immunological memory transferred by RV specific $CD4^+$ T lymphocytes. It provides a new way to indicate the effectiveness of rotavirus vaccines in animal or human beings.

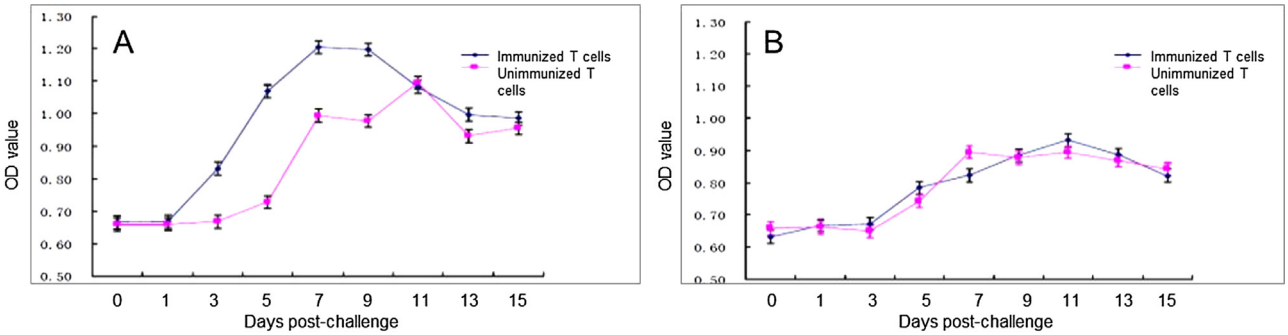

Fig. 5. Serum IgG changes in nude mice after transfused with $CD4^+$ (A) and $CD8^+$ (B) T cells isolated from LLR-immunized and unimmunized nude mice.

Conflict of interest

All the authors declare that no competing interests exist.

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Author contributions

ZLL, TG designed and coordinated study; ZLL, JLD, QCY, YYL, YL performed experiments; ZLL, JLD analyzed data; JLD, ZLL, YYL, YL, QCY, YCL, TG contributed to the writing and editing of this manuscript; JLD, TG wrote the paper. All authors read and approved the final manuscript.

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