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Immunohistochemical analysis of T and B cell proliferation in the spleens of the immunized and infected *Rhesus macaques* with plague vaccines and *Yersinia pestis*

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ABSTRACT: In our previous study, more B and T cells were observed in the splenic tissues from the immunized *Rhesus macaques* with plague vaccines than from normal animals. However, whether these cells represent proliferating B and T cells elicited by plague vaccines is unclear. To answer this question, the proliferation of T and B cells was examined by means of double immunohistochemistry in the spleens from the *Rhesus macaques* immunized with subunit vaccine SV1 (20 μg F1 + 10 μg rV270), and those both immunized with SV1, SV2 (200 μg F1 + 100 μg rV270), live attenuated vaccine EV or alhydrogel and challanged with *Y. pestis*, respectively. Double staining results showed that higher B cell proliferation in germinal centers and more resting B cells in marginal zone (MZ) were observed in the tested animals than in the normal animal, and that more resting T cells in periarterial lymphatic sheath (PALS) of the spleen tissues from the tested animals than from the normal animal, indicating that naïve T cells might have undergone a proliferation process at earlier stages of the immunization. Germinal centers in the control spleen tissues were incomplete, which was attributed to the histopathological lesions caused by virulent *Y. pestis*. The size of germinal centers in the spleen tissues from the animals both immunized with SV1, SV2 or EV and infected with *Y. pestis* was larger than that from the animal only immunized with SV1. In conclusion, B cell proliferation, more resting B cells, more resting T cells and expanded germinal centers are the signs of eliciting specific humoral immunity and keeping immune memory response. These results were also consistent with results of our previous study that the immunized animals with SV1, SV2 or EV elicited higher antibody and IL-4 production.

KEY WORDS: plague vaccines; *Rhesus macaques*; immunohistochemistry; *Y. pestis*; lymphocyte proliferation

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Plague is a zoonotic disease caused by Gram-negative bacterium *Yersinia pestis* (*Y. pestis*), which is usually transmitted to humans from infected rodents via the bite of an infected flea[1]. Historically, plague was an awful infectious disease afflicting human populations and re-shaping the human civilization, leading to millions of deaths. Plague has been classified as a re-emerging disease recently by the World Health Organization[2] and has attracted a considerable attention because of its potential misuse as an agent of biological warfare or bioterrorism[3].

Both live attenuated and killed whole cell vaccines against plague have been used in humans since the early part of the 20th century. However, killed whole cell vaccines against *Y. pestis* only have a short protection against bubonic plague and are needed for frequent boosting to maintain immunity[4]. Live attenuated vaccine EV was effective against bubonic and pneumonic plague, but it showed side effects of varying severity and has not been used in the Western world[4-6]. The DNA vaccine based on *Y. pestis* F1 and LcrV antigens alone or in combination was efficacious against both bubonic and pneumonic plague[7-9]. However, DNA vaccines usually elicit lower and slower immune responses than conventional vaccines, and gene gun immunization that delivers DNA-coated particles

into the dermis of the skin needs to be used for improving immune responses[8,10]. In contrast, subunit vaccines have obvious advantages over the traditional vaccines and DNA vaccines in terms of safety of use. At present, the plague subunit vaccines, including F1 and V antigen, are being developed[11].

To develop a safe and effective plague subunit vaccine, we have extracted the highly-purified natural F1 antigen from *Y. pestis* EV by a new purification strategy[12] and prepared a non-tagged rV270 protein containing amino acids 1 to 270 of LcrV by thrombin digestion[13]. The subunit vaccine comprising a dose level of 20 μg F1 and 10 μg rV270 that were adsorbed to 25% (v/v) alhydrogel in PBS buffer (SV1) has been identified as the optimal formula in mice, which provided a good protective efficacy against *Y. pestis* challenge in mice, guinea pigs and rabbits[14].

To further observe the dose-dependent efficacy and safety of the subunit vaccine F1 + rV270, a much higher dose level of SV2 (200 μg F1 + 100 μg rV270 + alhydrogel in PBS) was designed. *Rhesus macaques* were used to evaluate the protective efficacy of the vaccines SV1, SV2 or live attenuated vaccine EV (not contains alhydrogel adjuvant) against virulent *Y. pestis* challenge. During the animal experiment, one of four immunized animals in SV1 group was dead after transportation from Beijing to Qinghai, which may caused by high altitude reaction. Complete protection was observed in other immunized animals against subcutaneous challenged with 6×10^6 CFU of virulent *Y. pestis* strain 141, whereas the control animals succumbed to a same dose of *Y. pestis* 141 challenge within 3 to 5 days. We compared the antibody responses among SV1, SV2 and EV in Chinese-origin *Rhesus macaques*. There was no significant anti-F1 IgG titer difference among three groups of immunized animals after immunization. Post-mortem analysis of *Y. pestis* load in different organs of the animals that survived from the challenge showed that *Y. pestis* has been eliminated from the survived animals, whereas *Y. pestis* have been isolated from organs of the two control animals that died of challenge.

In the present study, we examined T and B lymphocyte proliferation of spleen samples obtained from one dead animal immunized with SV1, the other immunized *Rhesus macaques* with SV1, SV2, EV or aluminum hydroxide adjuvant in PBS buffer after challenging with virulent *Y. pestis* strain 141, and one normal animal by means of immunohistochemistry. To our knowledge, this is the first report that T and B lymphocyte proliferation was identified in spleen tissues after immunization of plague subunit vaccine or infection of *Y. pestis*.

Materials and methods

Vaccines and animals

The subunit vaccine F1 + rV270 comprised native F1 and rV270 antigens that were adsorbed to 25% (v/v) aluminum hydroxide adjuvant in PBS buffer. Adsorption of the proteins to the adjuvant was checked by subtracting protein in the supernatant from the total amount of proteins added. The live attenuated vaccine EV was obtained from the Lanzhou Institute of Biological Products (LIBP), China. Adult male and female Chinese *Rhesus macaques* were obtained from Laboratory Animal Center, Academy of Military Medical Science, China (licensed by Ministry of Health in General Logistics Department of Chinese People's Liberation Army). All the animals were 3-6 years old and weighed between 3 and 6 kg. The animal experiments were conducted strictly in compliance with the Regulations of Good Laboratory Practice for nonclinical laboratory studies of drug issued by the National Scientific and Technologic Committee of People's Republic of China.

Animal immunizations

Fourteen Chinese *Rhesus macaques* were divided into four groups, including three experimental groups and one control group. Each one of three experimental groups contained four animals (two male and two female), and the alum-immunized control group had two animals (one male and one female). Three experimental groups of animals were intramuscularly injected in the forelimbs with the vaccines SV1, SV2 or EV [1/2 of the human dose (8×10^8 cells)], respectively. Each animal of the control group was intramuscularly given 25% aluminum hydroxide adjuvant only. After the first immunization, all the animals were boosted with

an identical dose at the same injection sites on day 21.

Challenge with *Y. pestis*

Rhesus macaques were challenged with the virulent *Y. pestis* 141 strain, which was isolated from *Marmota himalayana* in Qinghai-Tibet Plateau and has a median lethal dose (MLD) of 5.6 colony-forming Unit (CFU) for BALB/c mice, 17.8 CFU for guinea pigs and New Zealand White rabbits by the subcutaneous route. All the immunized animals, except for one dead animal before being challenged, were challenged on week 10 after the primary immunization with 6×10^6 CFU by the subcutaneous route, and then closely observed for 14 days. All the animal experiments were performed in ABSL-3 laboratory.

Immunohistochemistry (IHC)

Samples from one dead animal in SV1 group and two control animals were collected immediately after death and fixed in neutral buffered 10% formalin. Other animals, including one normal animal and other experimental animals, were necropsied immediately after euthanasia at 14-day time point and the harvested samples were fixed in neutral buffered 10% formalin. IHC staining was performed following the user's manual of the DS-0001 Polymer kit (ZSGB-Bio). Briefly, after paraffin-embedded tissue sections were deparaffinized and rehydrated, the sections were submitted to expose antigen in citrate buffer solution by microwaving for 20 min, and incubated with 3% H_2O_2 in methanol for 10 min to block endogenous peroxidase activity. The sections were incubated with rabbit anti-human CD79a monoclonal antibody and mouse anti-human Ki67 monoclonal antibody for examining proliferating B lymphocytes, whereas rabbit anti-human CD3 monoclonal antibody and mouse anti-human Ki67 monoclonal antibody were used for examining proliferating T lymphocytes. Negative control sections were incubated with PBS instead of primary antibody. The sections were incubated with the HRP-labeled goat anti-mouse IgG or AP-labeled goat anti-rabbit IgG (ZSGB-Bio) for 30 min at 37 ℃. The slides were stained with 3, 3'-diaminobenzidine tetrahydrochloride (DAB), and then with AP-Red. Finally, the sections were rinsed, counterstained, dehydrated, cleaned, mounted and examined by light microscopy.

Results

Evaluation of B cell proliferation in the spleens

The spleen sections were double stained by rabbit anti-human CD79a monoclonal antibody and mouse anti-human Ki67 monoclonal antibody to detect the B cells and proliferating cells, respectively. The B lymphocytes were visualized as those expressing CD79a (mauve surface stain), and proliferating cells were visualized as those expressing Ki67 (straw yellow surface stain). Double staining results showed that CD79a was expressed at a low level (Figure 1a, yellow arrows) and no evident Ki67 antigen expression was observed in folliculi of the spleens from the normal animal (Figure 1a). Compared with the normal animal, high-level expression of CD79a and Ki67 antigens was observed in germinal centers of spleens from the animal only immunized with SV1 (Figure 1e, blue arrow) or those both immunized with SV1 (Figure 1b, blue arrow), SV2 (Figure 1c, blue arrow) or EV (Figure 1d, blue arrow) and challenged by *Y. pestis*. In addition, single CD79a antibody staining could be seen in marginal zone of spleens from the animal only immunized with SV1 (Figure 1e, yellow arrow) or those both immunized with SV1 (Figure 1b, yellow arrow), SV2 (Figure 1c, yellow arrow) or EV (Figure 1d, yellow arrow) and challenged by *Y. pestis*. We also found that the size of germinal centers in the animal only immunized with SV1 was smaller than that in animals both immunized with SV1, SV2 or EV vaccine and infected with *Y. pestis*. Double staining of the spleen tissues from the control animals (Figure 1f) showed that germinal centers were impaired, but high-level expression of CD79a was found in marginal zone (B-cell area, yellow arrow) of the spleens.

Single staining with Ki67 or CD79a was used as the standard control to evaluate the results of double staining. The staining with Ki67 monoclonal antibody showed high-level Ki67 expression in germinal center (Figure 1g, blue arrows), whereas the staining with CD79a monoclonal antibody showed high-level CD79a expression both in germinal center (Figure 1h, green arrow) and mar-

ginal zone (Figure 1h, yellow arrows) of the spleen tissue from the immunized animal. Negative control spleen tissue that was incubated with PBS instead of CD79a and Ki67 monoclonal antibody did not show staining by IHC with the HRP-labeled goat anti-mouse IgG and AP-labeled goat anti-rabbit IgG (Figure 1i).

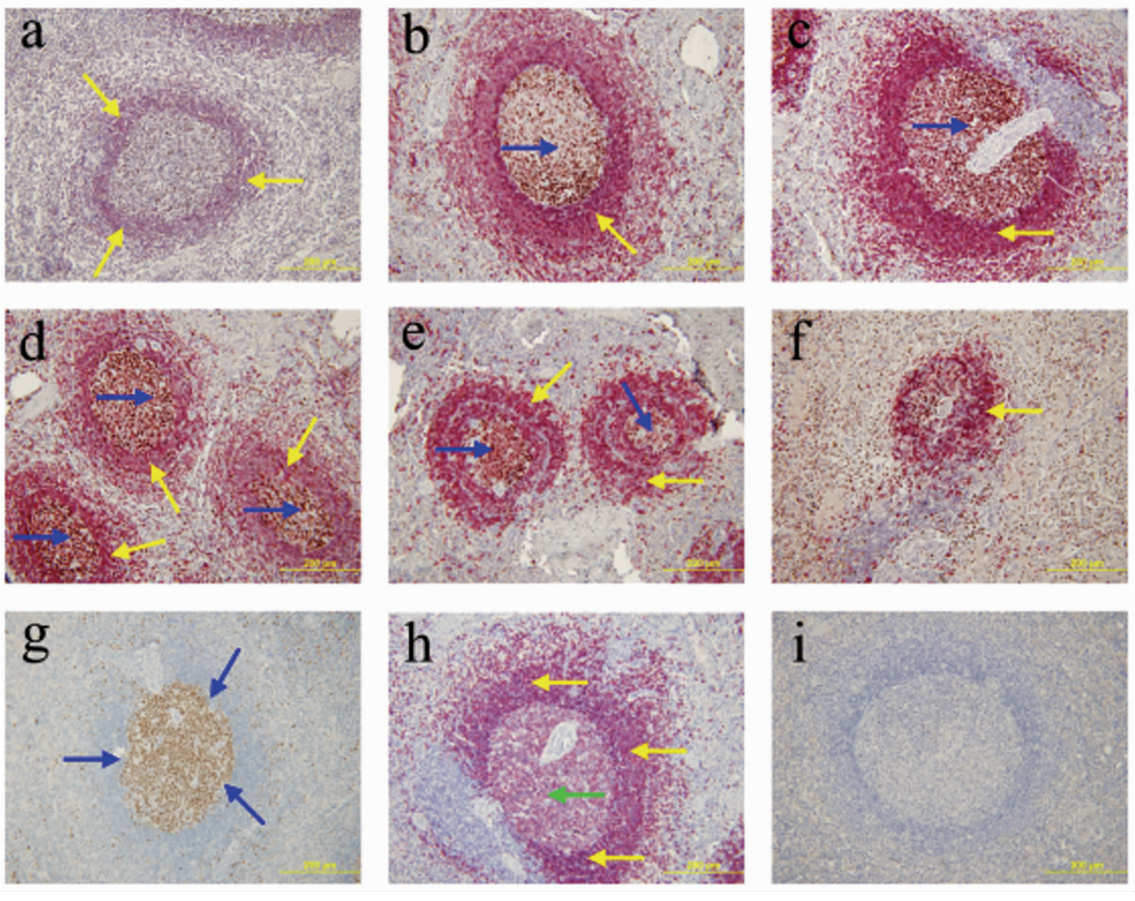

Fig. 1 Double staining of the spleen sections with rabbit anti-human CD79a monoclonal antibody and mouse anti-human Ki67 monoclonal antibody to examine the proliferating B cells

The spleen sections were from the normal *Rhesus macaque* (a), the animal immunized with SV1 and infected with *Y. pestis* (b), the animal immunized with SV2 and infected with *Y. pestis* (c), the animal immunized with EV and infected with *Y. pestis* (d), the immunized animal with SV1 (e), the control animal infected with *Y. pestis* (f). Single staining with Ki67 or CD79a was used as the standard control. The staining with Ki67 monoclonal antibody for evaluation of proliferating cells (g), the staining with CD79a monoclonal antibody for examining B cells (h), negative control spleen tissue that was incubated with PBS instead of CD79a and Ki67 monoclonal antibody (i).

The intensity of Ki67 and CD79a antigen expression in paraffin-embedded spleen tissue sections by IHC staining was subjectively scored using following system according to Confer's report[15] and summarized in Table 1.

Evaluation of T cell proliferation in the spleens

The spleen sections were double stained using rabbit anti-human CD3 monoclonal antibody and mouse anti-human Ki67 monoclonal antibody to determine T cells and the proliferating cells, respectively. T cells expressing CD3 were visualized as mauve surface stain, and proliferating cells were visualized as those expressing Ki67 (straw yellow surface stain). Double staining results showed that CD3 was expressed at a low level in periarterial lymphatic sheath (PALS) T-cell area (Figure 2a, yellow arrows) and no evident Ki67 antigen expression was observed in white pulp of the spleen from the normal animal (Figure 2a). Compared with the normal animal, high-level expression of CD3 or Ki67 antigen was observed in PALS T-cell areas (yellow arrows) or germinal center B-cell areas (blue arrows) of the spleens from the animal

only immunized with SV1 (Figure 2e) and those both immunized with SV1 (Figure 2b), SV2 (Figure 2c) or EV (Figure 2d) and challenged by *Y. pestis*. Double staining of the spleen tissues from the control animals (Figure 2f) showed high-level expression of CD3 in T-cell area (yellow arrows) of the spleens, but germinal centers were impaired.

Single staining with Ki67 or CD3 monoclonal antibody that was used as the standard control shows high-level Ki67 (Figure 2g) or CD3 (Figure 2h) expression in B-cell area (blue arrows) or T-cell area (yellow arrows) of the spleen tissue from the immunized animal. Negative control spleen tissue that was incubated with PBS instead of CD3 and Ki67 monoclonal antibody did not show staining by IHC with the HRP-labeled goat anti-mouse IgG and AP-labeled goat anti-rabbit IgG (Figure 2i).

The intensity of Ki67 and CD3 antigen expression in paraffin-embedded spleen tissue sections by IHC staining was subjectively scored as described above and also summarized in Table 1.

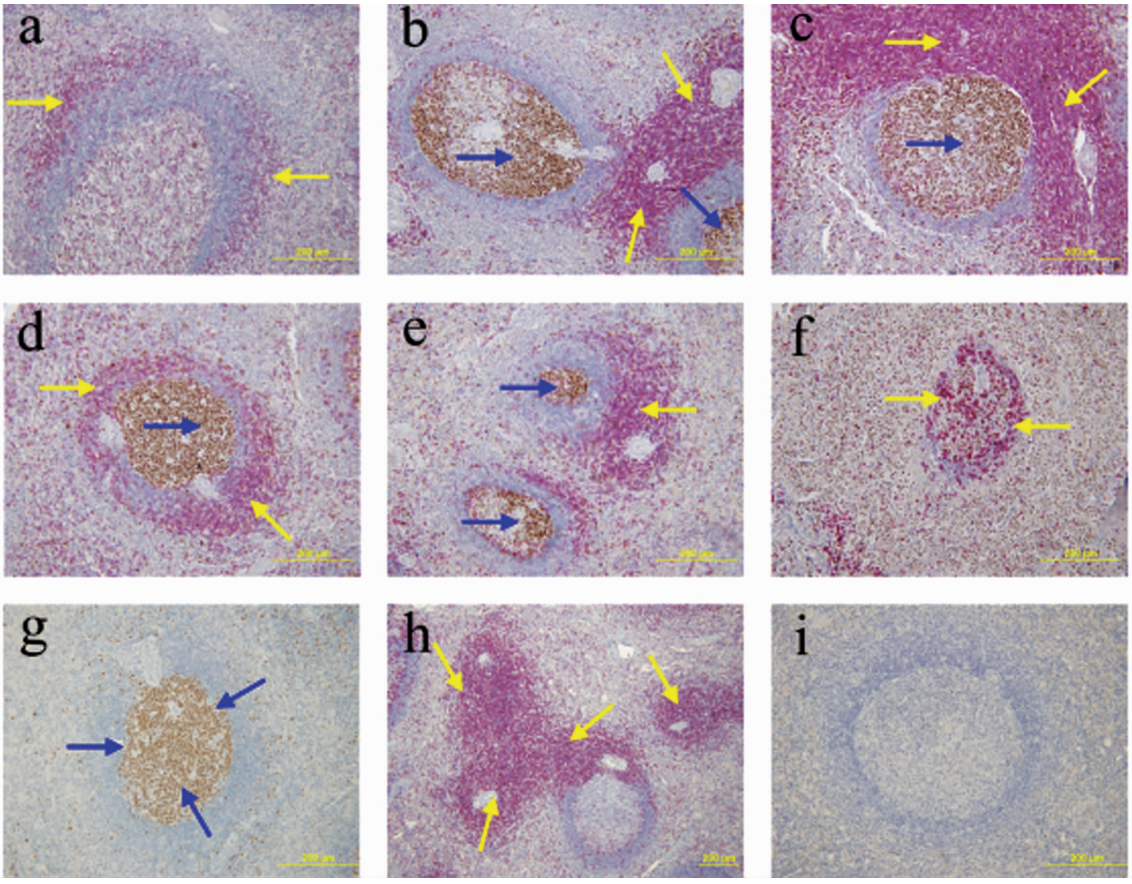

Fig. 2 Double staining of the spleen sections with rabbit anti-human CD3 monoclonal antibody and mouse anti-human Ki67 monoclonal antibody to examine the proliferating T cells

The spleen sections were from the normal *Rhesus macaque* (a), the animal immunized with SV1 and infected with *Y. pestis* (b), the animal immunized with SV2 and infected with *Y. pestis* (c), the animal immunized with EV and infected with *Y. pestis* (d), the immunized animal with SV1 (e), the control animal infected with *Y. pestis* (f). Single staining with Ki67 or CD3 was used as the standard control. The staining with Ki67 monoclonal antibody for evaluation of proliferating cells (g), the staining with CD3 monoclonal antibody for examining T cells (h), negative control spleen tissue that was incubated with PBS instead of CD3 and Ki67 monoclonal antibody (i).

Discussion

Y. pestis is a facultative intracellular bacterial pathogen that can survive within macrophages and go on to express various virulence determinants until the latest stages of infection[16]. It is generally accepted that an ideal plague vaccine should have both humoral and cellular immune responses. Humoral immunity relies on antibodies to neutralize extracellular bacteria and toxins, while cellular immunity contributes to elimination of intracellular

Tab. 1 T and B cell proliferation responses in the spleen sections of *Rhesus macaques* immunized with plague vaccines and challenged with *Y. pestis*

Groups of *Rhesus macaques*	B lymphocytes			T lymphocytes		
	CD79a	Ki67	CD79a + Ki67	CD3	Ki67	CD3 + Ki67
Negative control	−	−	×	−	−	×
Normal	+	−	×	+	−	×
Control	++	++	++	++	++	×
SV1	+++	+++	+++	++	++	×
SV1 + Infection	+++	+++	+++	+++	+++	×
SV2 + Infection	+++	+++	+++	+++	+++	×
EV + Infection	+++	+++	+++	++	+++	×

Note: The scoring system is as follows: "−", no detectable antigen; "+", antigen faintly detected; "++", moderate expression; "+++", high-level antigen staining; "×", no combined staining.

pathogens. It has been demonstrated that cell-mediated immunity is important for a plague vaccine to protect against pulmonary *Y. pestis* infection[17]. In our previous study, analysis of anti-F1 and anti-rV270 IgG1/IgG2 subclasses in the immunized mice with SV1[14] and determination of serum cytokines in the immunized *Rhesus macaques* with SV1, SV2 or EV showed that EV could induce both humoral-and cell-mediated immunity[18], whereas effective cellular immunity was not demonstrated in subunit vaccines in alhydrogel. These results are consistent with the other reports that subunit vaccines effectively induce robust responses for IgG1, suggesting a Th2 humoral-mediated immunity, whereas live attenuated vaccine EV responded with the predominant production of IgG1 and IgG2a isotypes, indicating both Th2 and Th1 responses[19-20]. The potential of the live attenuated vaccine to elicit a Th1 response is the characteristic of cell-mediated immunity[6, 17, 21].

In our previous study, a single immunohistochemical staining with CD79a (a B cell marker) for B cells or CD3 (a T cell marker) for T cells was used to identify proliferating B or T cells in paraffin wax sections of the spleens from the immunized *Rhesus macaques* with plague vaccines. The results indicated that the spleen tissues from the immunized animals with plague vaccines exhibit statistically more B and T cells than those from normal animal that was neither immunized nor infected with *Y. pestis* (data not shown). However, it is unclear that whether these cells represent proliferating B and T cells. A nuclear antigen Ki-67 expressed in the G1, G2, S, and M phases but not the G0 phase of the cell cycle has been widely used as a marker of proliferating or cycling cells[22]. In the present study, we use expression of the Ki-67 antigen combining with that of CD79a or CD3 to identify proliferating B or T cells in response to immunization or infection by immunohistochemistry. To our knowledge, this is the first report that T and B lymphocyte proliferations were determined in spleen tissues after immunization with plague subunit vaccines or infection with virulent *Y. pestis*.

The spleen tissues from the *Rhesus macaques* immunized only with SV1 and from those both immunized with SV1, SV2 or EV and challenged with virulent *Y. pestis* showed the formation of germinal centers in splenic follicle. Except for incomplete germinal centers in the control tissues, all the germinal centers are intact and are surrounded by a concentric ring of marginal zone lymphocytes. Double staining with CD79a and Ki67 indicated that in addition to greater proliferating B cells are localized in the germinal centers, a large numbers of resting B cells are recruited to the marginal zone of splenic follicle. These results are consistent with other reports that within GC, activated B cells undergo vigorous proliferation, somatic mutation of IgV region genes, Ig isotype switching, and selection after interaction with specific antigens[23-24]. Marginal zone B cells in spleens are thought to be part of the recirculating memory B-cell pool. After marginal zone B cells migrate into the germinal center, they can present the antigen to the germinal center B cells. The follicular center cells bound to the presented antigen can proliferate and form the germinal center reaction, thus expanding the pool of B cells responding to the antigen and differentiating into plasma cells secreting antigen specific immunoglobulin and new memory B

cells[25]. It is evident that germinal center formation, greater proliferating B cells in the germinal centers and a large numbers of resting B cells in the marginal zone of splenic follicle are the signs of eliciting specific humoral immunity and keeping immune memory response.

Our results also showed that the size of germinal centers in the spleens of animals immunized with SV1, SV2 or EV and then infected with virulent *Y. pestis* was significantly increased compared with that of the animal only immunized with SV1. The germinal center reaction is the basis of T-dependent humoral immunity against foreign pathogens. Germinal centers represent a unique collaboration between proliferating antigen-specific B cells, T follicular helper cells, and the specialized follicular dendritic cells that constitutively occupy the central follicular zones of secondary lymphoid organs. The primary function of germinal centers is to produce the high-affinity antibody-secreting plasma cells and memory B cells that ensure sustained immune protection and rapid recall responses against previously encountered foreign antigens[26]. Given that in our previous study, a significant anti-F1 antibody titer boost was observed in three groups of the immunized Chinese-origin *Rhesus macaques* after challenge with virulent *Y. pestis*[18]. There is a positive correlation between the level of antibody production and the expansion of germinal centers after reencountering the same antigens. This correlation is consistent with a report that rechallenge with T cell-dependent Ags induces memory B cells to re-enter germinal centers and undergo further expansion and differentiation into plasma cells (PCs) and secondary memory B cells[27].

Additionally, in our previous study of immunological responses and protective efficacy of SV1, SV2 and EV in *Rhesus macaques*, three tested groups of *Rhesus macaques* had significant higher IL-4 level than that in the control group. It has been demonstrated that IL-4 is an important activator of humoral immunity and implicate in both antimicrobial host defense and pathogenesis of diseases with an inflammatory component[28]. Therefore, antibody production in the immunized *Rhesus macaques* with SV1, SV2 or EV is based on proliferating B cells in germinal center and the production of cytokine IL-4. Antibody titer is the indicator reflecting the state of humoral immunity, and IL-4 is a Th2 cytokine that promotes antibody dependent immune responses[29].

T cell proliferation is an indicator reflecting the state of cellular immunity[30]. In a previous report on lymphocyte proliferation, the ability of the peptides or peptide conjugates of *Y. pestis* F1 antigen to induce a cell-mediated immunity response was evaluated *in vitro* by detecting the splenocyte proliferative response in outbred mice. High T cell proliferation was observed after peptide-primed spleen cells were stimulated *in vitro* with the peptides and peptide conjugates of F1 antigen[31]. To identify whether proliferation of T cells is elicited in response to immunization or infection in secondary lymphoid tissue, the spleen tissues from the only immunized *Rhesus macaques* with SV1 and from both immunized animals with SV1, SV2 or EV and challenged with virulent *Y. pestis* were analyzed by immunohistochemistry. Double staining of spleen tissues with anti-CD3 monoclonal antibody and anti-Ki67 monoclonal antibody showed no proliferating T cell in PALS (T-cell area), but there are more resting T cells recruited to T-cell area compared with normal spleen tissue. These results indicated that T lymphocyte proliferation may occur at earlier stages of immunization or infection because the tissues were only examined 70 days after the immunization or 14 days after infection with *Y. pestis*. Although T-cell proliferation was not observed by double staining method in PALS of the spleen tissues, we can indirectly assess the extent of T-cell proliferation by comparison with the normal tissue. More resting T cells in PALS indicated that naïve T cells in spleens have ever undergone a proliferation process after they encounter antigen first in the T-cell zone of spleens. Normally memory T cells similar to naïve T cells stay quiescent in the G_0 phase of the cell cycle, which have longer life span than naïve T cells[32-33]. Therefore, the resting T cells in PALS of spleens may be mainly composed of memory T cells. Following antigen stimulation, quiescent T cells exit the G0 phase and enter into the cell cycle to undergo cell division. Cell division drives clonal expansion to generate a pool of T cells that are capable of recognizing a specific antigen[33].

The germinal centers in the immunized animals are intact and are surrounded by a concentric

ring of marginal zone lymphocytes, whereas they are incomplete in the control animals. This result is attributed to the histopathological lesions caused by virulent *Y. pestis*. In our previous study[34], the spleen tissues from the *Rhesus macaques* both immunized with SV1, SV2 or EV and infected with virulent *Y. pestis* and from the control animals were examined by histopathological methods. Compared with the spleen tissues of normal animal, no change in histopathology was found in all examined tissues of the immunized animals with SV1, SV2 and EV, whereas the control animals showed evident alterations in the spleens. The control spleen tissues had the reduced number of white pulp, acinus lienalis and lymphocytes and displayed splenic cord dropsy.

References

[1]Perry RD, Fetherston JD. *Yersinia pestis* — etiologic agent of plague[J]. Clin Microbiol Rev, 1997, 10(1): 35-66.

[2]Williamson ED. Plague vaccine research and development[J]. J Appl Microbiol, 2001, 91(4): 606-608. DOI: 10.1046/j.1365-2672.2001.01497.x

[3]Riedel S. Plague: from natural disease to bioterrorism[J]. Proc (Bayl Univ Med Cent), 2005, 18(2): 116-124.

[4]Russell P, Eley SM, Hibbs SE, et al. A comparison of Plague vaccine, USP and EV76 vaccine induced protection *against Yersinia* pestis in a murine model[J]. Vaccine, 1995, 13(16): 1551-1556.

[5]Morton M, Garmory HS, Perkins SD, et al. A *Salmonella enterica* serovar Typhi vaccine expressing *Yersinia pestis* F1 antigen on its surface provides protection against plague in mice[J]. Vaccine, 2004, 22(20): 2524-2532. DOI: 10.1016/j.vaccine.2004.01.007

[6]Williamson ED, Eley SM, Griffin KF, et al. A new improved sub-unit vaccine for plague: the basis of protection[J]. FEMS Immunol Med Microbiol, 1995, 12(3-4): 223-230. DOI: 10.1111/j.1574-695X.1995.tb00196.x

[7]Wang S, Heilman D, Liu F, et al. A DNA vaccine producing LcrV antigen in oligomers is effective in protecting mice from lethal mucosal challenge of plague[J]. Vaccine, 2004, 22(25-26): 3348-3357. DOI: 10.1016/j.vaccine.2004.02.036

[8]Garmory HS, Freeman D, Brown KA, et al. Protection against plague afforded by immunisation with DNA vaccines optimised for expression of the *Yersinia pestis* V antigen[J]. Vaccine, 2004, 22(8): 947-957. DOI: 10.1016/j.vaccine.2003.09.020

[9]Grosfeld H, Cohen S, Bino T, et al. Effective protective immunity to *Yersinia pestis* infection conferred by DNA vaccine coding for derivatives of the F1 capsular antigen[J]. Infect Immun, 2003, 71(1): 374-383. DOI: 10.1128/IAI.71.1.374-383.2003

[10]Bennett AM, Phillpotts RJ, Perkins SD, et al. Gene gun mediated vaccination is superior to manual delivery for immunisation with DNA vaccines expressing protective antigens from *Yersinia pestis* or Venezuelan Equine Encephalitis virus[J]. Vaccine, 1999, 18(7-8): 588-596. DOI: 10.1016/S0264-410X(99)00317-5

[11]Leary SE, Griffin KF, Garmory HS, et al. Expression of an F1/V fusion protein in attenuated *Salmonella typhimurium* and protection of mice against plague[J]. Microb Pathog, 1997, 23(3): 167-179. DOI: 10.1006/mpat.1997.0141

[12]Wang T, Qi ZZ, Wu BC, et al. A new purification strategy for fraction 1 capsular antigen and its efficacy against *Yersinia pestis* virulent strain challenge[J]. Protein Expr Purif, 2008, 61(1): 7-12. DOI: 10.1016/j.pep.2008.05.003

[13]Wang T, Qi ZZ, Wu BC, et al. Cloning and expression of *Yersinia pestis* rV270 antigen and efficacy against *Y. pestis* virulent strain challenge[J]. Lett Biotechnol, 2008, 19(5): 663-666. (in Chinese)

[14]Qi ZZ, Zhou L, Zhang QW, et al. Comparison of mouse, guinea pig and rabbit models for evaluation of plague subunit vaccine F1+rV270[J]. Vaccine, 2010, 28(6): 1655-1660. DOI: 10.1016/j.vaccine.2009.02.078

[15]Confer AW, Fulton RW, Step DL, et al. Viral antigen distribution in the respiratory tract of cattle persistently infected with bovine viral diarrhea virus subtype 2a[J]. Vet Pathol, 2005, 42(2): 192-199. DOI: 10.1354/vp.42-2-192

[16]Lukaszewski RA, Kenny DJ, Taylor R, et al. Pathogenesis of *Yersinia pestis* infection in BALB/c mice: effects on host macrophages and neutrophils[J]. Infect Immun, 2005, 73(11): 7142-7150. DOI: 10.1128/IAI.73.11.7142-7150.2005

[17]Parent MA, Berggren KN, Kummer LW, et al. Cell-mediated protection against pulmonary *Yersinia pestis* infection[J]. Infect Immun, 2005, 73(11): 7304-7310. DOI: 10.1128/IAI.73.11.7304-7310.2005

[18]Qiu YF, Liu YR, Qi ZZ, et al. Comparison of immunological responses of Plague vaccines F1 + rV270 and EV in Chinese-origin *Rhesus macaque*, *Macaca mulatta*[J]. Scand J Immun, 2010, 72(5): 425-433. DOI: 10.1111/j.1365-3083.2010.02456.x

[19]Williamson ED, Eley SM, Stagg AJ, et al. A sub-unit vaccine elicits IgG in serum, spleen cell cultures and bronchial washings and protects immunized animals against pneumonic plague[J]. Vaccine, 1997, 15(10): 1079-1084. DOI: 10.1016/S0264-410X(96)00303-9

[20]Do Y, Park CG, Kang YS, et al. Broad T cell immunity to the LcrV virulence protein is induced by targeted delivery to DEC-205/CD205-positive mouse dendritic cells[J]. Eur J Immunol, 2008, 38(1): 20-29. DOI: 10.1002/eji.200737799

[21]Philipovskiy AV, Smiley ST. Vaccination with live *Yersinia pestis* primes CD4 and CD8 T cells that synergistically protect against lethal pulmonary *Y. pestis* infection[J]. Infect Immun, 2007, 75(2): 878-885. DOI: 10.1128/IAI.01529-06

[22]Gerdes J, Lemke H, Baisch H, et al. Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67[J]. J Immunol, 1984, 133(4): 1710-1715.

[23]Rajewsky K. Clonal selection and learning in the antibody system[J]. Nature, 1996, 381(6585): 751-758. DOI: 10.1038/381751a0

[24]Liu YJ, Malisan F, Bouteiller OD, et al. Within germinal centers, isotype switching of immunoglobulin genes occurs after the onset of somatic mutation[J]. Immunity, 1996, 4(3): 241-250. DOI: 10.1016/S1074-7613(00)80432-X

[25]Kurtin PJ. Marginal zone B cells, monocytoid B cells, and the follicular microenvironment. Determinants of morphologic fea-

tures in a subset of low-grade B-cell lymphomas[J]. Am J Clin Pathol, 2000, 114(4): 505-508.

[26]Gatto D, Brink R. The germinal center reaction[J]. J Allergy Clin Immunol, 2010, 126(5): 898-907. DOI: 10.1016/j.jaci.2010.09.007

[27]Frölich D, Giesecke C, Mei HE, et al. Secondary immunization generates clonally related antigen-specific plasma cells and memory B cells[J]. J Immunol, 2010, 185(5):3103-3110. DOI: 10.4049/jimmunol.1000911

[28]Papadogiannakis EI, Kontos VI, Tamamidou M, et al. Determination of intracellular cytokines IFN-γ and IL-4 in canine T lymphocytes by flow cytometry following whole-blood culture [J]. Can J Vet Res, 2009, 73(2): 137-143.

[29]Duramad P, McMahon CW, Hubbard A, et al. Flow cytometric detection of intracellular TH1/TH2 cytokines using whole blood: validation of immunologic biomarker for use in epidemiologic studies[J]. Cancer Epidemiol Biomarkers Prev, 2004, 13(9): 1452-1458.

[30]Yang LH, Hu YL, Xue JB, et al. Compound Chinese herbal medicinal ingredients can enhance immune response and efficacy of RHD vaccine in rabbit[J]. Vaccine, 2008, 26(35): 4451-4455. DOI: 10.1016/j.vaccine.2008.06.075

[31]Sabhnani L, Manocha M, Sridevi K, et al. Developing subunit immunogens using B and T cell epitopes and their constructs derived from the F1 antigen of *Yersinia pestis* using novel delivery vehicles[J]. FEMS Immunol Med Microbiol, 2003, 38(3): 215-229. DOI: 10.1016/S0928-8244(03)00170-6

[32]Wu HQ, Neilson JR, Kumar P, et al. miRNA Profiling of naïve, effector and memory CD8 T cells[J]. PLoS One, 2007, 2(10): e1020. DOI: 10.1371/journal.pone.0001020

[33]Bird JJ, Brown DR, Mullen AC, et al. Helper T cell differentiation is controlled by the cell cycle[J]. Immunity, 1998, 9(2): 229-237. DOI: 10.1016/S1074-7613(00)80605-6

[34]Tian G, Qiu YF, Qi ZZ, et al. Histopathological observation of immunized *Rhesus macaques* with plague vaccines after subcutaneous infection of *Yersinia pestis*[J]. PLoS One, 2011, 6(4): e19260. DOI: 10.1371/journal.pone.0019260

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免疫组化分析鼠疫疫苗免疫和鼠疫菌攻毒后恒河猴脾组织中 T 和 B 淋巴细胞增殖

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摘　要:目的　在我们先前的研究中发现,鼠疫疫苗免疫的猕猴脾组织中 B 细胞和 T 细胞数量明显增加。然而,是否这些细胞是由鼠疫疫苗引起的增殖性 B 细胞和 T 细胞还不得而知。**方法**　为回答这个问题,本研究使用免疫组化双标记方法检测了猕猴脾组织中 T 细胞和 B 细胞的增殖。应用 Ki67 抗体以及 T 细胞和 B 细胞特异性单克隆抗体的免疫组化双标法,对脾组织 T 细胞和 B 细胞增殖进行检测。脾组织分别来自于亚单位疫苗 SV1(20 μg F1 + 10 μg rV270)免疫的猕猴以及分别通过 SV2 (200 μg F1 + 100 μg rV270)、减毒活疫苗 EV 和铝佐剂免疫并分别攻毒的猕猴。**结果**　与正常动物相比,受试动物脾组织生发中心有较多的 B 细胞增殖,边缘区有较多的静止 B 细胞,在动脉周围淋巴鞘区有较多的静止 T 细胞,提示原始 T 细胞可能在免疫早期发生增殖。经 SV1、SV2 或 EV76 免疫并攻毒的动物脾组织生发中心较仅用 SV1 免疫动物的生发中心扩大。此外,铝佐剂免疫并攻毒的猕猴脾组织生发中心不完整,这可能归因于强毒株鼠疫菌感染引起的病理损伤。B 细胞增殖、静止 B 细胞增加、静止 T 细胞增多及生发中心扩大是诱导特异性体液免疫和保持免疫记忆反应的标志。**结论**　这些结果与我们先前的发现的 SV1、SV2 或者 EV76 免疫动物激发较高的抗体和 IL-4 分泌相一致。

关键词:鼠疫疫苗;恒河猴;免疫组化;鼠疫耶尔森氏菌;淋巴细胞增殖

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