



Immune Responses Induced by Recombinant *Bacillus Subtilis* Expressing the Spike Protein of Transmissible Gastroenteritis Virus in pigs

Reporter: Mou Chunxiao

PI: Yang Qian

Nanjing Agriculture University, China



Backgrounds

- **Transmissible gastroenteritis virus (TGEV) causes enteritis and diarrhea, which leads to severe dehydration and mortality as high as 100% in neonatal piglets, and results in huge economic losses in pig industry.**
- **The digestive tract is the primary entry site of TGEV. The TGEV entering route would be cut off if the mucosal immunity in the intestine was well established.**
- **Intramuscular vaccination is the widely used strategy to prevent TGEV infection. It can stimulate well systemic immunity, but fails to induce effective mucosal immunity. Besides, it causes stress response to animals.**

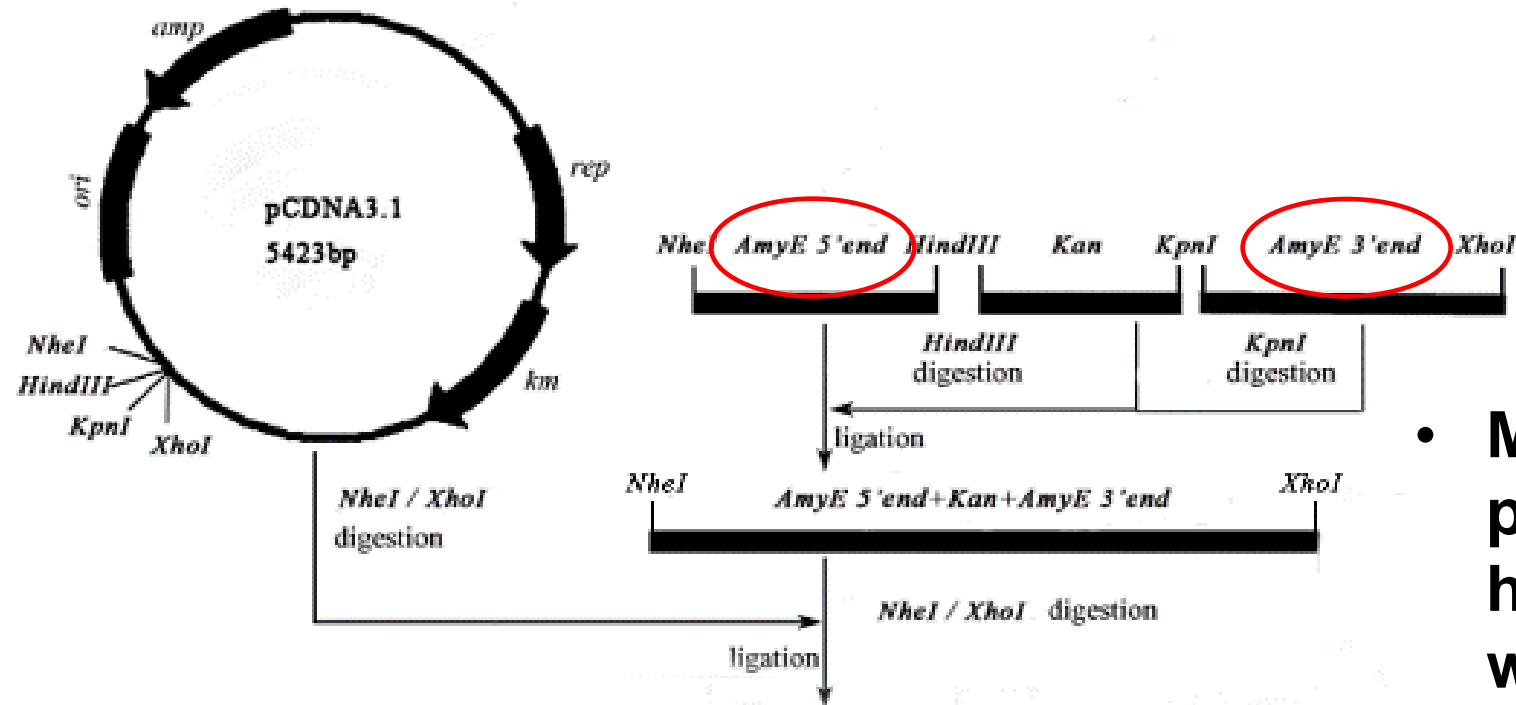
- **Oral delivery has received more attentions in vaccine development because of mucosal immunological response as well as its easy and safe administration.**
- **The degradation by digestive enzymes and acids may compromise the immunogenicity of sampled antigens, limiting the application of oral administration.**
- **“Live attenuated TGEV vaccine” , such as recombinant poxvirus vaccine, produces a good antibody response against TGEV invasion. However, live viruses are unsafe and bear risks of reversion to virulence.**

- ***Bacillus subtilis***, an aerobic probiotics, was widely used as feed additive in pig farms. The spores offer unique resistance properties and can survive under extreme conditions.
- ***B. subtilis*** could induce non-specific mucosal immune responses against infection, increase IgA production.
- The TGEV particle consists of a canonical set of M, E, S, and N proteins. S protein is responsible for viral binding to host cells, and is also the major protein to induce neutralizing antibodies against TGEV.

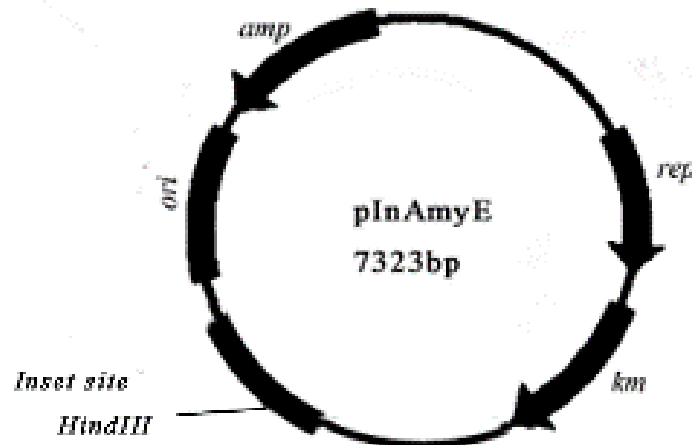
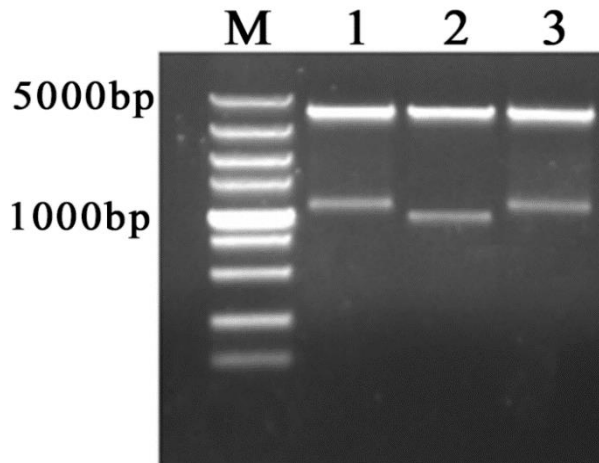
Objective

- To construct the recombinant *B. subtilis* expressing TGEV S protein, induce mucosal antibodies at the first line of defense. This might be a valuable strategy for preventing the invasion and infection of TGEV.

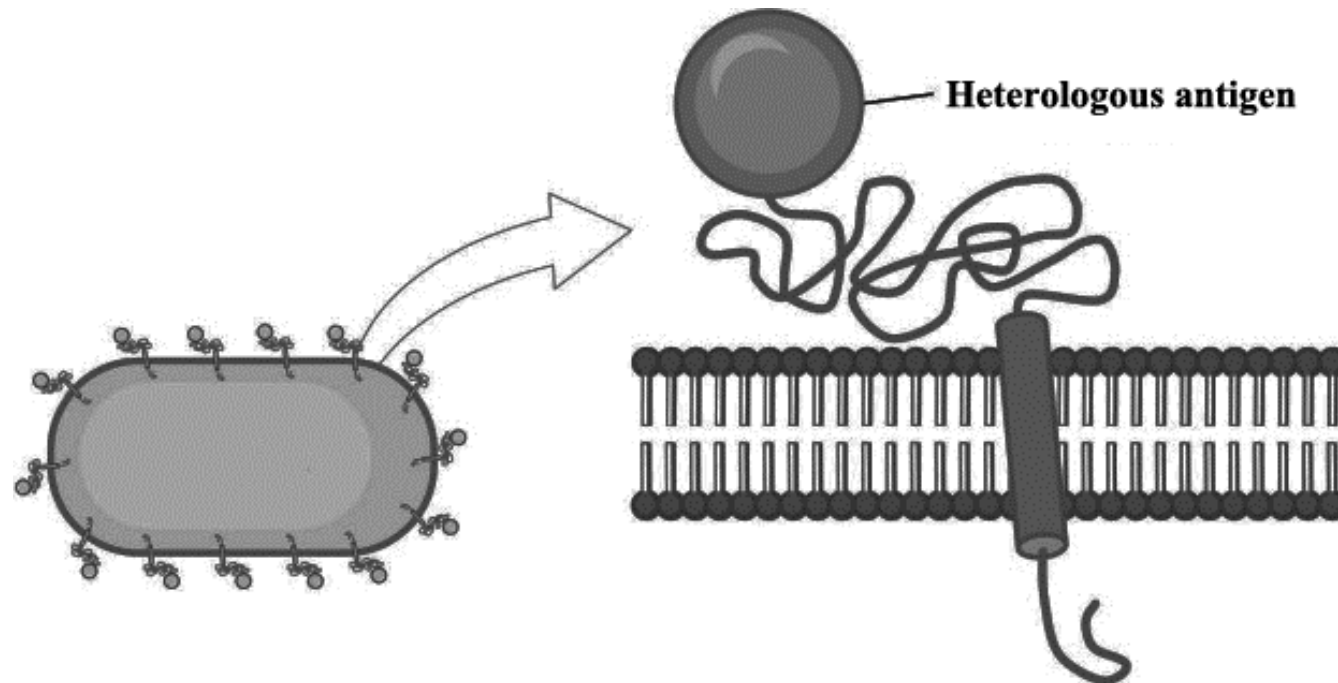
1. Construction of integrative vector *pInAmyE*



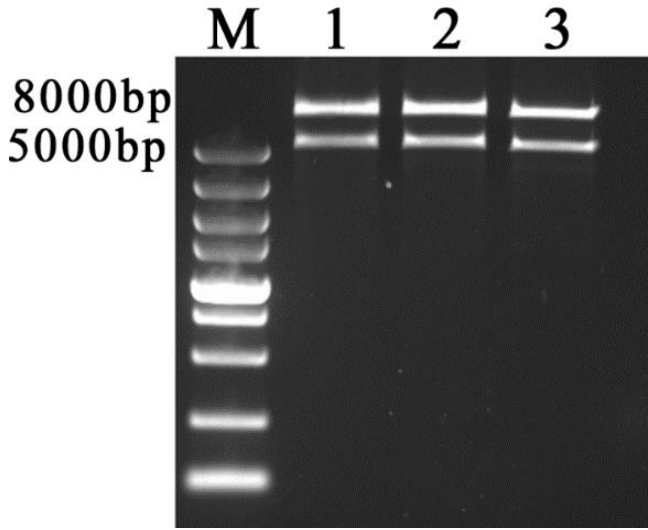
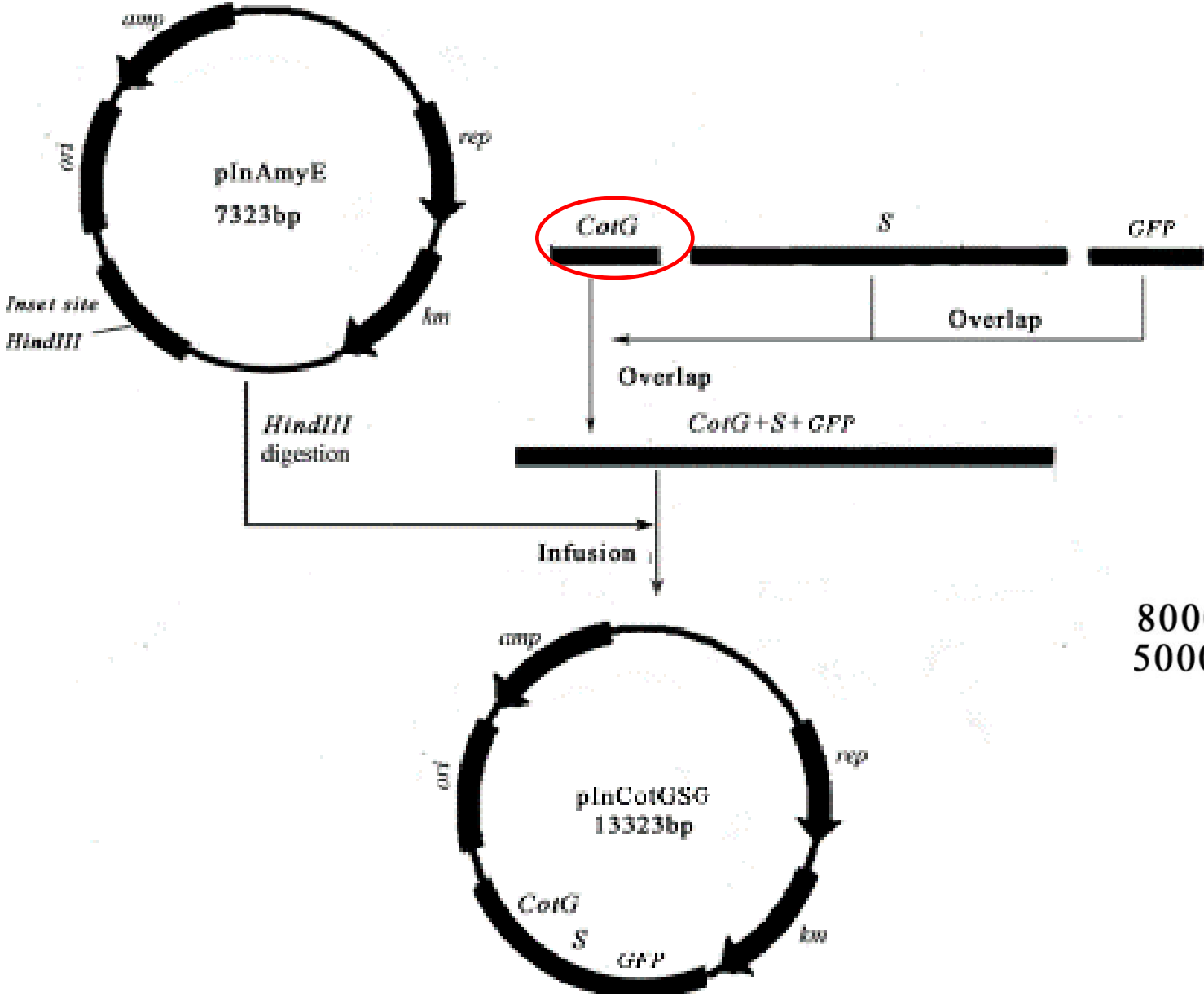
- Many studies use shuttle plasmids to express heterologous proteins, which compromise the missing of plasmids.
- To overcome this hurdle, integrating the target gene into *B. subtilis* genome will be a good choice.



- **Surface display is a powerful technique that uses natural microbial functional components to express heterologous peptides and proteins on the cells. In this way antigen proteins can be recognized by immune cells easily.**
- ***B. subtilis* could be used as a vehicle for heterologous antigen expression, especially using the spore surface display system.**

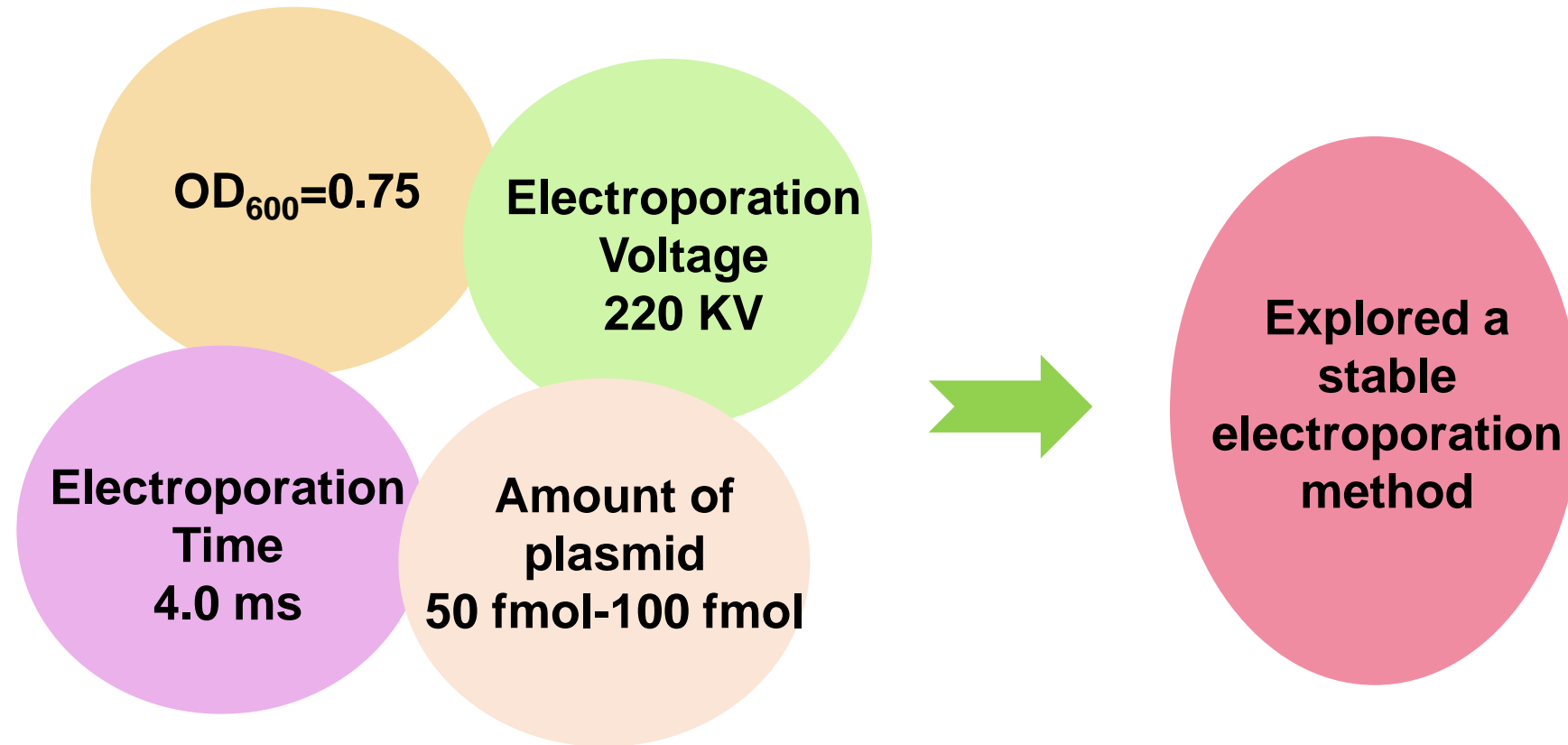


2. Construction of recombinant plasmid *plnCotGSG*



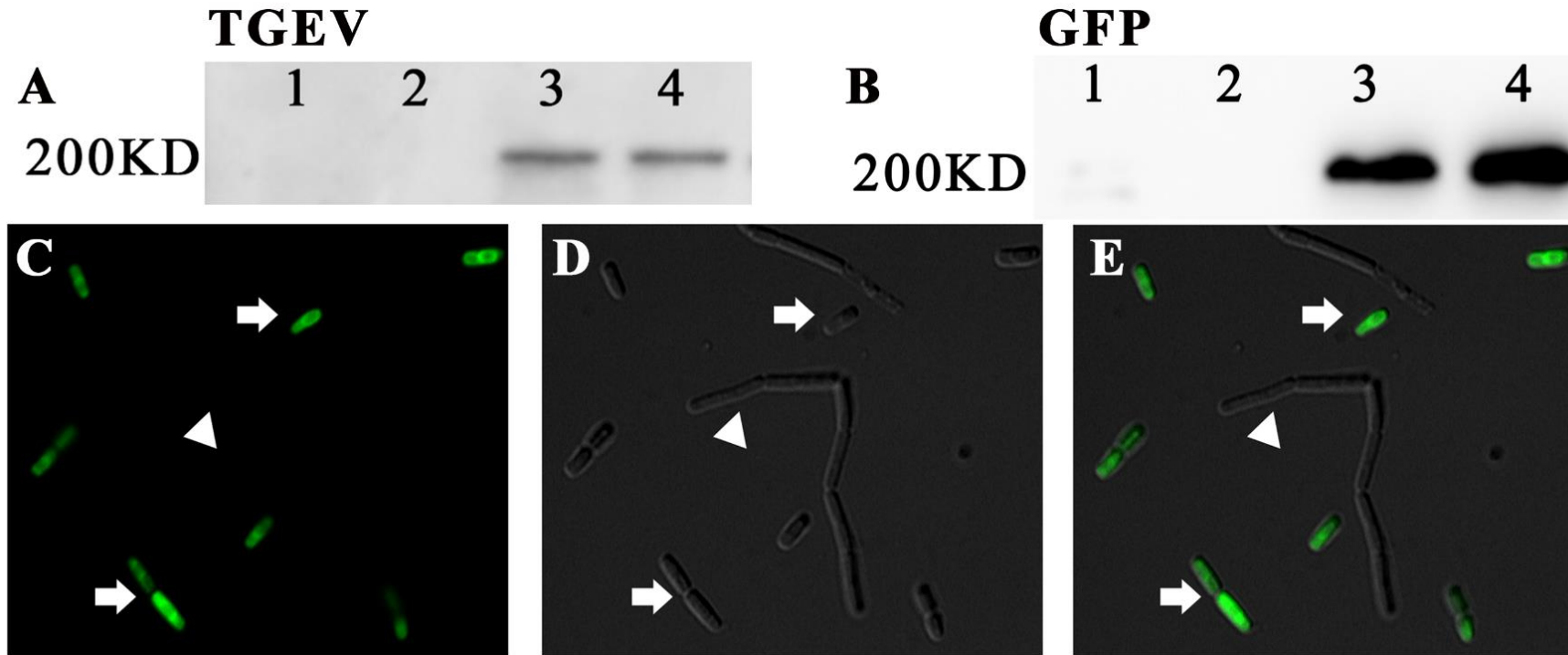
- **It is much more difficult to transform plasmids into gram positive bacteria, such as *B. subtilis*, than negative bacteria because of the thick cell walls.**
- **And it is hardly to complete the process by ordinary chemical transform method. After getting the recombinant plasmid, then we explored the electroporation method.**

3. Electroporation of recombinant plasmid *plnCotGSG*



The *plnCotGSG* was transformed into *B. subtilis* WB800 by electroporation, the recombinant *B. subtilis* strain was named as *B. subtilis* CotGSG.

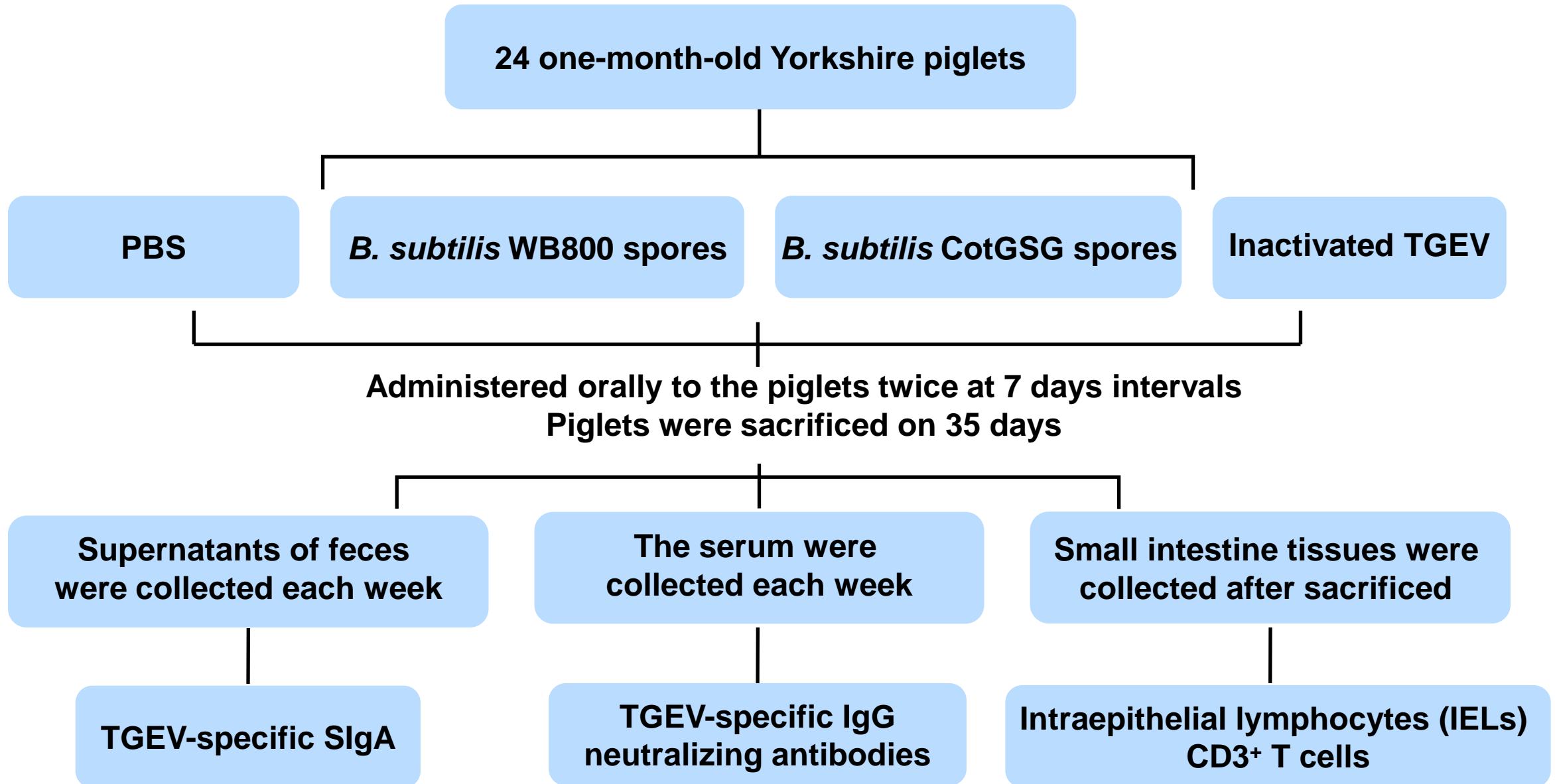
4. Analysis of fusion protein expression



The protein could be well detected with the antibody of TGEV or GFP by western blot, and the *B. subtilis* CotGSG spores covered green fluorescent clearly.

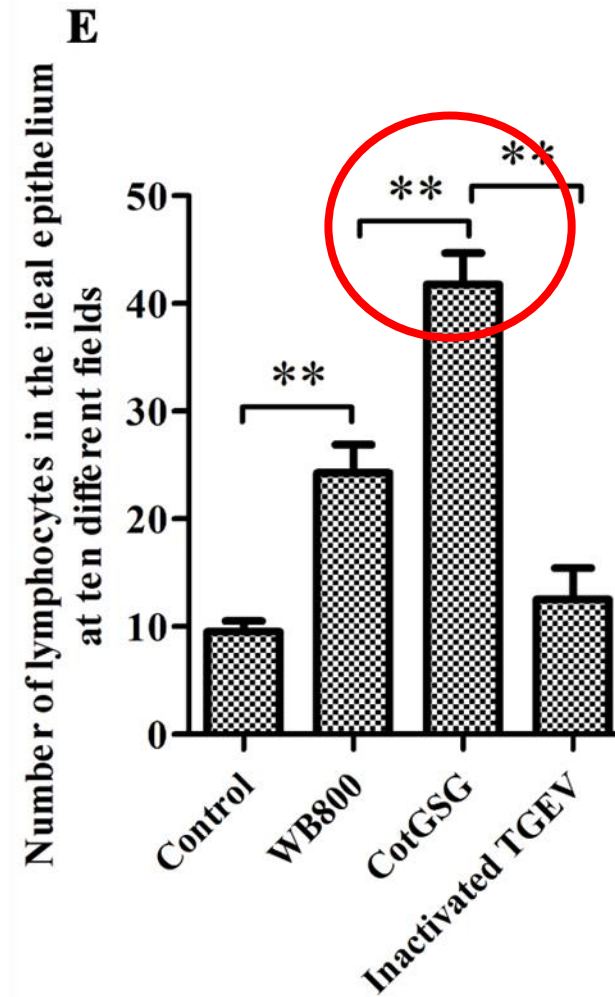
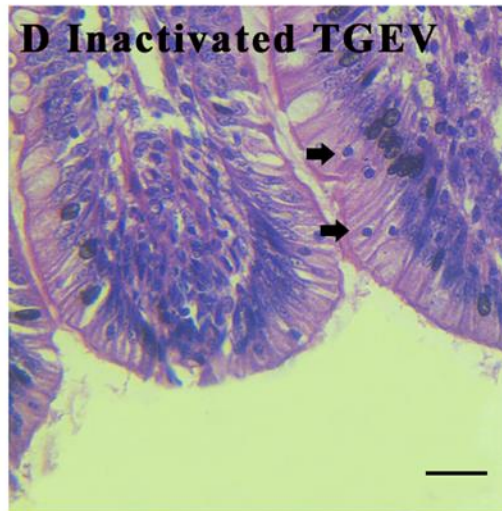
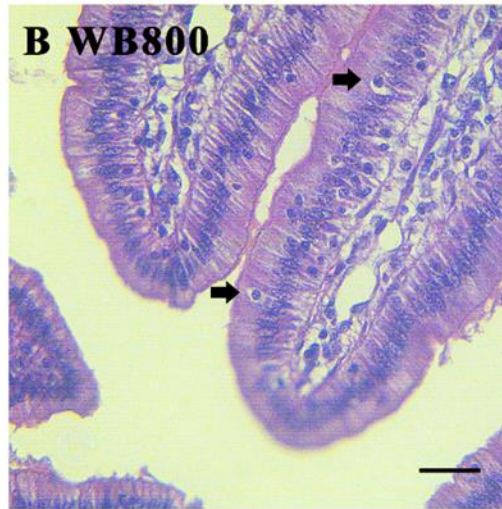
TGEV S protein was successfully displayed on the surface of spores.

5. Animal experiment

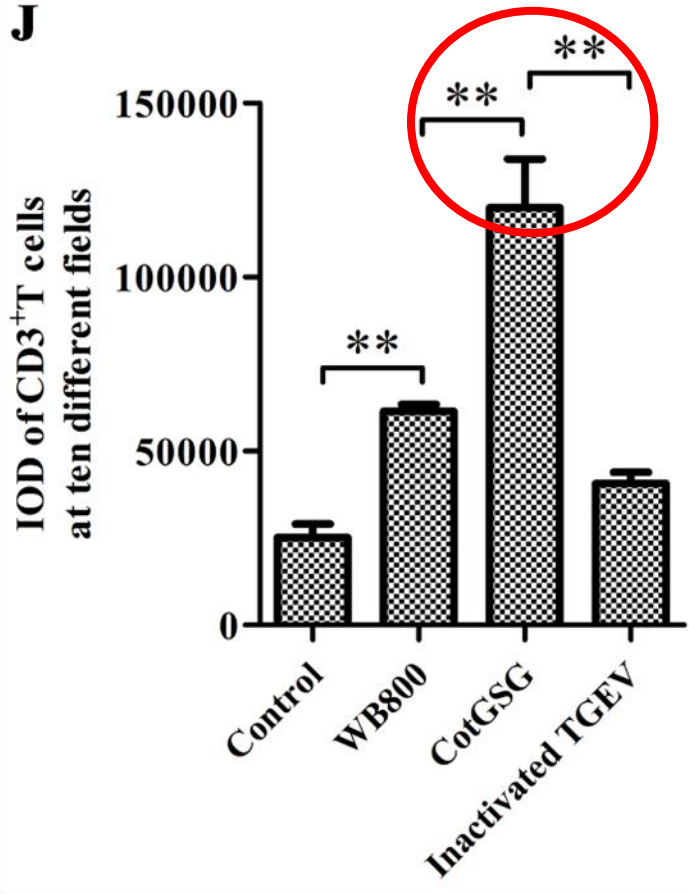
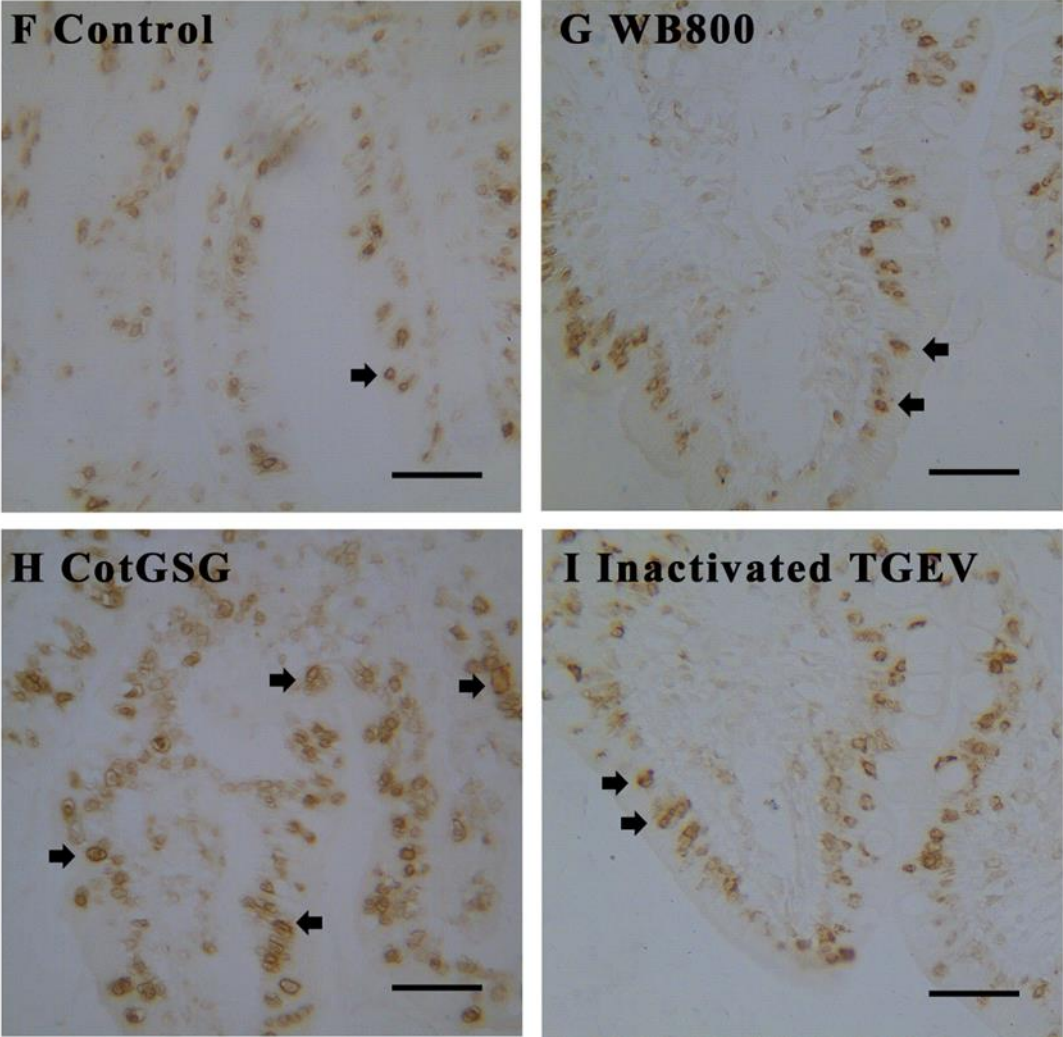


6. Local cellular immune responses after oral immunization

The change of intraepithelial lymphocytes (IELs) in the small intestine

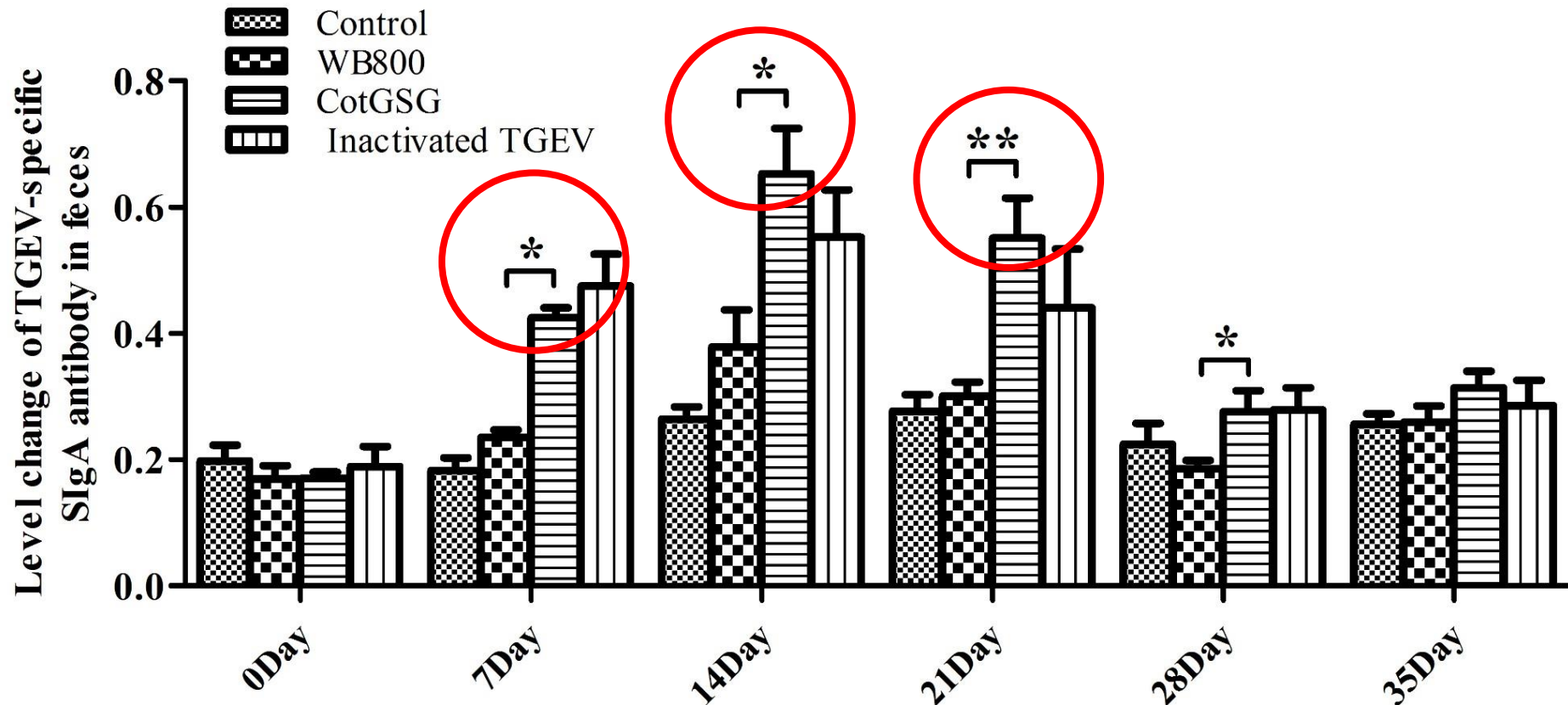


The change of CD3⁺ T lymphocytes in the small intestine



7. Local humoral immune responses after oral immunization

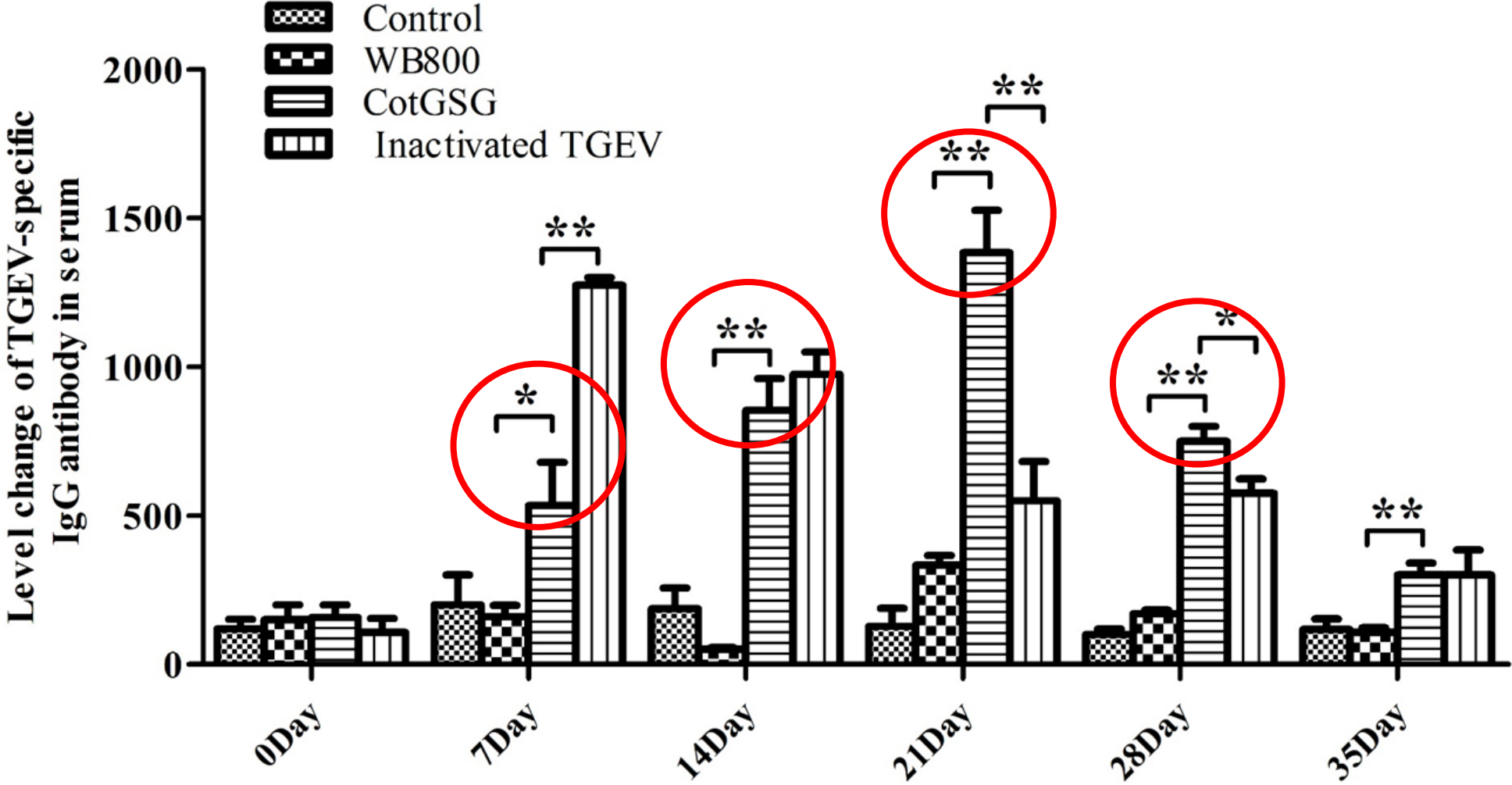
The change of TGEV-specific IgA antibody titers (OD₄₅₀)



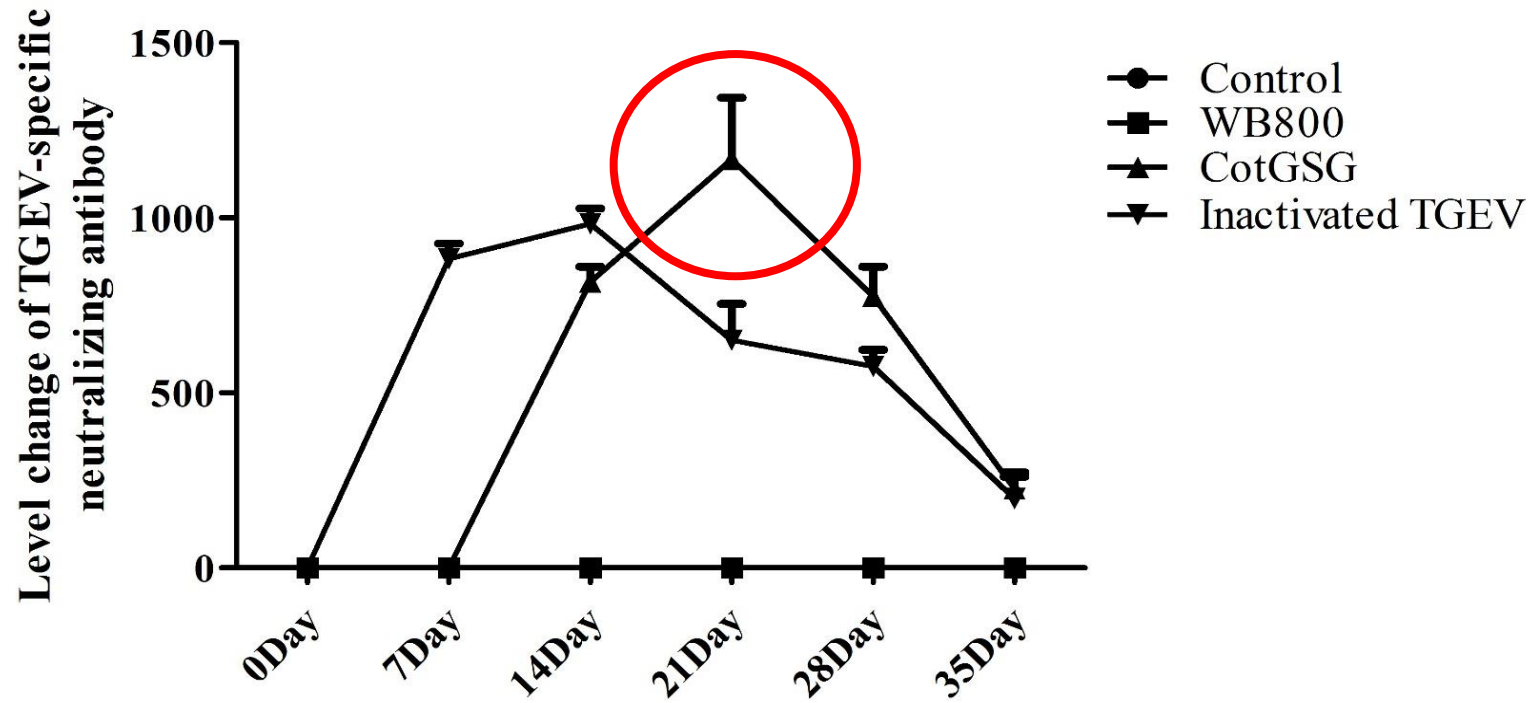
***B. subtilis* CotGSG spores could effectively induce local immune responses in piglets.**

8. Systemic immune responses after oral immunization

The change of serum TGEV-specific IgG antibody titers (the maximum dilutions)



The change of serum TGEV-specific neutralizing antibodies titers

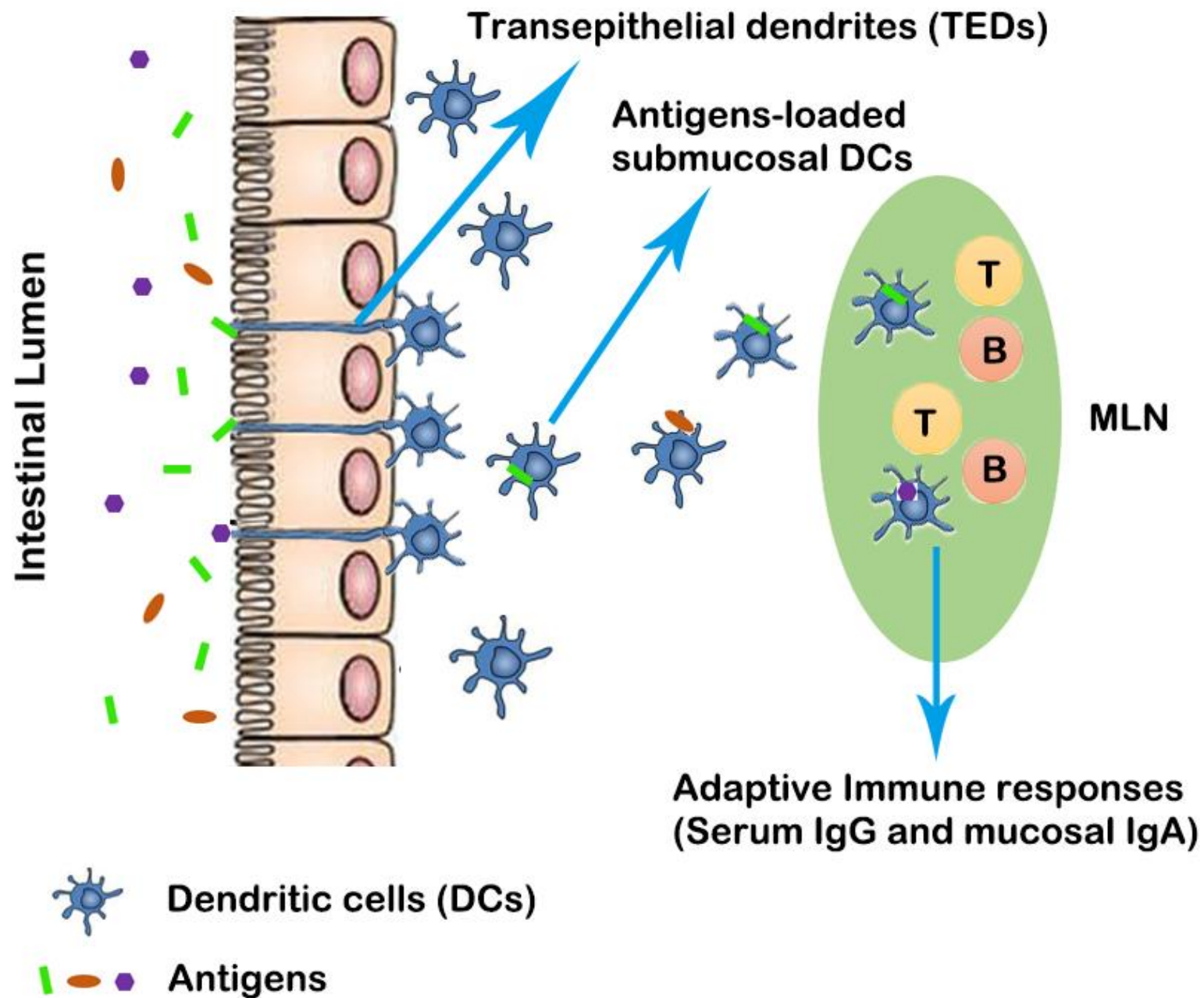


Serum collected from piglets in CotGSG group showed powerful ability to induce neutralizing antibodies against TGEV.

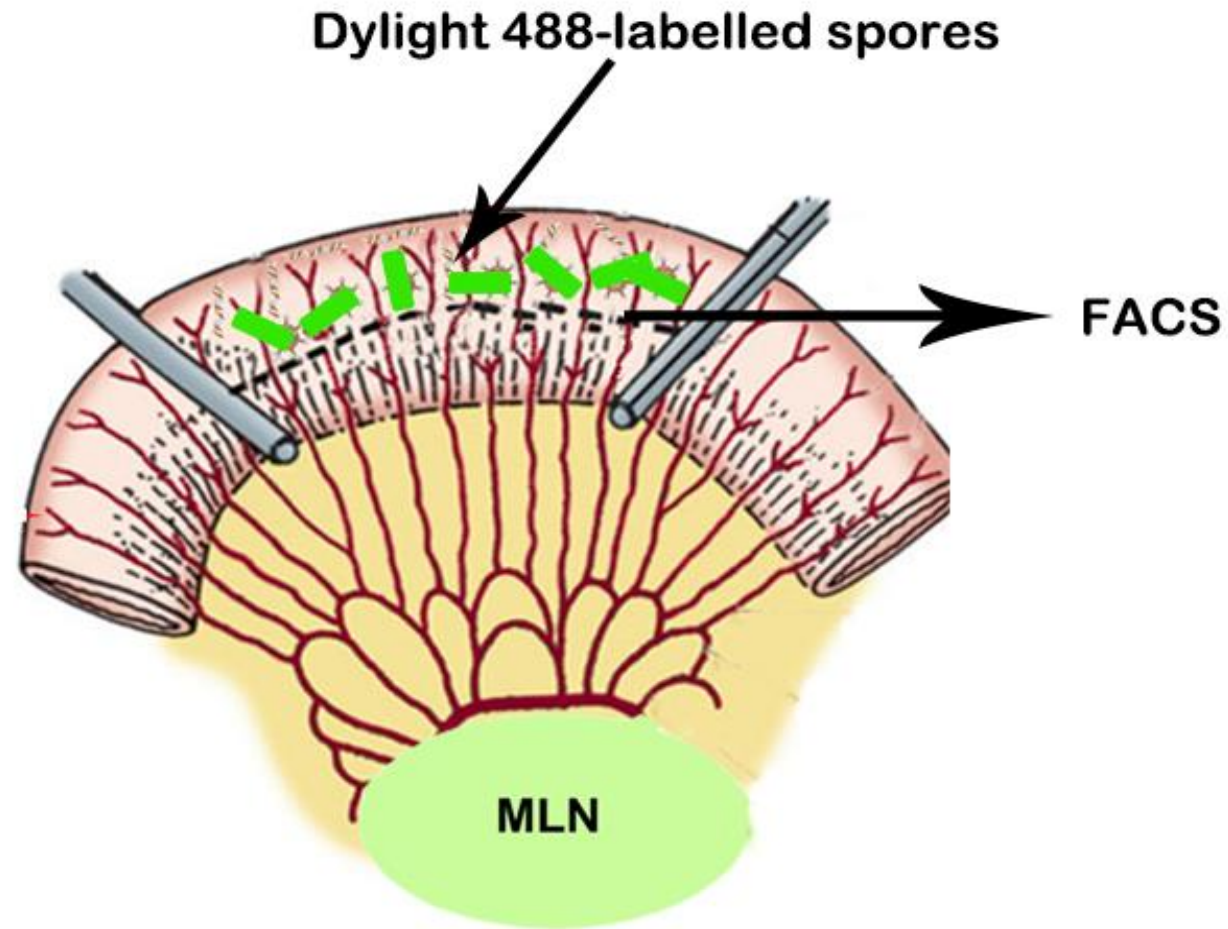
***B. subtilis* CotGSG spores could effectively induce systemic immune responses in piglets.**

**How did *B. subtilis* CotGSG spores induce
such an effective immune response**

?

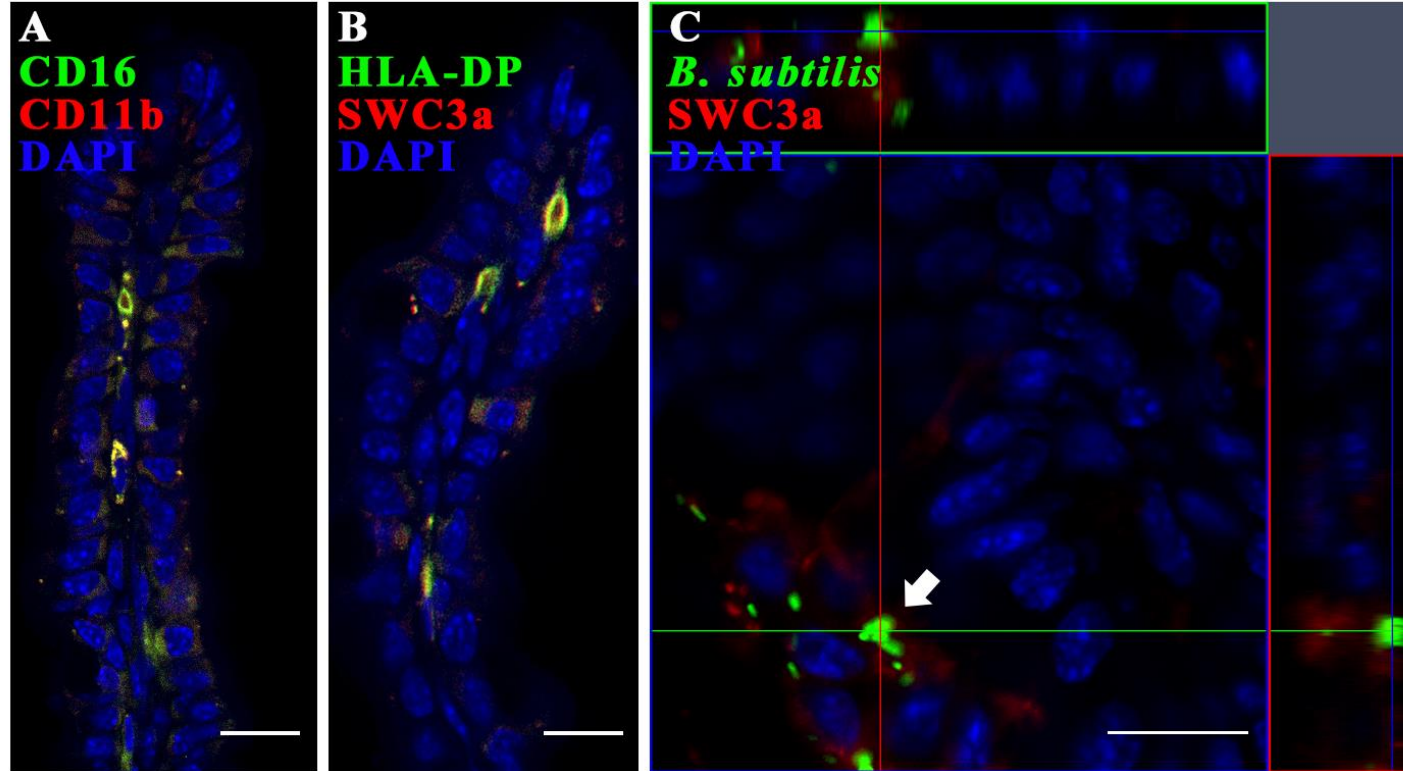


9. Ligated loop experiment

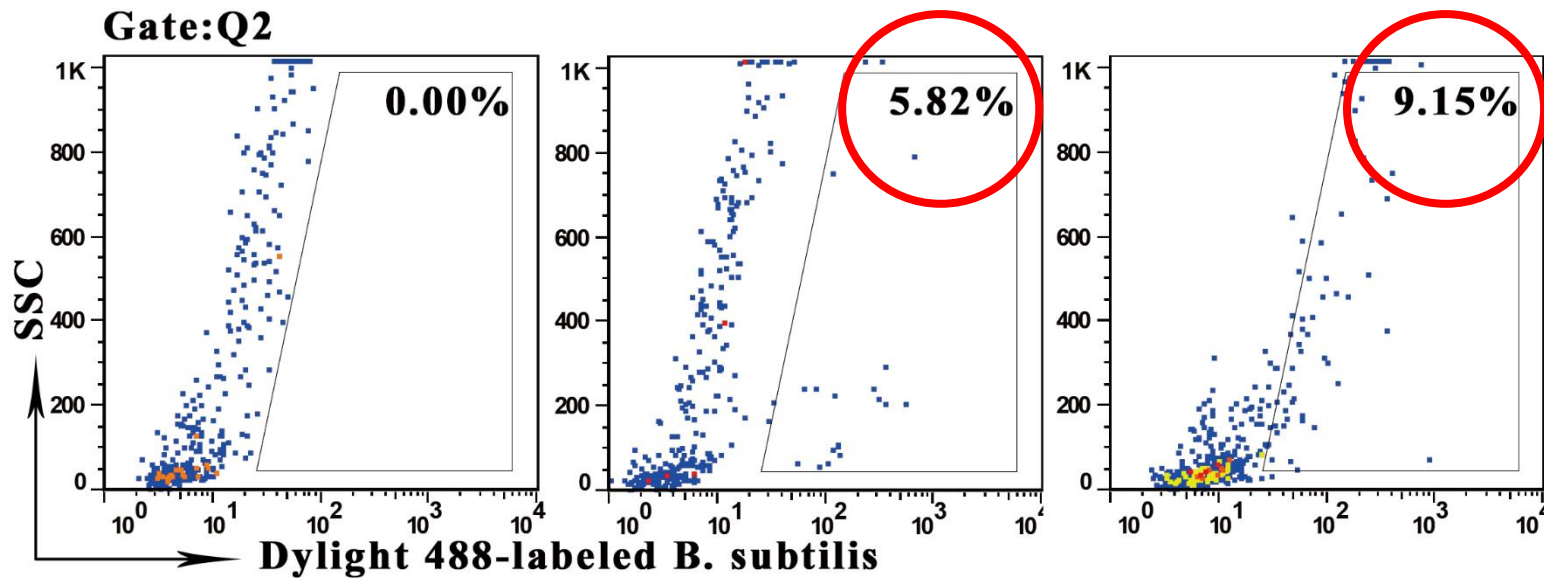
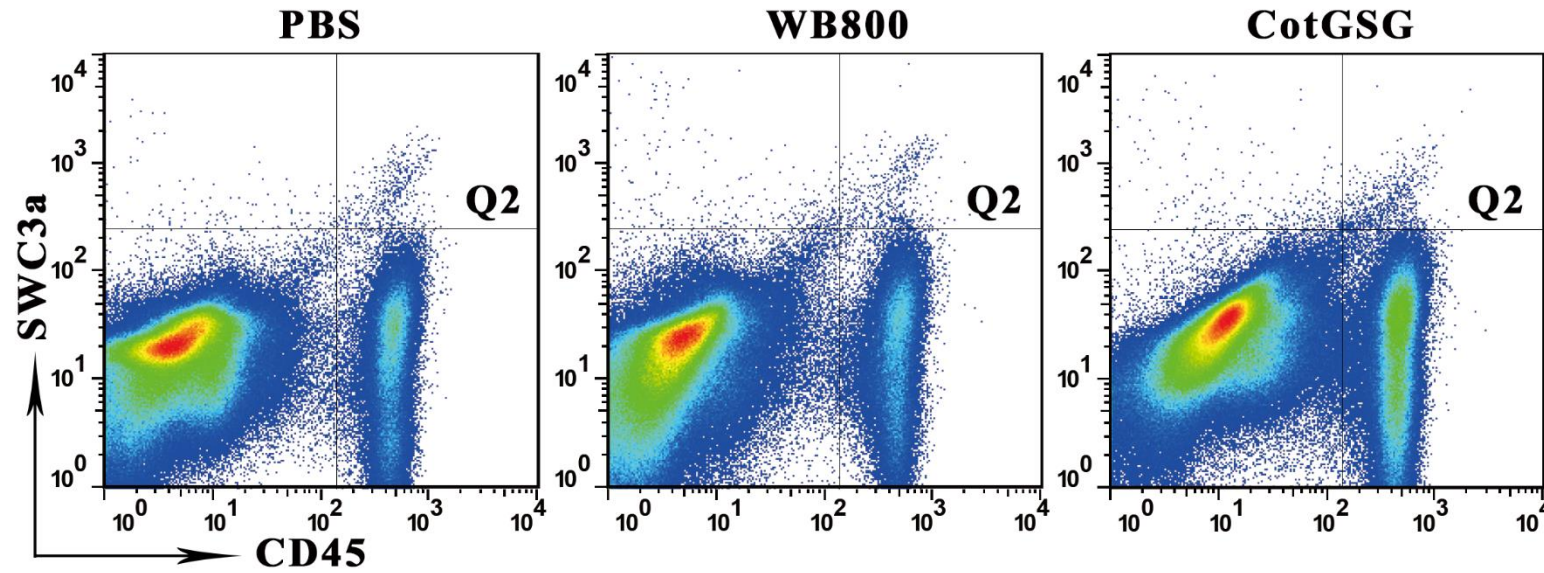


Ileum segments containing the Peyer's patches (PPs) were injected into :
PBS, Dylight 488-labelled *B. subtilis* WB800 spores or CotGSG spores.
2 h later, segments were removed.

10. Analysis of *B. subtilis* sampled by DCs

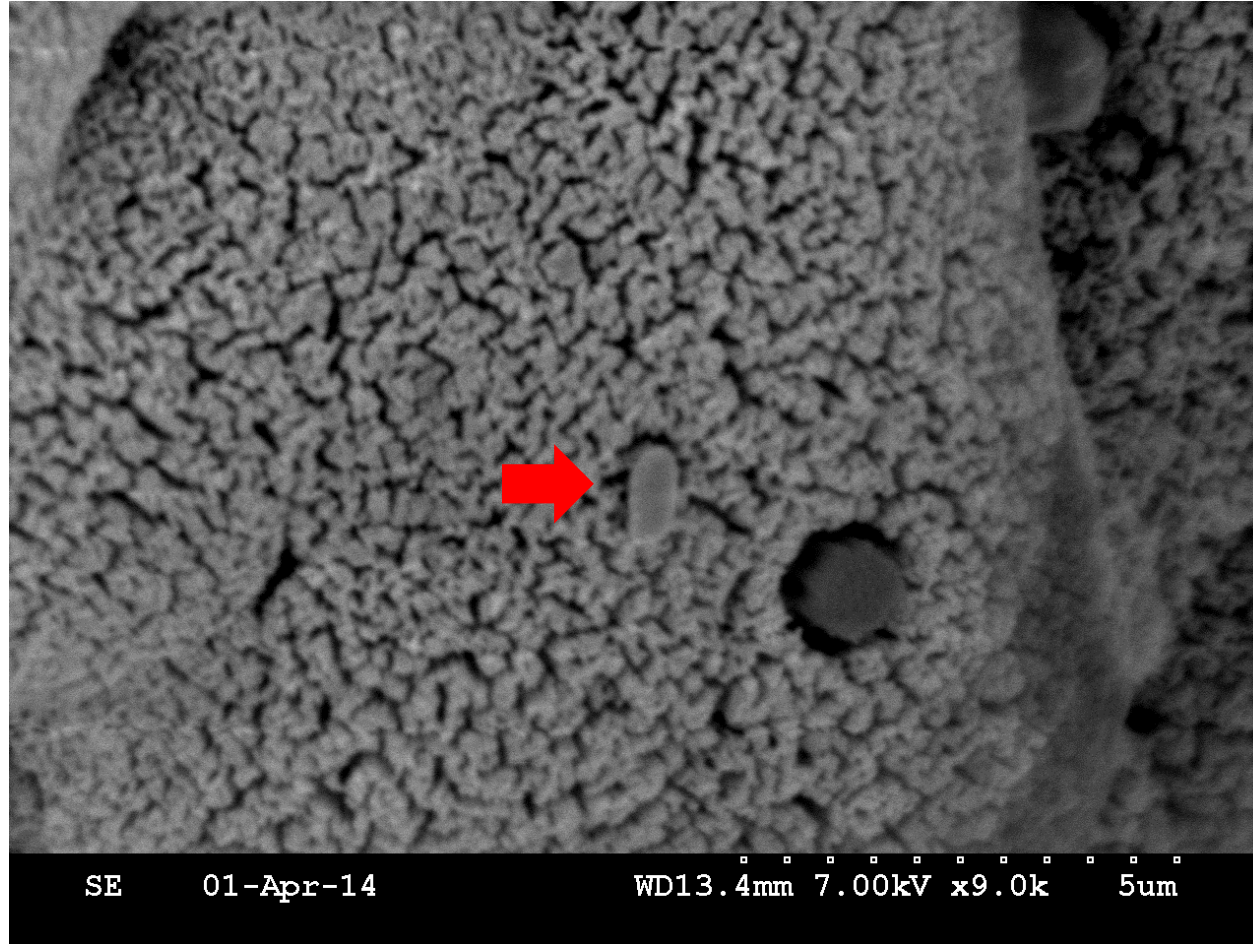


Labelled DCs with HLA-DP⁺ SWC3a⁺ or CD16⁺ CD11b⁺, and Dylight 488-labelled spores were found inside the DCs.

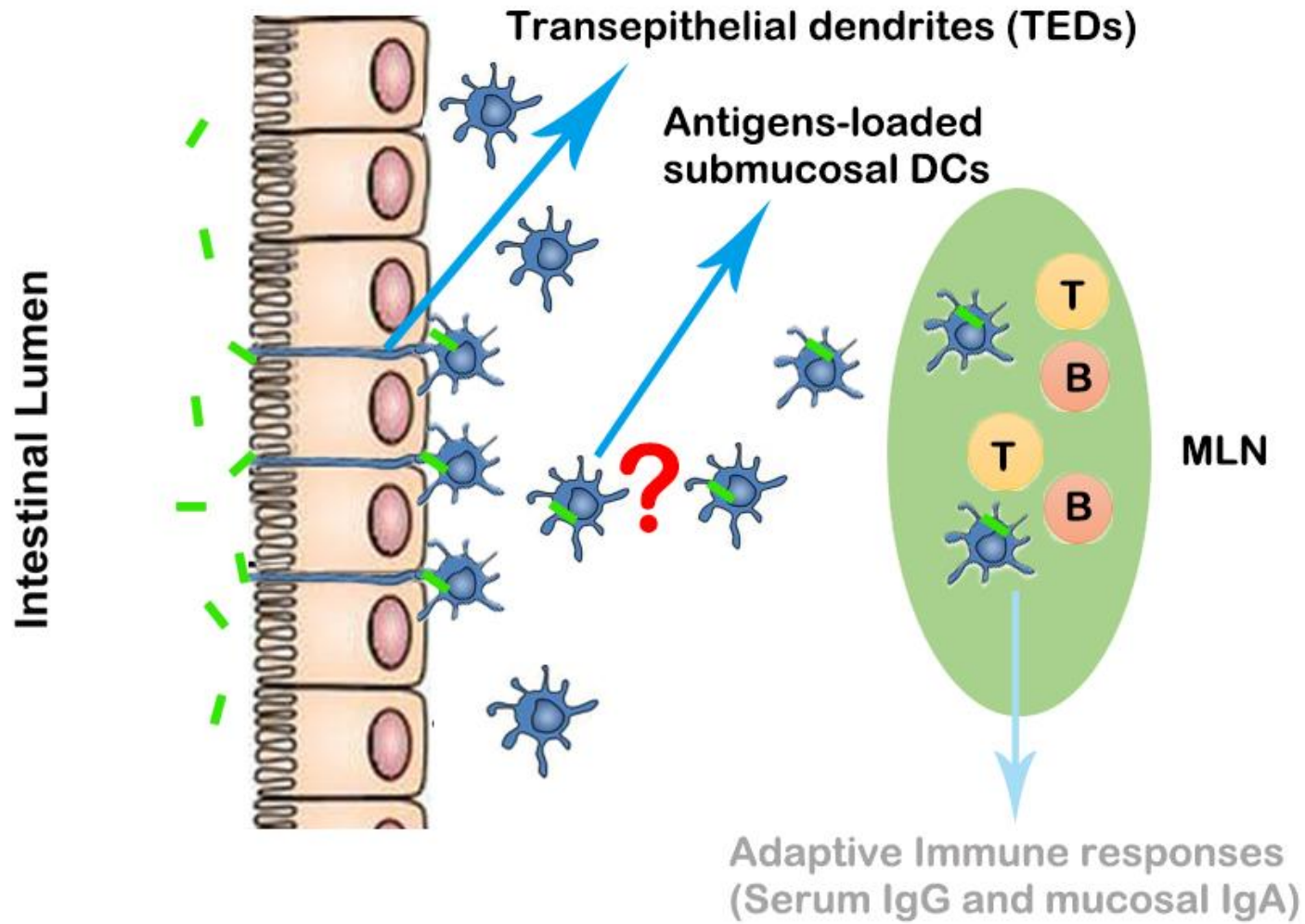


- Stained the intestinal epithelium-associated cells with SWC3a and CD45, and analyzed by FACS.
- The percentage of DCs sampled *B. subtilis* were increased in *B. subtilis* spores treatments.

Observation of epithelium through scanning electron microscope



**From the photo a bacteria was entering the intestinal epithelium.
It could be inferred that the luminal bacteria may be sampled by DCs.**

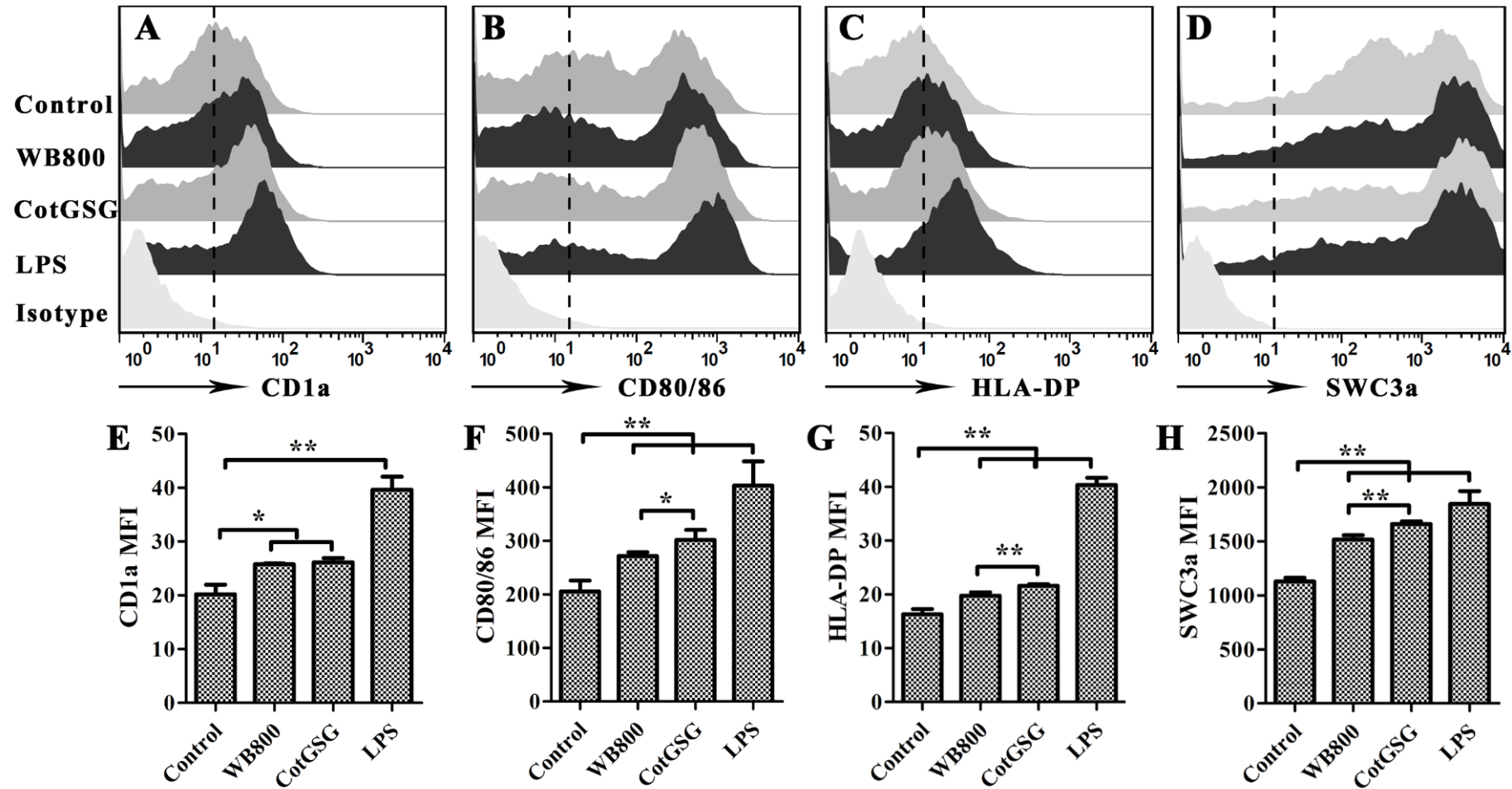


Dendritic cells (DCs)



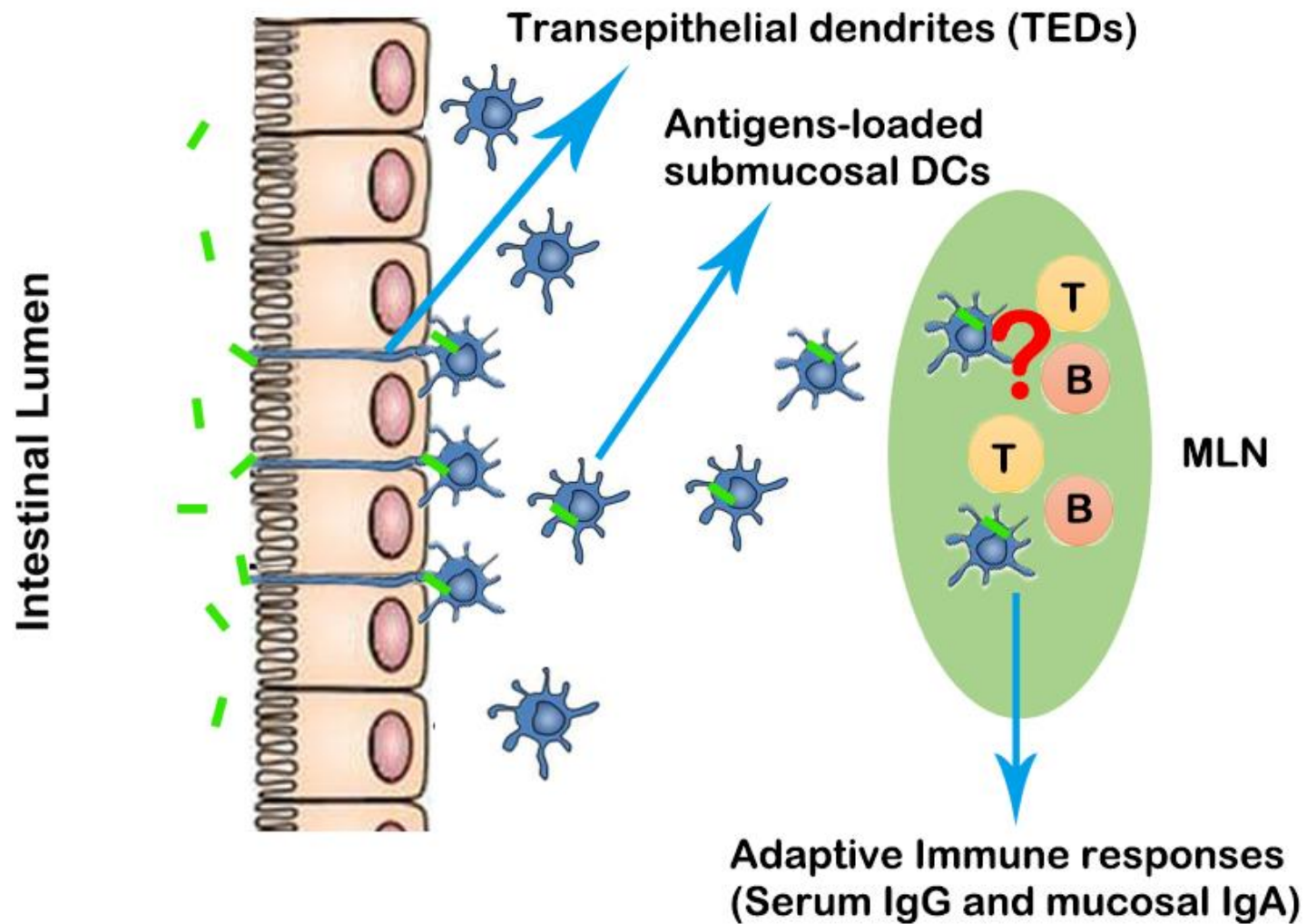
Dylight 488-labelled *B. subtilis*

11. Analysis of the maturation of BM-DCs



The effects of different *B. subtilis* on bone marrow derived DCs maturation were detected by FACS.

B. subtilis spores could enhance the maturation of BM-DCs.

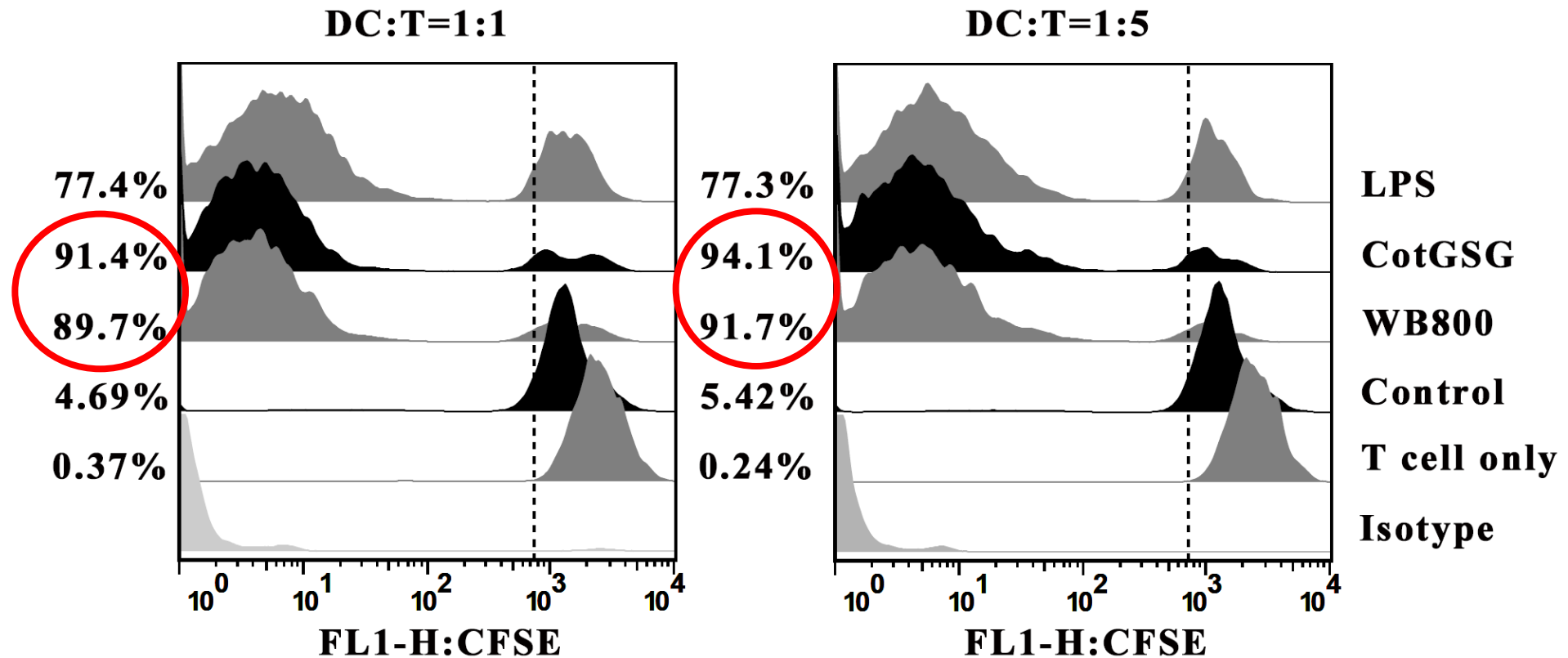


Dendritic cells (DCs)



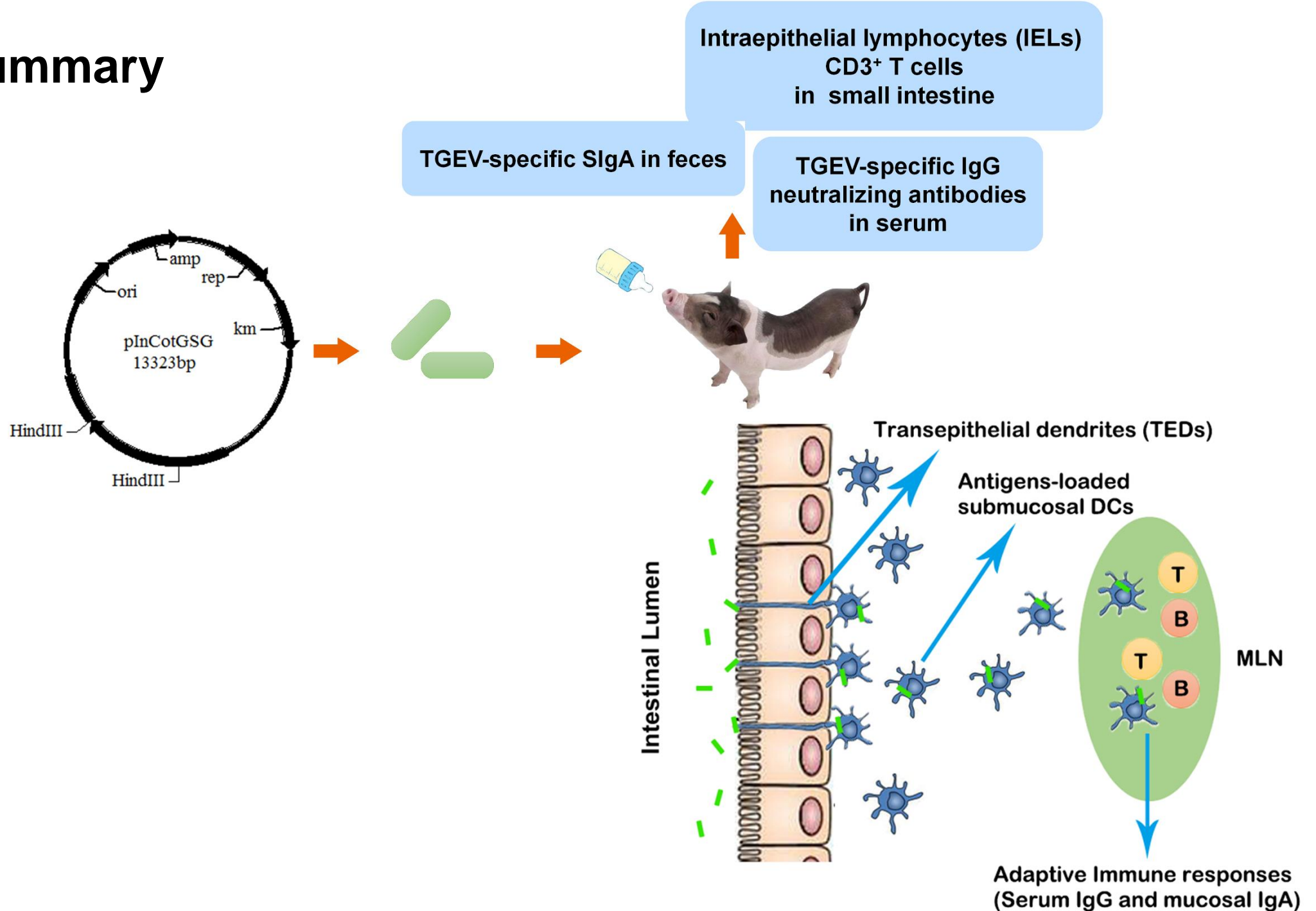
Dylight 488-labelled B. subtilis

12. Mixed lymphocyte reaction



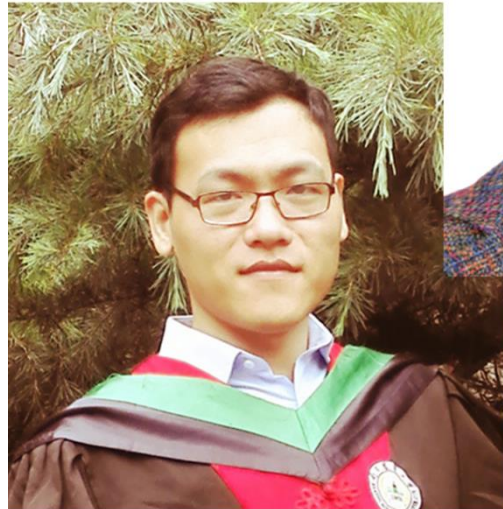
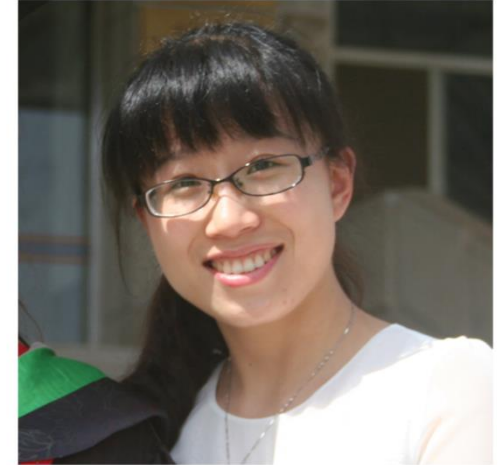
DCs isolated from *B. subtilis* spores group could significantly promote the proliferation of allogeneic T cells.

Summary



Acknowledgement

- This work was supervised by professor Yang Qian.
- Dr. Zhu Liqi coordinated this work.



CpG DNA assists the whole inactivated H9N2



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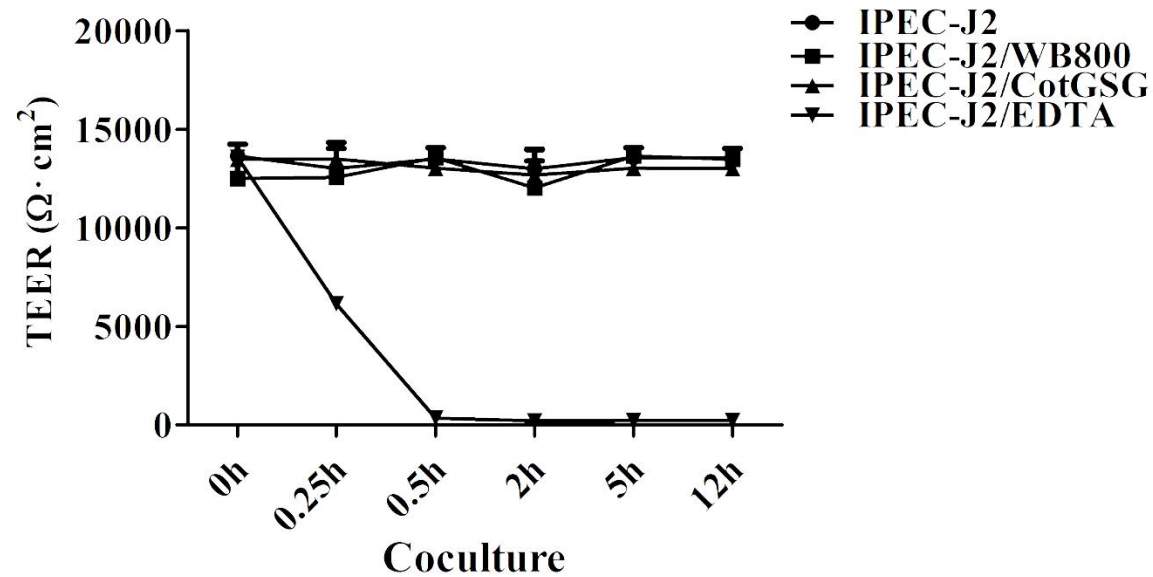
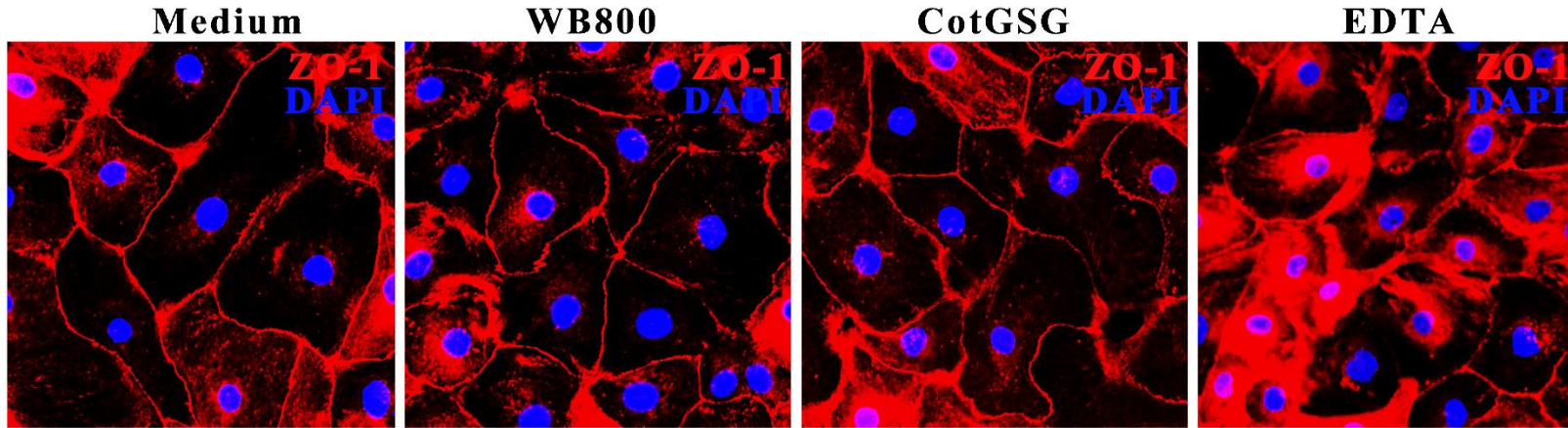
Accepted: 21 March 2016

Published: 15 April 2016

Xiaojuan Chen, Chongzhi Tu, Tao Qin, Liqi Zhu, Yinyan Yin & Qian Yang

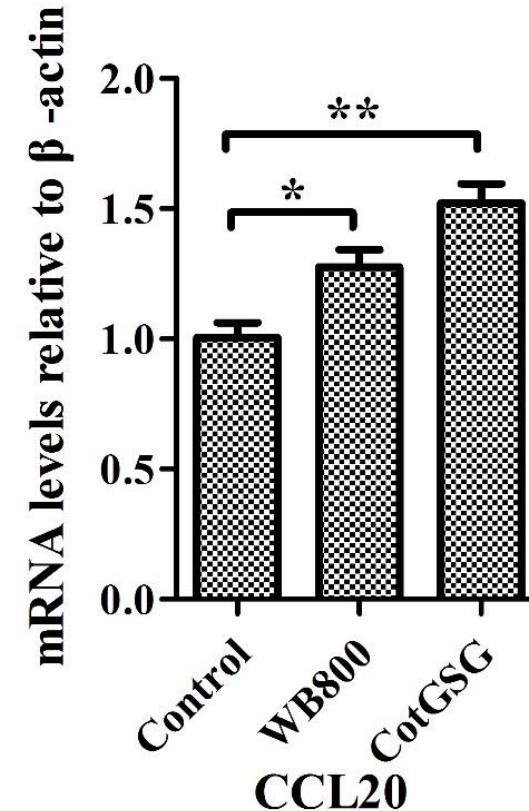
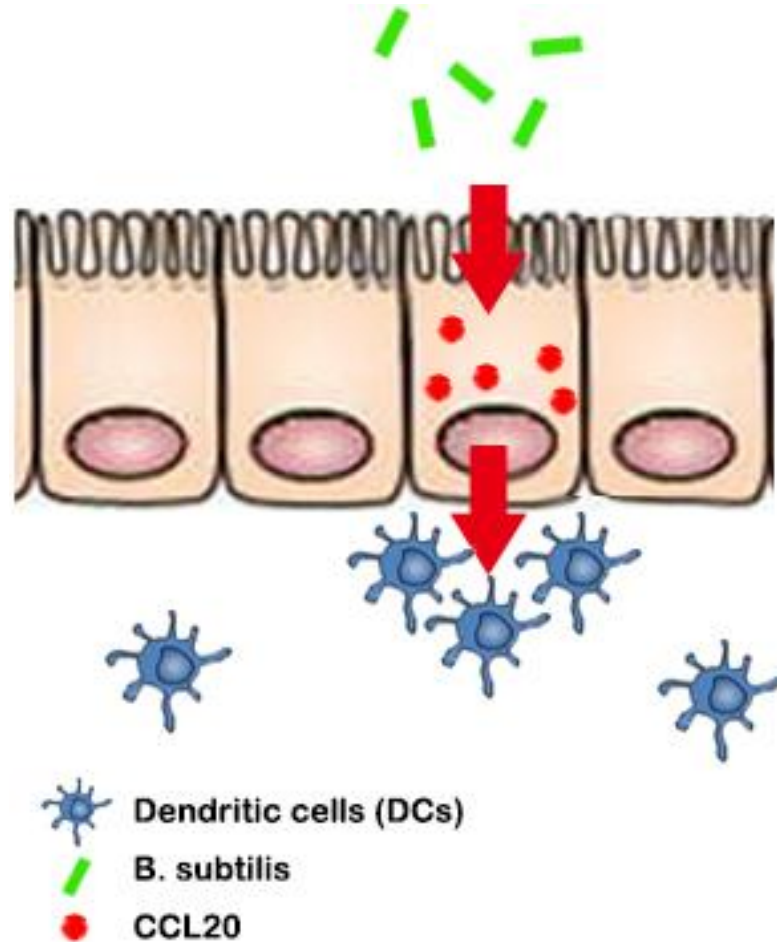
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Analysis of epithelial barrier integrity



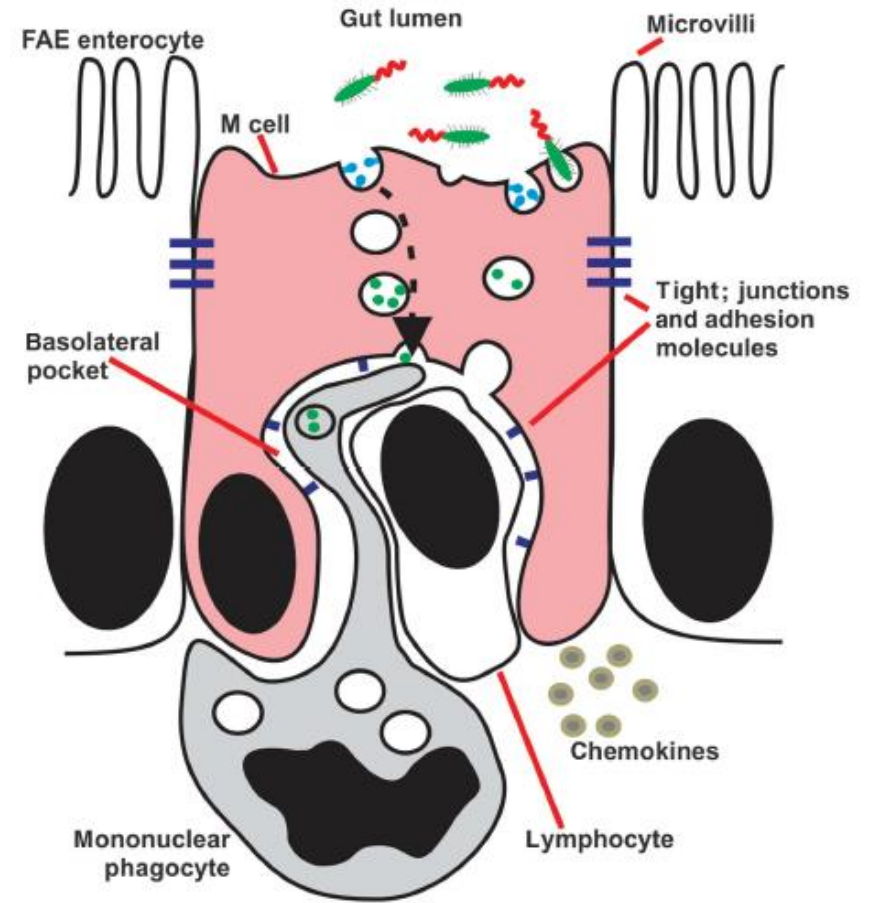
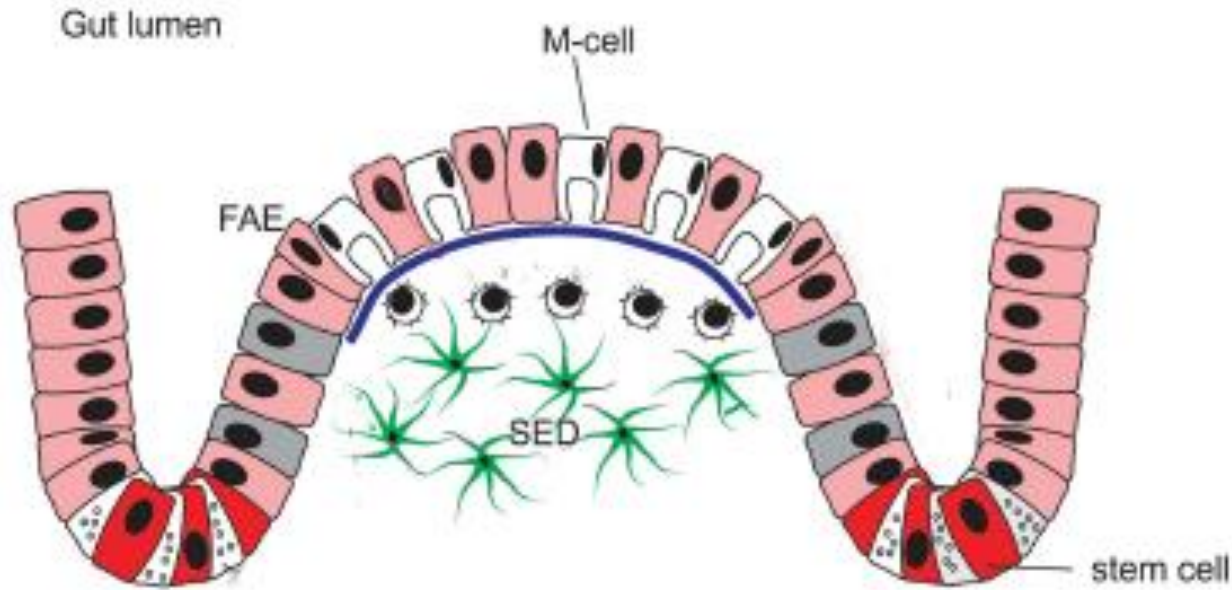
***B. subtilis* spores were not involved in altering epithelial barrier integrity to promote their uptake by DCs.**

Analysis of the mRNA expression of CCL20



In ileum segments, the mRNA expression of CCL20 was upregulated by *B. subtilis* spores.

B. subtilis spores could induce the expression of CCL20 and recruit more DCs into the intestinal epithelium.

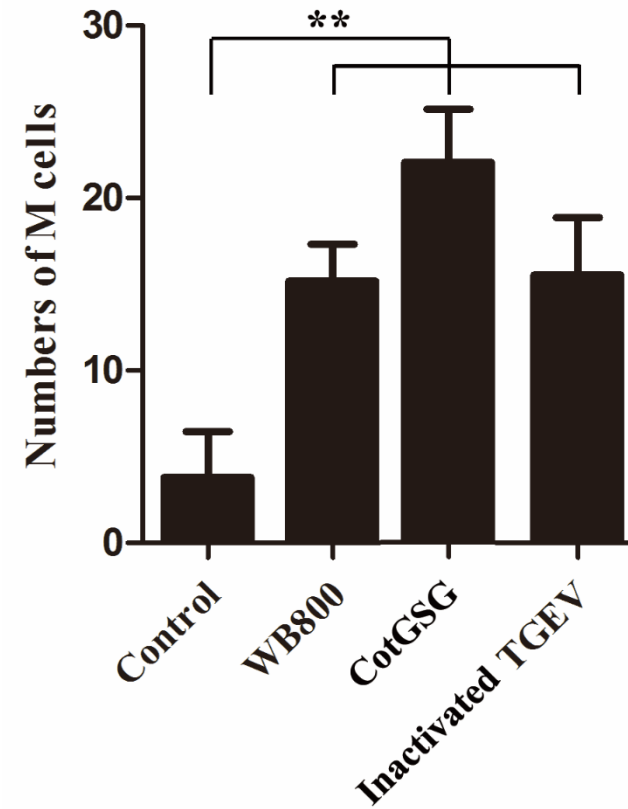
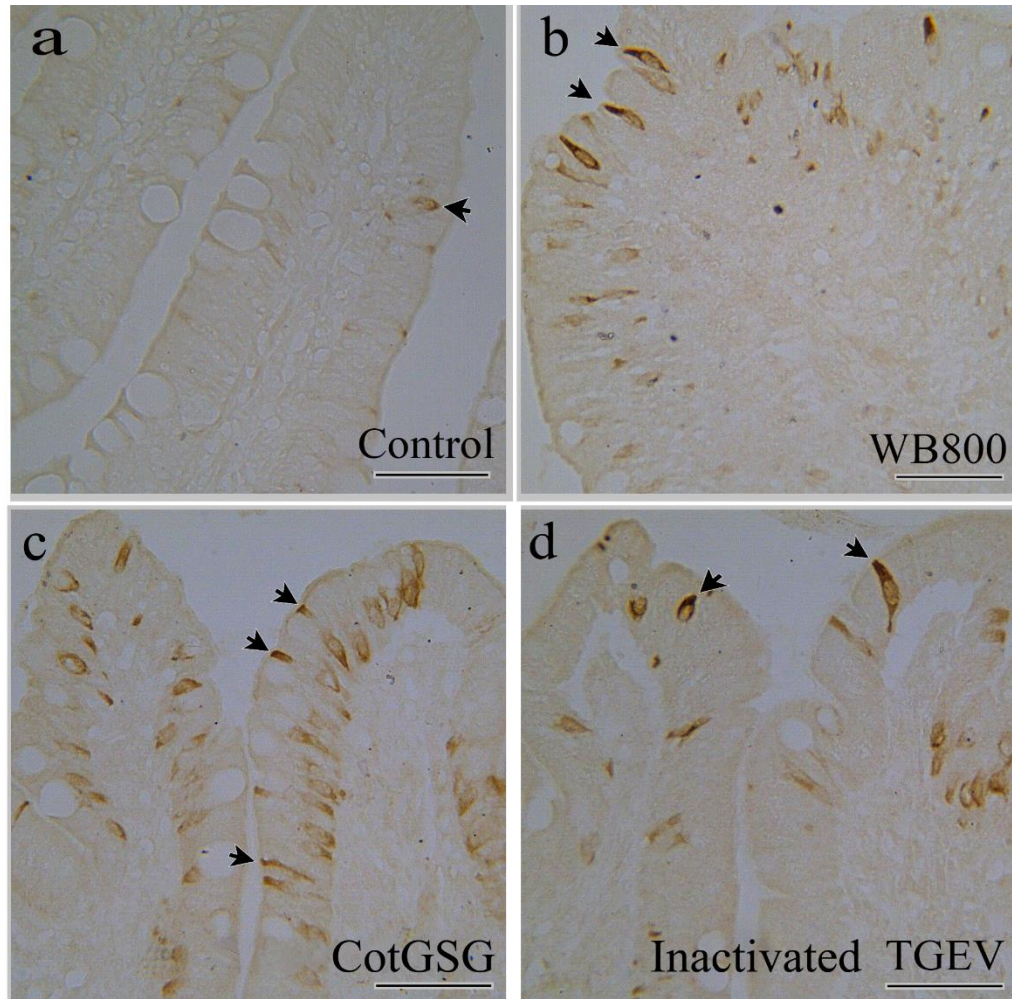


The M-cells, which are located in the follicle-associated epithelium (FAE) on PPs, can transport antigens and effectively interact with underlying immune cells.

M-cells act as gatekeepers in the mucosal immune system, delivering vaccines to M-cells can guarantee a potent oral immunity.

Local cellular immune responses after oral immunization

The change of M cell in the small intestine



These indicated *B. subtilis* spores could induce intestinal epithelial cells to transform into M cells or induce stem cells to differentiate into M cells.

And we will continue to study the mechanism in future experiments.

The serum TGEV-specific neutralizing antibodies titers were detected by plaque test.