Plasma transfusion promoted reprogramming CD4+ T lymphocytes immune response in severe sepsis mice model through modulating the exosome protein Galectin 9

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DOI:
10.21203/rs.3.rs-21029/v1

SUBJECT AREAS
Critical Care & Emergency Medicine
KEYWORDS
fresh frozen plasma (FFP) transfusion, CD4+ T lymphocytes, exosome protein Galectin 9, severe sepsis
Abstract
Sepsis is a life-threatening disease resulting in excessive stimulation of the host’s immune cells. In the study, the purpose is to investigate the roles of fresh frozen plasma (FFP) transfusion in shaping the CD4⁺ T lymphocytes immune response through modulating the secreted exosome protein Galectin 9 in mice with severe sepsis. We firstly identified the protein Galectin 9 highly accumulated in the blood plasma of severe sepsis mice with western blot, then with transmission electron microscopy (TEM) and protein analysis, we found protein Galectin 9 was a secreted exosome protein in sepsis mice. Thereafter, we treated the severe sepsis mice with antibiotic-Cefuroxime Axetil, meanwhile, one group mice received FFP transfusion, the other one group mice received normal saline. Surprisingly, the FFP transfusion reduced the secretion of exosome protein Galectin 9 and there was crosstalking between the exosome protein Galectin 9 and CD4⁺ T lymphocytes in mice with severe sepsis. Results showed that the proliferation of Th1, Th17 were promoted and obviously inhibited Treg cells maintenance in the sepsis mice receiving FFP transfusion. Correspondingly, this immune reprogrammed activity shaped the inflammatory cytokines secretion with increase of IL-1β, IL-6 and IFN-γ, whilst, decrease of IL-10. Taken together, it was suggested that FFP transfusion promoted reprogramming CD4⁺ T lymphocytes immune response through inhibiting the secretion of exosome protein Galectin 9 in mice with severe sepsis to relieve immunosuppression.

Background
Sepsis is a life-threatening disease with an estimation of 27% morbidity and 26% mortality globally¹. It’s occurred with dysregulated host immune response to bacterial infection, which made the initial hyperinflammatory phase invert to immunosuppression and immune paralysis status². Studies had showed that the major histocompatibility complex II molecules, such as human leukocyte antigen-DR (HLA-DR) in antigen-presenting cells (APCs-dendritic cells, macrophage cells, et al ) played important roles in the innate immune response and activating adapted immune responses in sepsis³, ⁴. While, CD4⁺ T lymphocytes are the predominant effector cells in adaptive immune response of sepsis⁵, hence, in recent years, more and more researchers paid attention to the
functions or roles of CD4⁺ T lymphocytes in sepsis⁶, ⁷. The CD4⁺ T lymphocytes can differentiate into several subtypes, such as, T helper (Th) cells (Th1, Th2, Th17) and regulatory T cells-iTreg cells⁸. The Th1 cells are responsible for cell-mediated immunity and release IL-2 and IFN-γ to promote themselves differentiation and enhance the endogenous phagocytosis or clearance of pathogens in monocytes and macrophages, while, Th2 cells participate in humoral immune response and cleared the extracellular infections, such as parasite⁹. Moreover, the Th17 cells play important roles in the clearance of extracellular pathogens and recruiting, activating neutrophils by chemotaxis¹⁰. Besides, Treg cells could inhibit excessive inflammatory response by secreting IL-10 and TGF-β, which also suppressed the activities of monocytes, dendritic cells and macrophages¹¹. Studies had displayed that the ratio of Th1/Th2¹² and Th17/Treg¹³ were all decline in severe sepsis patients. Hence, keeping the balance of T helper cells and regulatory T cells is a way to alleviate immunosuppression in severe sepsis.

The protein Galectin-9 is an important immune modulator in both innate and adaptive immune responses¹⁴. It mediated the innate immune cells, such as macrophages and DCs via binding the T cell immunoglobulin and mucin domain containing molecule 3 (Tim-3)¹⁵ to initiate inflammatory response. Conversely, it suppressed Th1 cell immune responses via binding Tim-3 receptor, which thus induced Th1 cell apoptosis and exhaustion¹⁶. Moreover, studies identified that protein Galectin-9 not only inhibited Th17 expansion but also promoted the proliferation of regulatory T cells¹⁷, it was found interacting with CD44 receptor to promote FOXP3 expression, which thus induced the differentiation and expansion of Treg (iTreg) cells¹⁸. Importantly, our preliminary experiment found the protein Galectin-9 existing in the blood samples of severe sepsis mice model, which maybe participated in the immunosuppression activity in severe sepsis. Therefore, to our knowledge, we made further studies about it and revealed it to be an exosome protein in the blood samples of severe sepsis mice model.

As fresh-frozen plasma (FFP) transfusion and red blood cell transfusion is controversial in patients
with septic shock\textsuperscript{19,20}. Hence, in this study, we attempted to investigate the roles of FFP transfusion in severe sepsis mice model.

Materials And Methods

Ethics statement

All animal experiments were performed according to the guidelines of the animal ethical organization and obtained the permission of Shanghai Gongli Hospital, the Second Military Medical University

Materials and reagents

Lipopolysaccharides from Escherichia coli 0111:B4 (catlog: L2630, sigma), antibiotic-Cefuroxime Axetil (Chengdu beite pharmaceutical co. LTD),

Total Exosome Isolation Reagent (catlog: 4478359, Invitrogen\textsuperscript{™}), ExoAb Antibody Kit (catlog: EXOAB-KIT-1, SBI), anti-mouse Galectin 9 (catlog: ab69630, abcam),

Transmission Electron Microscope (catlog: HT7700, HITACHI), Mouse lymphocyte isolation medium (catlog: LTS1092, shanghai yanjin bio. co. ltd), RPMI-1640 medium (Thermo Fisher Scientific, USA), Fetal Bovine Serum (gibico), Fixation/Permeabilization Solution (catlog: 554722, BD ), Wash Buffer (catlog: 554723, BD), Flow Cytometry Staining Buffer (catlog: 00-4222-57, eBioscience\textsuperscript{™}), anti-Mouse CD16/CD32 (catlog: MFCR00, Invitrogen), anti-mouse CD4-FITC (catlog: 11-0040-85, eBioscience\textsuperscript{™}), anti-mouse IFN-\(\gamma\)-PE (catlog: 12-7319-42, eBioscience\textsuperscript{™}), anti-mouse IL-17-PE-Cy7 (catlog: 25-7042-82, eBioscience\textsuperscript{™}), anti-mouse CD25-APC (catlog: RM6005, Invitrogen), anti-mouse Foxp3-PE-Cy5.5 (catlog: 35-4776-41, eBioscience\textsuperscript{™}), Mouse IL-1\(\beta\) ELISA Kit (catlog: 70-EK212/3-96, MultiSciences), Mouse IL-6 ELISA Kit (catlog: 70-EK206/3-96, MultiSciences), Mouse IL-10 ELISA Kit (catlog: 70-EK210/3-96, MultiSciences), Mouse IFN-\(\gamma\) High Sensitivity ELISA Kit (catlog: 70-EK280HS-96, MultiSciences)

Infectious severe sepsis mice model establishment and FFP transfusion

The forty number C57/BL6 mice were brought from Shanghai bangyao biotechnology co. LTD\textsuperscript{[No.A0001]}, 10-12 weeks old, they were maintained in specific-pathogen-free conditions in the animal care facility, after one week adapted cultivation, ten mice were anesthetized with 2% isoflurane and sacrificed, then collected the blood samples from the mice aorta abdominalis
immediately for FFP transfusion, while, the other thirty mice were administered with Escherichia coli 0111:B4 (100×106 colony forming units [CFUs] by intraperitoneal injection. Besides, the methods of bacteria cultivation were listed below\textsuperscript{21}: the Escherichia coli 0111:B4 were stored in 20% glycerol at -70°C, then, obtained some bacteria in frozen with inoculation loops and cultured them on LB agar (sigma) for 12 hours (h), 37°C, afterwards, the colonies were counted with scratch inoculation method after overnight incubation. Thereafter, judging from the mice breath, mobility and food/water intake to estimate endotoxemia three times daily for up to seven days, while, the mice appeared with tremble, high fever and difficult breathing, they were estimated severe sepsis. Then, the infectious sepsis mice model were all gavaged antibiotic-Cefuroxime Axetil (75mg/d, per mice) and randomly divided into two groups, one group mice were received FFP transfusion via the lateral tail vein, while, the other group model mice were received equivalent saline injection. After FFP transfusion and the mice recovered from endotoxemia, the mice were anesthetized with 2% isoflurane and sacrificed, then collected the blood samples from all the mice aorta abdominalis immediately.

**Exosome isolation and identification**

The collected blood samples of mice were centrifugated at 3000g/min to obtain the serum, then the Total Exosome Isolation Reagent were added to the serum with reverse blending and placed it for one night, 4 °C, afterwards, the mixed medium were centrifuged with 3000g/min, 5 min for three times, while, the precipitates were the exosome, the precipitates in three tubes were extracted the proteins, then analyzed the marker proteins CD63, CD81 with ExoAb Antibody Kit, besides, the exosome protein Galectin 9 was also detected with western blot. While, the precipitates in other tubes were resuspended with PBS, then visualized it with Transmission Electron Microscope.

**Flow cytometry**

The lymphocyte were isolated from the collected blood samples of mice with mouse lymphocyte isolation medium at 3000g/min centrifugation. Then, the obtained lymphocytes were cultured in RPMI-1640 medium with FBS and counted. Later,
3-5×10^6 cells were put in each tube and they were permeabilized with Fixation/Permeabilization Solution for 30 min away from light, then, the anti-Mouse CD16/CD32 (1μg/10^6 cell) were added in each tube for 30 min, 4 °C away from light.

Afterwards, the cells were washed with wash buffer, and one tube wasn’t received antibody incubation, the other tubes were dividedly received double staining antibodies dilution buffer: anti-mouse CD4-FITC mixed with anti-mouse IFN-γ-PE, anti-mouse CD4-FITC mixed with anti-mouse IL-17-PE-Cy7, anti-mouse CD25-APC mixed with anti-mouse Foxp3-PE-Cy5.5 for 30 min, 4 °C incubation away from light, Thereafter, the cells were washed twice and re-suspended in Flow Cytometry Staining Buffer for Flow Cytometry analysis with instrument Calibur FACS (Bioscience, BD, USA).

**Elisa assay**

The collected blood samples of mice were centrifugated at 3000g/min to obtain the plasma for three times, then the plasma was analyzed the production of cytokines IL-1β, IL-6, IL-10 and IFN-γ with the enzyme linked immunosorbent assay (ELISA) kits, finally, the microplate reader (Bio-Tek, Epoch) was used to measure optical density at 450 nm, all the procedures were accorded with the reagents manufacturers’ instructions.

**Statistical analysis**

The data were analyzed with one-way ANOVA method in SPSS19.0 software. The significance was defined as P<0.05. All graphs were depicted with Graphpad prism 6.0 presenting data with mean ± SEM. Data are representative of at least two or three independent experiments.

**Results**

**FFP transfusion inhibited the secretion of exosome protein Galectin 9 in infectious severe sepsis mice model**

In the severe sepsis mice model, we firstly detected the protein Galectin 9 in mice blood compared with the normal control mice ([**figure 1A**](#)), then we further identified the protein Galectin 9 was a secreted exosome protein ([**figure 1 B, C**](#)), besides, FFP transfusion in the sepsis mice model inhibited the secretion of exosome protein Galectin 9 compared with the sepsis mice without FFP transfusion ([**figure 1D, E**](#)).
FFP transfusion facilitated the reprogramming of CD4$^+$ T cell immune response through regulating the exosome protein Galectin 9

The protein galectin-9 was a member of the β-galactoside binding lectin family locating on the cell membrane, cytoplasm, and nucleus. Interestingly, in the study, results showed that FFP transfusion promoted the reprogramming of T cell immune response. The CD4$^+$ T cell subtypes of Th1, Th17 and Treg cells maintenance changed greatly. FFP transfusion promoted the proliferation of Th1 cells and Th17 cells (figure 2A, B), whilst, obviously inhibited the differentiation and expansion of Treg cells (figure 2A, C). Moreover, this immune reprogrammed activity mediated the secretion of inflammatory cytokines, the pro-inflammatory cytokines production of IL-1β, IL-6 and IFN-γ were all increased at different levels, whilst, the anti-inflammatory cytokine IL-10 production was reduced (figure 3).

Discussion
In this study, we studied the sepsis disease in hypoinflammatory status. In the severe sepsis mice model, we identified the protein galectin-9 to be an exosome protein, which was found exerting therapeutic effects on polymicrobial sepsis through expanding NKT cells and pDC-like macrophages to modulate the inflammatory response$^{22}$. Whilst, in our study, results showed that FFP transfusion facilitated the reprogramming of CD4$^+$ T cell immune response through inhibiting the secretion of exosome protein Galectin 9. The differentiation and expansion of Th1 and Th17 cells was recovered after FFP transfusion, whilst, this activity of Treg cells was evidently suppressed. This finding was consistent with the study of Wu HP$^{23}$ and Li J$^{24}$ that the proportion of Th1/Th2 was inversed and the counts of Th1, Th2, Th17 and Treg cells were all decreased in severe sepsis or septic shock. Furthermore, the inflammatory cytokines, IL-1β, IL-6, IFN-γ and IL-10 were all modulated with FFP transfusion. The pro-inflammatory cytokines, IL-1β, IL-6 and IFN-γ were reversed, whilst, the production of IL-10 was reduced. This effect was possibly due to the activity of exosome protein Galectin 9 inducing Th1 cell apoptosis, exhaustion and inhibiting Th17 cells expansion being weakened, thus, IFN-γ mutually influenced Th1 cell
differentiation and itself secretion\textsuperscript{25}, meanwhile, IL-1\textbeta{} and IL-6 favored Th17 cells differentiation to suppress TGF-\textbeta{}-driven induction of Foxp3 T cells\textsuperscript{26,27}. In addition, FFP transfusion reduced the secretion of exosome protein Galectin 9, which could indirectly reduced the differentiation and expansion of Treg (iTreg) cells\textsuperscript{18}. However, owing to the severe sepsis mice all treated antibiotics, hence, it may assist balancing the destroyed immune system. Whilst, during our study process, the severe sepsis mice without antibiotics treatment were all died, so we get the conclusion that FFP transfusion facilitated the reprogramming of CD4\textsuperscript{+} T cell immune response through inhibiting the secretion of exosome protein Galectin 9.

Galectin-9 protein had been found highly expressed in eosinophils, DC, macrophages, T-lymphocytes, endothelial cells, Kupffer cells, intestinal epithelial cells, and vascular endothelial cells, etc\textsuperscript{28}. Besides, it’s a highly modulatory molecule in immune function that it interacted with multiple receptors, such as, Tim-3, cell surface protein disulfide isomerase (PDI), IgE, 4-1BB, (CD137 and tumor necrosis factor receptor superfamily, member 9 (TNFRSF9)), and CD44\textsuperscript{16,28,29}. In the study of Wang, et al\textsuperscript{30} clearly proved Tim-3 to be a potential therapeutic target for the treatment of sepsis. Therefore, in our study, we should further investigate the interacted receptor of Galectin-9 exosome in CD4\textsuperscript{+} T cells. In addition, the master transcription factor and secondary transcription factors\textsuperscript{8}, such as Th1,T-bet/STAT4; Th2, GATA3/STAT5; Th17, ROR\gamma{}t/STAT3; iTregs, Foxp3/STAT5 had identified playing important roles in the differentiation and maintenance of CD4\textsuperscript{+} T cells, hence, how the cytokines regulate the transcription factors in CD4\textsuperscript{+} T cells awaiting reveal. On the other hand, in our study, FFP transfusion in sepsis mice didn’t induce unpredictable adverse effects, whilst, FFP transfusion in clinical study often occured adverse effects, such as acute lung injury\textsuperscript{31}. This reason may be due to the species variation and the amount of FFP transfusion, accordingly, we thought further study are necessary to identify whether the truth of our finding is similar in clinical sepsis patients. Taken together, FFP transfusion promoted reprogramming CD4\textsuperscript{+} T lymphocytes immune response through inhibiting the
secretion of exosome protein Galectin 9 in mice with severe sepsis to relieve immunosuppression.

Declarations

**Ethics approval and consent to participate**

All animal experiments were performed according to the guidelines of the animal ethical organization and obtained the permission of Shanghai Gongli Hospital, the Second Military Medical University.

**Consent to publish**

All of the authors have Consented to publish this research.

**Availability of supporting data**

All data generated or analyzed during this study are included in this published article.

**Competing interests**

All authors declare no conflict of interest.

**Funding**

This work is supported by Key Disciplines Group Construction Project of Pudong Health Bureau of Shanghai (No. PWZxq 2017-10) and National Natural Science Foundation of China (No. 81870147).

**Authors’ contributions**

Each author has made an important scientific contribution to the study and has assisted with the drafting or revising of the manuscript.

**Acknowledgements**

We would like to acknowledge the reviewers for their helpful comments on this paper.

**References**

1. K. Thompson, B. Venkatesh and S. Finfer, *Internal Medicine Journal*, 2019, **49**, 160-170.

2. R. S. Hotchkiss, G. Monneret and D. Payen, *Lancet Infectious Diseases*, **13**, 260-268.

3. S. Cajander, A. Bäckman, E. Tina, K. Strålin, B. Söderquist and J. Källman, *Critical Care*, **17**, R223.

4. S. Cajander, E. Tina, A. Bäckman, A. Magnuson, K. Strålin, S. Bo and J. Källman, 2016, **11**, e0154690.
5. C. Ammer-Herrmenau, U. Kulkarni, N. Andreas, M. Ungelenk, S. Ravens, C. Hübner, A. Kather, I. Kurth, M. Bauer and T. Kamradt, PloS one, 2019, 14, e0211716-e0211716.
6. K. M. Ramonell, W. Zhang, A. Hadley, C.-W. Chen, K. T. Fay, J. D. Lyons, N. J. Klingensmith, K. W. McConnell, C. M. Coopersmith and M. L. Ford, PloS one, 2017, 12, e0188882-e0188882.
7. P. Huang, Y. Zhou, Z. Liu and P. Zhang, Mediators Inflamm, 2016, 2016, 1701059-1701059.
8. J. Zhu, H. Yamane and W. E. Paul, Annu Rev Immunol, 2010, 28, 445-489.
9. W. E. Paul and J. Zhu, Nature Reviews Immunology, 10, 225-235.
10. X. Song, H. Gao and Y. Qian, Advances in Experimental Medicine & Biology, 2014, 841, 99-151.
11. X. Chen and J. J. Oppenheim, Journal of Leukocyte Biology, 95, 723-731.
12. N. R. Ferguson, H. F. Galley and N. R. Webster, 25, 106-109.
13. J. Guo, W. Tao, D. Tang and J. Zhang, 2017, 40, 607.
14. Y. Li, J. Feng, S. Geng, S. Geng, H. Wei, G. Chen, X. Li, L. Wang, R. Wang and H. Peng, 48, 670-677.
15. A. C. Anderson, D. E. Anderson, L. Bregoli, W. D. Hastings, N. Kassam, C. Lei, R. Chandwaskar, J. Karman, E. W. Su and M. Hirashima, Science, 318, 1141-1143.
16. C. Zhu, A. C. Anderson, A. Schubart, H. Xiong, J. Imitola, S. J. Khoury, X. X. Zheng, T. B. Strom and V. K. Kuchroo, 6, 1245-1252.
17. M. Seki, S. Oomizu, K.-m. Sakata, A. Sakata, T. Arikawa, K. Watanabe, K. Ito, K. Takeshita, T. Niki and N. Saita, Clinical Immunology, 127, 78-88.
18. C. Wu, T. Thalhamer, R. F. Franca, S. Xiao and V. K. Kuchroo, Immunity, 2014, 41, 270-282.
19. N. Reiter, N. Wesche and A. Perner, Danish Medical Journal, 2013, 60, A4606.
20. L. B. Holst, 2016, 63.

21. E. B. Okeke, I. Okwor, Z. Mou, P. Jia and J. E. Uzonna, *Shock*, 40, 65-73.

22. T. Kadowaki, A. Morishita, T. Niki, J. Haras, M. Sato, J. Tani, H. Miyoshi, H. Yoneyama, T. Masaki and T. Hattori, *Critical Care*, 17, R284.

23. H. P. Wu, K. Chung, C.-Y. Lin and B.-Y. Jiang..., *Inflammation Research*, 62, 751-763.

24. *Inflammation*, 38, 995-1002.

25. J. Zheng, Y. Liu, G. Qin, K.-T. Lam and J. Guan, *European Journal of Immunology*, 2011.

26. Y. Chung, S. H. Chang, G. J. Martinez, X. O. Yang, R. Nurieva, H. S. Kang, L. Ma, S. S. Watowich, A. M. Jetten and Q. Tian, 30, 576-587.

27. Y. Carrier, W. Gao, T. Korn, T. B. Strom, M. Oukka, H. L. Weiner, V. K. Kuchroo and E. Bettelli, *Nature*, 441, 235-238.

28. S. Merani, W. Chen and S. Elahi, *Reviews in Medical Virology*, 25, 175-186.

29. S. Madireddi, S.-Y. Eun, S.-W. Lee, I. Nem?ovi?ova, A. K. Mehta, D. M. Zajonc, N. Nishi, T. Niki, M. Hirashima and M. Croft, *Journal of Experimental Medicine*, 211, 1433-1448.

30. F. Wang, H. Hou, L. Xu, M. Jane, J. Peng, Y. Lu, Y. Zhu and Z. Sun, *Human Immunology*, 75, 470-478.

31. R. P. Dellinger, M. M. Levy, J. M. Carlet, J. Bion, M. M. Parker, R. Jaeschke, K. Reinhart, D. C. Angus, C. Brun-Buisson, R. Beale, T. Calandra, J.-F. Dhainaut, H. Gerlach, M. Harvey, J. J. Marini, J. Marshall, M. Ranieri, G. Ramsay, J. Sevransky, B. T. Thompson, S. Townsend, J. S. Vender, J. L. Zimmerman, J.-L. Vincent, C. International Surviving Sepsis Campaign Guidelines, N. American Association of Critical-Care, P. American College of Chest, P. American College of Emergency, S. Canadian Critical Care, M. European Society of Clinical, D. Infectious, M. European Society of Intensive
Figures

Figure 1

Western blot analyzed the protein Galectin 9 in sepsis mice blood samples, &P<0.05 indicated significant difference vs normal control group (A); Transmission Electron Microscope (TEM) detected protein Galectin 9 to be an exosome protein, the arrow pointed the exosomes (B), the exosome surface protein CD63 and CD81 (C); Western blot analyzed exosome protein Galectin 9 in sepsis mice blood samples with or without plasma transfusion, #P<0.05 indicated significant difference vs sepsis model group (D, E).
The T cell subtypes of Th1, Th17 and Treg cells maintenance changed with or without plasma transfusion by Flow cytometry analysis (A). **P<0.05 of Th1, Th17 and Treg cells showed significant difference vs sepsis model group (B, C), besides, data were presented as mean ± SEM.
Figure 3

Elisa assay analyzed the secretion of inflammatory cytokines, the increase of IL-1β, IL-6 and IFN-γ, whilst, the decrease of IL-10 in sepsis mice model with plasma transfusion vs sepsis mice model, &P<0.05 indicated significant difference, besides, data were presented as mean ± SEM.