KAMILA REIS SANTOS

Evaluation of the cellular immune response of recombinant *Staphylococcus aureus* proteins that predict cure and prevention associated with granulocyte and macrophage colony stimulating factor (GM-CSF)

São Paulo
2021

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Thesis submitted to the Postgraduate Program in Veterinary Clinic of the School of Veterinary Medicine and Animal Science of the University of São Paulo to obtain the Doctor's degree in Sciences.

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Area:

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Advisor:

Prof. Dr. Alice M. M. P. Della Libera

De acordo:_____

Prof. Dr. Alice Della Libera

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CERTIFICADO

Certificamos que a proposta intitulada "Avaliação da resposta imune celular de proteínas recombinantes de Staphylococcus aureus preditoras de cura e prevenção associados ou não ao fator estimulante de colônia de granulócitos e macrófagos (GM-CSF).", protocolada sob o CEUA nº 6276100519 (ID 006643), sob a responsabilidade de **Alice Maria Melville Paiva Della Libera** *e equipe; Kamila Reis Santos* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo (CEUA/FMVZ) na reunião de 03/06/2019.

We certify that the proposal "Evaluation of the cellular immune response of recombinant proteins of Staphylococcus aureus predictors of cure and prevention associated or not to the colony stimulating factor of granulocytes and macrophages (GM-CSF).", utilizing 30 Isogenics mice (30 females), protocol number CEUA 6276100519 (ID 006643), under the responsibility of **Alice Maria Melville Paiva Della Libera** *and team; Kamila Reis Santos* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the School of Veterinary Medicine and Animal Science (University of São Paulo) (CEUA/FMVZ) in the meeting of 06/03/2019.

Finalidade da Proposta: Pesquisa

Vigência da Proposta: de 08/2019 a 08/2021 Área: Clínica Médica Veterinária

Origem:	Biotério do Departamento de Medicina Veterinária Preventiva e Saúde Animal						
Espécie:	Camundongos isogênicos	sexo:	Fêmeas	idade:	6 a 6 semanas	N:	30
Linhagem:	C57bl/6J			Peso:	100 a 500 g		

Local do experimento: O experimento será realizado no biotério do Departamento de medicina preventiva e saúde animal da universidade de São Paulo. As amostras serão processadas no laboratório de zoonoses bacterianas.

São Paulo, 21 de abril de 2020

Prof. Dr. Marcelo Bahia Labruna
Coordenador da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

Camilla Mota Mendes
Vice-Coordenador
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

São Paulo, 29 de junho de 2020
CEUA N 6276100519

IImo(a). Sr(a).
Responsável: Alice Maria Melville Paiva Della Libera
Área: Clínica Médica Veterinária

Título da proposta: "Avaliação da resposta imune celular de proteínas recombinantes de Staphylococcus aureus preditoras de cura e prevenção associados ou não ao fator estimulante de colônia de granulócitos e macrófagos (GM-CSF).".

Parecer Consubstanciado da Comissão de Ética no Uso de Animais FMVZ (ID 006463)

A Comissão de Ética no Uso de Animais da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo, no cumprimento das suas atribuições, analisou e **APROVOU** a Emenda (versão de 23/junho/2020) da proposta acima referenciada.

Resumo apresentado pelo pesquisador: "O pedido de emenda se dá pela necessidade de aumentar um grupo experimental no referido projeto. O presente estudo é composto por cinco grupos experimentais, sendo um controle e quatro com diferentes protocolos experimentais. Porém, identificamos um gargalo na resposta imunológica devido ao uso de o fator estimulante de colônia de granulócitos e macrófagos (GM-CSF), sendo necessário fazer a inclusão de um grupo controle que receberá somente a vacina de DNA recombinante contendo GM-CSF, ou seja, o controle do efeito desta vacina sem a ação das proteínas recombinantes de S. aureus. Gostaríamos de ressaltar que a emenda anterior foi aprovada no dia 12 de março de 2020, no início do período de quarentena, dessa forma não foram pedidos os animais para realização da nova fase experimental. Dessa forma pedimos que que seja aprovada a inclusão de mais 6 animais, totalizando 36 animais para o estudo.".

Comentário da CEUA: "Não existem óbices para a aprovação da inclusão de outros 6 animais. Recomendamos que a pesquisadora verifique com a CiBIO (Comissão interna de Biossegurança) sobre as aprovações necessárias para a utilização de DNA recombinante e se estão de acordo com as normativas da CTNBIO".

Prof. Dr. Marcelo Bahia Labruna
Coordenador da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

Camilla Mota Mendes
Vice-Coordenadora da Comissão de Ética no Uso de Animais
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EVALUATION FORM

Autor: SANTOS, Kamila Reis

Title: Evaluation of the cellular immune response of recombinant *Staphylococcus aureus* proteins that predict cure and prevention associated or not with granulocyte and macrophage colony stimulating factor (GM-CSF)

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Date: _____/_____/_____

Committee Members

Prof. _____

Institution:_____ Decision: _____

Prof. _____

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Prof. _____

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Prof. _____

Institution:_____ Decision: _____

Prof. _____

Institution:_____ Decision: _____

DEDICATION

I dedicate this thesis to my intellectual mentors, Fernando Nogueira de Souza and Alice Della Libera.

ACKNOWLEDGEMENTS

To my parents, **Francisco and Valéria**, and sisters **Jéssica and Thais**, for supporting me since the beginning of this journey, and they are the most important part of my life, thank you for helping me to build each part of this dream.

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“"And those who were seen dancing were considered insane by those who could not hear the music."

Friedrich Nietzsche

ABSTRACT

SANTOS, K. R. **Evaluation of the cellular immune response of recombinant *Staphylococcus aureus* proteins that predict cure and prevention associated with granulocyte and macrophage colony stimulating factor (GM-CSF)** [Avaliação da resposta imune celular de proteínas recombinantes de *Staphylococcus aureus* preditoras de cura e prevenção associados ou não ao fator estimulante de colônia de granulócitos e macrófagos (GM-CSF)] 2021. 99 f. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2021.

Staphylococcus aureus is a notorious pathogen associated with chronic intramammary infections in dairy cows, besides being extremely critical due to its contagiousness and refractoriness to antimicrobial treatment, beyond critical issues related to food security and public health. A more comprehensive understanding of these mechanisms, including those that may not be known or fully appreciated at present. With this in mind, the present thesis is divided into three chapters. First, the first aims explored the expression of CD62L and CD44 by bovine PBMCs and WC1.1^{+} γδ T cells under *S. aureus* cell culture stimulation condition. Thus, we were able to identify that *S. aureus* was associated with high expression of CD44 in overall PBMC and WC1.1^{+} γδ T cells, and they could generate a memory WC1.1^{+} γδ T cells, preferably central memory cells. The second chapter comprises the evaluation of the immunogenic of three *S. aureus* -associated preventive recombinant proteins called: F0F1 ATP synthase subunit α (SAS), succinyl-diaminopimelate (SDD) and cysteinyl-tRNA synthetase (CTS). associated with granulocyte-macrophage colony-stimulating factor (GM-CSF) plasmid DNA vaccine in a murine model. In this chapter, we observe that these recombinant *S. aureus* proteins associated with GM-CSF trigger type 3 immunity, mainly by the TCRVγ4+ subpopulation, that could confer a robust protective type 3 immunity. The last chapter deals with the evaluation of the immunogenicity of three *S. aureus* -cure associated, so-called: elongation factor-G (EF-G), enolase (ENO) and phosphoglycerate kinase (PGK) in association with the granulocyte macrophage colony stimulating factor (GM-CSF) plasmid DNA vaccine in a murine model. The results demonstrated that these recombinant proteins alone are also capable to foster type 3 immunity in response to *S. aureus*, but by both αβ and γδ lymphocytes instead of TCRVγ4+ subpopulation. In this case, GM-CSF did not improve the immunogenicity of these *S aureus* recombinant proteins, and instead favor type 2 immune response pattern. Although, our study strongly indicated promising outcomes, more studies are needed in experimentally *S. aureus* challenged animals to validate our results.

Keywords: Memory cells. Vaccine. Type 3 immunity. Dairy cattle. Mastitis.

RESUMO

SANTOS, K. R. **Avaliação da resposta imune celular de proteínas recombinantes de *Staphylococcus aureus* preditoras de cura e prevenção associados ao fator estimulante de colônia de granulócitos e macrófagos (GM-CSF)** [Evaluation of the cellular immune response of recombinant Staphylococcus aureus proteins that predict cure and prevention associated or not with granulocyte and macrophage colony stimulating factor (GM-CSF)] 2021. 99 f. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2021.

Staphylococcus aureus é um patógeno notório associado a infecções intramamárias crônicas em vacas leiteiras, além de ser extremamente crítico por sua contagiosidade e refratariedade ao tratamento antimicrobiano, além de questões críticas relacionadas à segurança alimentar e saúde pública. Uma compreensão mais abrangente desses mecanismos, incluindo aqueles que podem não ser conhecidos ou totalmente apreciados no momento. Com isso em mente, a presente tese está dividida em três capítulos. Primeiramente, o primeiro capitulo objetivou explorou a expressão de CD62L e CD44 por PBMCs bovinos e células T WC1.1^{+} γδ sob condição de estimulação de cultura de células de *S. aureus*. Assim, pudemos identificar que *S. aureus* está associado à alta expressão de CD44 em PBMC e células T WC1.1^{+} γδ em geral, podendo gerar células T de memória WC1.1^{+} γδ, de preferência células de memória central. O segundo capítulo compreende a avaliação da imunogenicidade de três proteínas recombinantes preventivas associadas a *S. aureus* denominadas: F0F1 ATP sintase subunidade α (SAS), succinil-diaminopimelato (SDD) e cisteinil-tRNA sintetase (CTS). associada à vacina de DNA plasmídico de fator estimulador de colônia de granulócitos-macrófagos (GM-CSF) em um modelo murino. Neste capítulo, notamos que essas proteínas recombinantes de *S. aureus* associadas ao GM-CSF desencadeiam imunidade tipo 3, principalmente pela subpopulação TCRVγ4^{+}, que poderia conferir uma imunidade protetora tipo 3 robusta. O último capítulo trata da avaliação da imunogenicidade de três proteínas de *S. aureus*, associadas à cura, chamadas: fator de alongamento-G (EF-G), enolase (ENO) e fosfoglicerato quinase (PGK) associada à vacina de DNA plasmídico de fator estimulador de colônia de granulócitos-macrófagos (GM-CSF) em um modelo murino. Os resultados demonstraram que essas proteínas recombinantes sozinhas também são capazes de promover imunidade tipo 3 em resposta a *S. aureus*, mas por ambos os linfócitos αβ e γδ em vez da subpopulação TCRVγ4 $^{+}$. Nesse caso, o GM-CSF não melhorou a imunogenicidade dessas proteínas recombinantes *S aureus* e, em vez disso, favoreceu o padrão de resposta imune tipo 2. Embora nosso estudo tenha indicado resultados promissores, mais estudos são necessários em animais desafiados experimentalmente com S. aureus para validar nossos resultados.

Palavras-chave: Células de memória. Vacina. Imunidade tipo 3. Gado leiteiro. Mastite.

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SUMMARY

1 INTRODUCTION

Bovine mastitis is known to be the disease of greatest concern in the dairy industry, because it is the most prevalent and most expensive disease. Much of the economic damage is linked to the expenses with treatments, often inefficient, which end up culminating in persistency infection, in addition to the losses generated by milk disposal (RUEGG, 2003; SCHUKKEN et al., 2003; AITKEN; CORL; SORDILLO, 2011; ZHAO et al., 2015). As we, the animals have a natural defense against pathogens. A more comprehensive understanding of these mechanisms, including those that may not be known or fully appreciated at present, and how they can be manipulated in favor of the host is the basis of this proposal to develop effective immunotherapeutic and vaccine to fight mastitis in a health-oriented animal production system. There are still many hiatuses related to the immune response of the host that need to be clarified and understood in an attempt to minimize the damage caused by this bacterium, regarding .... as a multifactorial disease, considering its complexity. (BRADFORD et al., 2015; OLIVEIRA et al., 2015).

Among the pathogens causing mastitis, *Staphylococcus aureus* stands out for being one of the most prevalent pathogens in intramammary infections (IMI), because it has the ability to evade the host's immune response, in addition to its importance tied to public health (SCALI et al., 2015; SACCO et al., 2020). Several studies have been developed in relation to *S. aureus*, highlighting in this case *S. aureus* resistant to methicillin (MRSA), making it a potential risk for human health, since it can be transmitted to humans, and entering the list of "priority pathological agents" by the World Health Organization (WHO) (JUHÁSZ-KASZANYITZKY et al., 2007; NORMANNO et al., 2007). According to the organization, if new strategies and approaches to reduce antimicrobial resistance do not emerge by 2050, about 10 million people will die each year from infections caused by antimicrobial-resistant super bacteria such as *S. aureus* (KRÖMKER; LEIMBACH, 2017).

This pathogen is a multi-host bacterial that has the ability to adapt and infect distinct species, making it a threat to public health and food safety. Recently, cattle were considered as the main animal reservoir for the emergence of human epidemic clones of *S. aureus* (RICHARDSSON et al., 2018). Thus, public health concerns about mastitis by *S. aureus* should be highlighted, leading to an urgent need for innovation and effective alternative approaches for the treatment and prevention of mastitis.

Vaccines for bovine *S. aureus* mastitis have been pursued for decades and approaches have focused mainly on opsonic antibody response aiming antibody-mediated bacterial clearance. A great challenge for the development of an effective vaccine relies on the on the combination and selection of antigens that could confer protective immunity. Although is focusing solely on the production of antibodies the solution to this vaccine? Currently we know that the immune response is enhanced when the union of innate with humoral response occurs converging in to three major types of cell mediate effector cells (ANNUNZIATO et al., 2015).

The type 1 immunity involves the T-bet transcription factor and the production of IFN-γ, innate lymphoid cell (ILC1), CD8 cytotoxic T cells (T_C1), and CD4 T_H1 cells, which protect against intracellular microbes through activation of mononuclear phagocytes. The type 2 comprises GATA-3 transcription factor, ILC2, T_C2, and CD4 T_H2 cells producing IL-4, IL-5, and IL-13, which induce mast cell, basophil, and eosinophil activation, as well as IgE and antibody production. And the type 3 that is mediated by RORγt transcription factor, ILC3s, T_C3 cells, and T_H17 cells producing IL-17, IL-22, or both, which activate mononuclear phagocytes but also recruit neutrophils and induce epithelial antimicrobial responses, thus protecting against extracellular bacteria and fungi cells (ANNUNZIATO et al., 2015).

In this context, what has been demonstrated is the importance in inducing type 3 immunity, mainly related to γδ T cell-derived IL-17, due to its critical role in the recruitment and activation of neutrophils. Studies conducted in murine and human models show that individuals with deficiency in some mechanisms of activation of these cells, or in the process of IL-17 release are more susceptible to infections by *S. aureus* (IWAKURA et al., 2008; NEMBRINI et al., 2009; CUA et al., 2010; PUEL et al., 2011).

As previously described, numerous studies have already been developed in an attempt to solve the mastitis problem, the vaccines already developed have added knowledge and help in the treatment of the disease, attenuating the damage caused by *S. aureus*, however, many vaccine studies in cattle prioritize the production of antibodies, while the response of T cells, especially unconventional T lymphocytes (in this case, gamma-delta), has been historically neglected, despite growing evidence of the role of this population in protective immunity. Moreover, new studies have shown that the use of new technologies such as the use of DNA vaccine and vaccines with recombinant proteins, can have a promising future and bring effective responses (PEREIRA et al, 2011; SCALI et al., 2015; RAINARD et al., 2017).

CHAPTER I

Short communication: Expression of L-selectin (CD62L) and CD44 on bovine lymphocytes and WC1.1^{+} γδ T cells under stimulation with *Staphylococcus aureus*

Will be submitted to: Frontiers in Veterinary Science

2 CHAPTER I - SHORT COMMUNICATION: EXPRESSION OF L-SELECTIN (CD62L) AND CD44 ON BOVINE LYMPHOCYTES AND WC1.1+ γδ T CELLS UNDER STIMULATION WITH STAPHYLOCOCCUS AUREUS

ABSTRACT

The present study explored the expression of CD62L and CD44 by bovine peripheral blood mononuclear cells (PBMCs) and WC1.1$^+$ γδ T cells under *S. aureus* cell culture stimulation. Here peripheral blood cells were isolated from ten dairy cows and co-cultured with *S. aureus*. Afterwards the identification of γδ T cell subpopulation and the expression of CD44, CD62L and proliferative (ki67$^+$) cells were evaluated by flow cytometry. Our results showed that the percentage of proliferative PBMCs and WC1.1$^+$ γδ T cells were higher when stimulated with *S. aureus*. The percentage of CD44$^+$ cells increased in *S. aureus* stimulated cultured PMBCs and WC1.1$^+$ γδ T cells, as well as the CD44 geometric meanfluorescence intensity (GMFI). The percentage of CD62L cells did not differ among groups in both PBMCs and WC1.1$^+$ γδ T cells. A higher GMFI of CD62L in proliferative PBMCs than nonproliferative ones upon stimulation with *S. aureus* were found, whereas no impact on the GMFI of CD62L was observed in WC1.1$^+$ cells. In summary, our study could identify that *S. aureus* was associated with high expression of CD44 in overall PBMC and WC1.1$^+$ γδ T cells, and they could generate a memory WC1.1$^+$ γδ T cells, preferably central memory cells.

Keywords: mastitis, adhesion, migration, inflammation, memory cells, dairy cattle.

CHAPTER 1

2.1 INTRODUCTION

Bovine mastitis the costliest disease for dairy farming and affect a high proportion of dairy cows. One of the most common bovine mastitis pathogens is *Staphylococcus aureus* that comprises a real issue for dairy farming due to its pathogenicity, contagiousness, likelihood of persistence of the intramammary infections, poor cure rates associated with current antimicrobial therapies, with unquestionable importance in veterinary and human medicines (Rainard et al., 2018; Cunha et al., 2020). Howbeit this bacterium is also known to be part of the skin microbiota (Da Costa et al., 2014; Santos et al., 2020).

Extravasation and subsequent trafficking of lymphocytes to the site of inflammation represent a hallmark of immunity against infections (Soltys and Quinn, 1999; Waters et al., 2003). In this concern, there is a consensus that T cells are imperative for protection against *S. aureus* infections, otherwise *S. aureus* can affect T cell response (Bröker et al., 2016). Among T cells, it has been showed that a subpopulation of $WC1.1^{+}$ γδ T cells is the major secreting IFN-γ (Rogers et al., 2005) and IL-17 cytokines (McGill et al., 2016), which is essential for protection against subsequent infections by *S. aureus* (Beekhuizen and van de Gevel, 2007; Cho et al., 2010; Murphy et al., 2014; Swintle et al., 2015). In this concern, almost nothing is known about this subpopulation of γδ T cells under *S. aureus* infections in dairy cattle.

CD44 is an adhesion molecule also known to participate in many cellular processes which includes the regulation of growth, survival, proliferation, differentiation, and motility. CD44 is an adhesion molecule that is expressed by most cells and mediates binding to extracellular matrix. This molecule is up-regulated in naïve T cells after activation via the T cell receptor and high levels is maintained on memory cells (Ponta et al., 2003; Baaten et al., 2010). Thus, CD44 proteins have essential functions in life and their absence or dysfunction cause pathogenic phenotypes (Ponta et al., 2003).

L-selectin (CD62L) is a type I transmembrane cell adhesion molecule expressed on most circulating leukocytes. The glycoprotein and cell adhesion molecule CD62L is an important cell surface marker to memory cell and is highly expressed in naïve T cells, these cells are responsible for the vigilance in the blood stream, and as soon as a pathogen enters the circulation, they migrate quickly to the lymph nodes (Ahlers, J. D.; Belyakov, I. M., 2010). The differentiation of naive cells into effector memory cells involves the downregulation of CD62L and the shutdown of the L-selectin gene, followed by an increase in CD44 expression in cell surface (Ivetic et al., 2019). Upon contact with the antigen, as T cells pass to expresses the CD44 molecule on its surface, this molecule is known to be expressed in cells of effective memory, when an expression of CD62L is linked to central memory cells (Samji and Khanna; 2017). Moreover, almost nothing is known about bovine γδ T cell differentiation during *S. aureus* infection, especially the WC1.1^{+} γδ T cells subpopulation.

Although the expression of CD62L and CD44 on bovine T cells have been studied in *Mycobacterium bovis* infection in cattle (Waters et al., 2003), and have been related to memory cell strategies, these markers have already been extensively revised for human and murine species, however, almost nothing is known about the regulation of these adhesion molecules on overall peripheral blood mononuclear cells (PMBCs) and WC1.1^{+} γδ T cells by *S. aureus* in dairy cattle. Thus, the present study explored the expression of CD62L and CD44 by bovine PBMCs and WC1.1^{+} γδ T cells under *S. aureus* cell culture stimulation condition.

2.2 MATERIALS AND METHODS

2.2.1 Animals and Sample Collection

The experiment was conducted at the Dairy Cattle Research Laboratory of the Department of Animal Nutrition and Animal Production, Pirassununga, Brazil, approved by the ethics committee on animal use of the School of Veterinary Medicine and Animal Sciences of the

University of São Paulo, under the protocol n. 6380210218. Here, we used ten clinically healthy midlactating Holstein dairy (days in milk = 171.3 ± 13.96; milk production = 29.4 ± 1.71), where 50 mL of peripheral blood were aseptically collected into heparin tube for the isolation of peripheral blood mononuclear cells (PBMCs).

2.2.2 Lymphocyte Isolation

For lymphocyte proliferation, PBMCs were isolated from each animal using Ficoll-Paque™ Plus® (cat. n. 17-1440-03, GE Heathcare, USA, density gradient 1.077 g mL^{-1}) following the instructions of the manufacturer. The PBMCs were placed in cell culture medium composed of RPMI-1640 (cat. n. R7638, Sigma Aldrich, St. Louis, USA) supplemented with 10% bovine serum fetal (SFB, cat. n. F9665 Sigma Aldrich, St. Louis, USA), 5 x 10^{-2} mM 2-mercaptoethanol (cat. n. 21985-023, Invitrogen, Grand Island, USA), 2 mM of L-glutamine (cat. n. 21051- 024, GIBCO™, Invitrogen, Grand Island, USA) and antibiotic-antimycotic solution of 100X (cat. n. 15240-062, Life Technologies, Pasley, United Kingdom). Cell viability was initially checked using trypan blue (cat. n. T8154-100ML, Sigma Aldrich, St. Louis, USA), which was always > 98%; then, the PBMCs were counted in a Neubauer chamber, and their concentration was adjusted to 2.2 × 10^6 viable cells mL^{-1}.

2.2.3 Preparation of *S. aureus* inoculum

An udder-adapted *S. aureus* (spa typing t605) strain originated from a case of persistence subclinical mastitis (Santos et al., 2020) was used. The staphylococci inoculum was prepared as previously described by Souza et al. (2016). The bacteria were resuspended in RPMI-1640 (cat. n. R7638, Sigma Aldrich, St. Louis, USA) with 10% heat-inactivated fetal bovine serum (Cultilab, Campinas, Brazil), and stored at - 80 ºC for at maximum 7 d. The bacterial concentration was adjusted to the final inoculum dose (2 x 10^8 staphylococci mL^{-1}) to obtain a multiplicity of infection (MOI) of 10. After adjusting the bacterial concentration, the inoculum

was heat-inactivated at 60 ºC for 1 h, after which 10 µL of the solution were plated on a plate containing blood agar and incubated at 37ºC for 24 h to confirm inactivation.

2.2.4 Cell culture

The cells were washed twice in cell culture medium and placed in plates of 96 U bottom wells (2×10^5 cells/90 µL/well) and cultured for 96 h (37 °C in an atmosphere humidified at 5% CO_2). To determine the effect of stimulation on the expression of CD62L and CD44, the cells were culture under unstimulated (basal, 10 µL of cell culture medium) and stimulated conditions with 10 µL of heat-inactivated *S. aureus* (MOI 1:10) or concanavalin-A type III (Con-A; cat. n. C2631, Sigma Aldrich, St. Louis, MO, USA), a widely used mitogen, at a final concentration of 10 µg mL^{-1} (Nieto Farias et al., 2018; Souza et al., 2020).

2.2.5 Identification of WC1.1+ γδ T cells and expression of CD62L and CD44

After the incubation period, the cells were harvest from the 96-well plates and transferred to 5 mL tubes, round bottom, 12 x 75 mm, for flow cytometry, and centrifuged at 250 x g at 4ºC for 8 minutes. After that, the supernatant was discarded, the identification of $WC1.1^+$ γδ T cells and those cells expressing CD62L and CD44 was performed by incubating the cells for 30 min at room temperature in the dark with the following monoclonal antibodies (mAbs): a phycoerythrin (PE)-conjugated mouse anti-human CD62L (cat n. MCA1076PE, Bio Rad, Hercules, California, EUA) that has cross-reactivity with bovine, mouse IgG3 anti-bovine CD44 (cat n. BOV2037, WSU, Pullman, WA, USA), and a primary mouse IgG1 anti-bovine $WC1.1^+$ cells (cat n. BOV2119, WSU, Pullman, WA, USA) γδ T cells. After the incubation period, the cells were washed with PBS at 250 x g at 4ºC for 8 minutes. The supernatant was discarded, and then the cells were labeled with the secondary antibodies goat-mouse IgG1 PE-

Texas Red® (cat n. M32017, Thermo Fisher, Massachusetts, EUA) and goat anti-mouse IgG3 PE-Cyanine 5.5 (PE-Cy5.5), (cat n. 1100-13, Southern Biotech, São Paulo, Brazil).

2.2.6 Lymphocyte proliferation

To determine if proliferation affects CD62L and CD44 expression, ki-67-stained PBMC were identified. Ki-67 is a nuclear protein that plays a role in the regulation of cell division. This protein is expressed during all active phases of cell division, although is absent in quiescent cells and during DNA repair (Soares et al., 2010). Thus, after immunophenotyping to identify the subpopulation of gamma-delta T cells ($WC1.1^{+}$) and cells expressing CD44 and CD62L, the cells were washed with 500 µL of permeabilization solution (PBS + 0.1% saponin + 0.09 % azide + 1 % SFB) and centrifuged at 250 x *g* at 4ºC for 8 minutes. After centrifugation, the cells were fixed using 500 µL of the fixation solution (paraformoaldeide 4%, 0.09% azide and PBS) and incubated for 15 minutes at room temperature in a dark place. Then the suspension was washed with 500 µL of permeabilization solution and centrifuged at 250 x *g* at 4ºC for 8 minutes. Afterwards, 10 µL of diluted (2 µL of Ki67 diluted in 198 µL of permeabilization solution) rabbit anti-Ki67 antibody solution (cat n. ab15580, abcam, Cambridge, UK) was added to the tubes and incubated for 1 h at 4 ºC. After the incubation period, 500 µL of the permeabilization solution was added and the samples centrifuged at 250 x *g* at 4ºC for 8 minutes. The supernatant was discarded and 1 µL of fluorescein isothiocyanate (FITC) goat anti-rabbit IgG H&L secondary antibody (cat n. ab6717, abcam, Cambridge, UK) was added to tubes and incubated again for 30 min at room temperature in a dark place. After incubation 500 µL of the permeabilization solution were added and the samples centrifuged at 250 x *g* at 4ºC for 8 minutes. After centrifugation, the samples were resuspended in 300 µL of PBS with 1% SFB and analyzed by flow cytometry (FACSCantoII™ flow cytometer, Becton Dickinson Immunocytometry System™, San Diego, USA). For this assay, 30,000 cells were examined in

each sample. Flow Jo Tree Star software (FlowJo - Treestar 10.5.3 for Windows, Tree Star Inc., Ashland, OR, USA) was used to analyze the data. An unstained control and single-stained samples were also prepared as compensation controls. Negative control samples were also stained with conjugated isotype control antibodies. In addition, cells were stained with fluorescence minus-one controls. Doublets were excluded using forward scatter (FSC) area versus FSC height.

2.2.7 Statistical analysis

Data distribution was initially evaluated by the Shapiro-Wilk test. The comparisons among groups (unstimulated control, and *S. aureus* and Con-A stimulated) with parametric data distribution were initially submitted to Repeated measures ANOVA, and the means were compared by the Tukey's multiple comparisons test. The variables with non-parametric distribution were compared by the Friedman test, followed by Dunn's test. Statistical analysis was carried out using GraphPad Prism 5.0 software® (GraphPad Software, Inc., San Diego, CA, USA). For the comparisons of the CD44 and CD62L expressions between proliferative and nonproliferative cells and among groups, the two-way ANOVA followed Bonferroni post-test was applied. Results are reported as mean $\pm$ SEM. Significance was set at $P < 0.05$), unless otherwise indicated.

2.3 RESULTS AND DISCUSSION

In the present study, we should noted that the percentage of proliferative ($Ki67^{+}$) cells were higher when stimulated with *S. aureus* ($P = 0.03$) than unstimulated control. In addition, a trend toward a higher proliferation upon mitogen Con-A ($P = 0.06$) stimulation was also observed.

Furthermore, a higher percentage of Ki67$^+$ cells among WC1.1$^+$ γδ T cells upon stimulated with *S. aureus* (P = 0.0004) and Con-A (P = 0.01) than unstimulated control was found.

CD44 is one of the universally used activation markers of T cells. After antigen encounter, T cells quickly up-regulate CD44 and its expression is also maintained in memory T cells. Beyond its usage as an activation and memory marker, CD44 mediates numerous other functions (Schumann et al., 2015). Here, the percentage of CD44$^+$ cells increased in *S. aureus* stimulated cultured PMBC (P = 0.02) and WC1.1$^+$ γδ T cells (P = 0.002) when compared to nonstimulated control cultures (Figure 1). The CD44 fluorescence intensity (i.e., GMFI) has also enhanced in overall PBMC when stimulated with *S. aureus* (P = 0.0009) (Supplemental Figure 1). A noteworthy observation of our findings is related to the fact high CD44 expression is important for IL-17 production (Schumann et al., 2015), which are crucial for protective immunity against *S. aureus* (Cho et al., 2010; Murphy et al., 2014). Furthermore, McGuill et al. (2016) showed that WC1.1$^+$ γδ T cells have a major contribution to IL-17 production that was even higher than T CD4$^+$ cells in response to bovine respiratory syncytial virus. Although, it is still unclear how specific populations of memory γδ T cells are generated, and in response of what antigens, cytokines, or other factors remains to be determined (Lalor et al., 2016). Furthermore, it is until unclear whether γδ T cells expansion is simply a mirror of the dynamic of the total lymphocyte response (Lalor et al., 2016). In contrast to our findings, Waters et al. (2003) reported that the percentage of cells under culture expressing CD44, regardless of the T cell subset, was not affected by *Mycobacterium bovis* purified protein derivate (PPD) stimulation. Indeed, these authors showed that CD44 increased on T CD4$^+$ cells upon PPD stimulation in *M. bovis* infected dairy cattle, but this effect was not observed in noninfected ones. Furthermore, PPD stimulation did not affect the expression of CD44 in T CD8$^+$ and γδ TCR$^+$ cells.

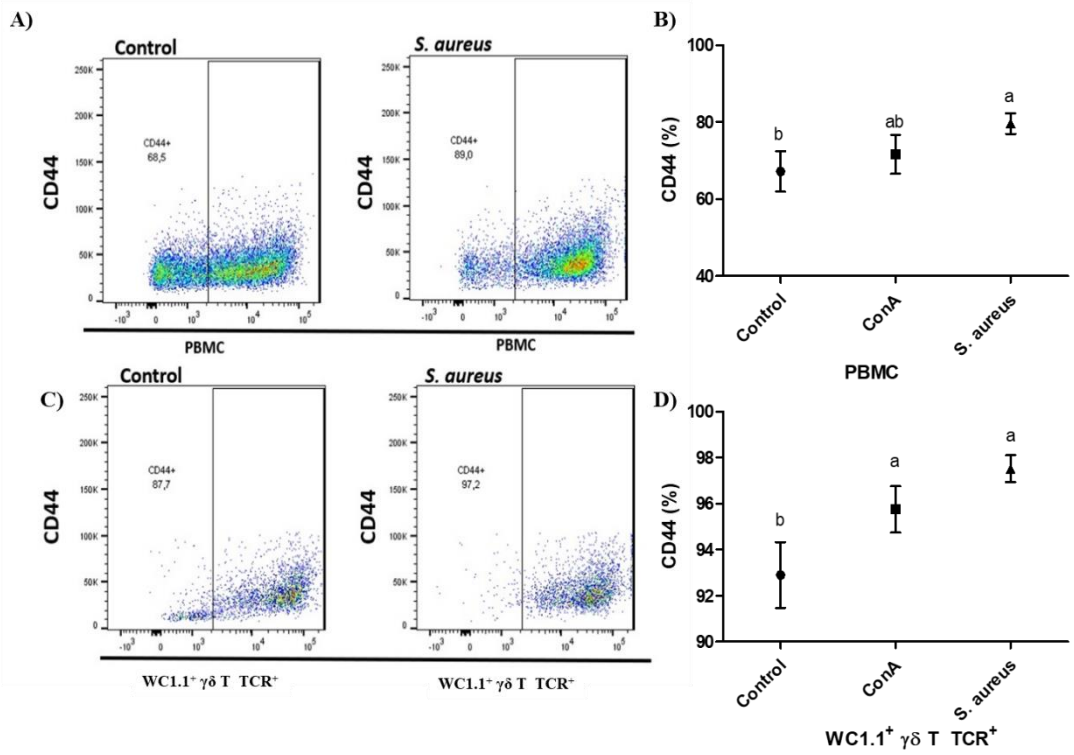

Figure 1. Representative dot plots from flow cytometry analysis demonstrating the CD44 population in peripheral blood mononuclear cells (PBMCs; A) and WC1.1$^+$ γδ T cells (C) in unstimulated control and upon stimulation with *S. aureus*. An increase in the percentage of CD44$^+$ in PBMCs (B) and WC1.1$^+$ γδ T cells (D) upon stimulation with *S. aureus* was found. Different letters indicated $P \leq 0.05$. Con-A: concanavalin-A type III.

Here, we detected a higher percentage of CD44$^+$ cells among proliferative (Ki67$^+$) overall PBMC than nonproliferative ones (Ki67$^-$ cells) in all groups ($P = 0.02$) (Figure 2). Curiously, a significant enhancement CD44 GMFI in proliferative overall PBMC (Ki67$^+$ cells) were detected just when stimulated with *S. aureus* when compared to nonproliferative cells (Ki67$^-$ cells) ($P = 0.02$) (Figure 2). This finding indicated that CD44 does occur upon proliferation under *S. aureus* stimuli. In agreement with our results, previous reports in human have shown that memory T cells have higher proliferation rates than naïve T cells (Macallan et al., 2017). Thus, our study indicated that memory cells in bovine are much shorter-lived than their naïve counterparts (CD44$^-$ cells; Zhao and Davis, (2010), and then memory cells is mainly maintained by long-lived clones in bovine rather than individual cells with long lifespan, as has previously demonstrated in mice and human (Macallan et al., 2017).

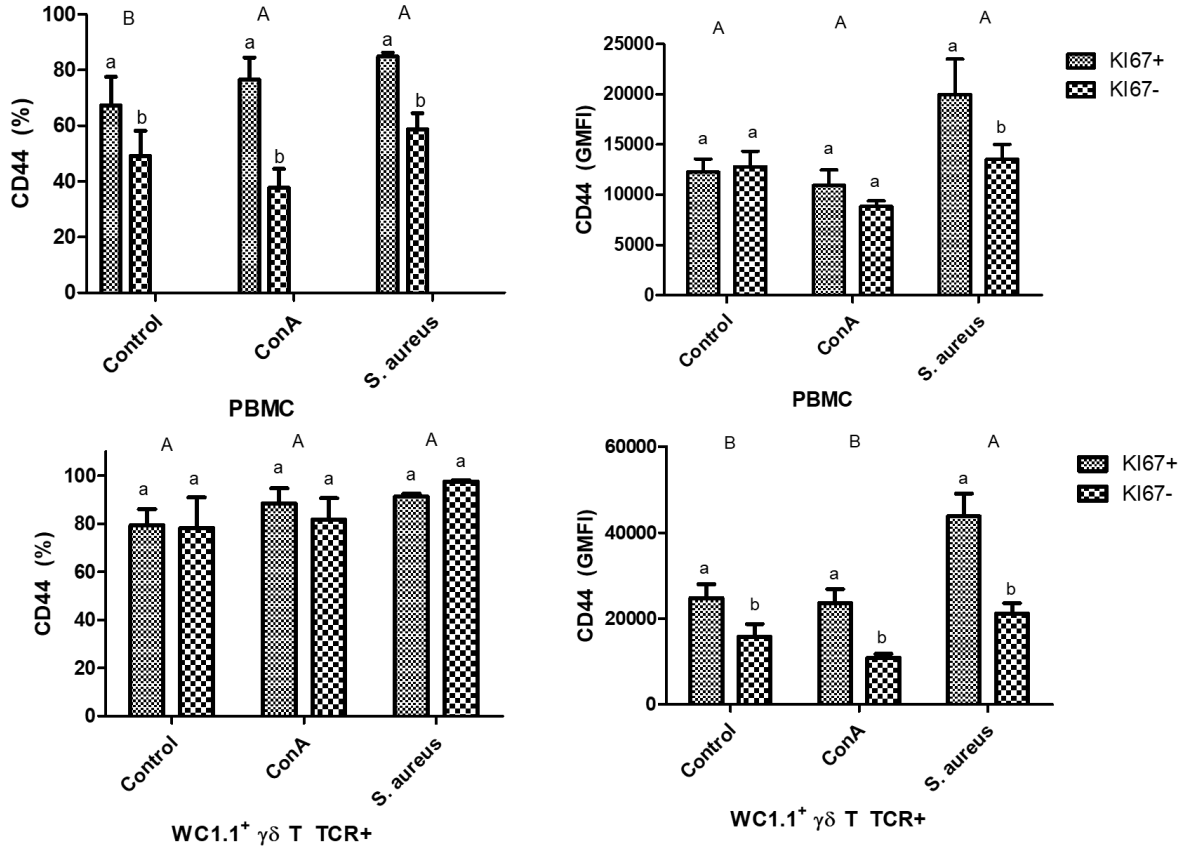

Figure 2. The percentage of CD44^{+} PBMCs was higher in proliferative (ki67^{+}) peripheral blood mononuclear cells (PBMC; A) than nonproliferative ones (ki67^{-}), although no statistical difference was observed in the percentage of CD44^{+} WC1.1^{+} γδ T cells between proliferative and nonproliferative (C). Here, the geometric mean fluorescence intensity (GMFI) was just higher in proliferative PBMCs upon stimulation with *S. aureus* (B), although the GMFI was higher in proliferative WC1.1^{+} γδ T cells than nonproliferative ones in all groups (D). Uppercase letters indicated statistical difference among unstimulated control, and upon stimulation with concanavalin-A (Con-A) and *S. aureus*. Lowercase indicated statistical difference proliferative (ki67^{+}) and nonprolifeative (ki67^{-}) cells. Different letters indicated $P \leq 0.05$.

On the other hand, no significant difference on the percentage of CD44^{+} cells among proliferative (ki67^{+}) and nonproliferative (Ki67^{-} cells) WC1.1^{+} γδ T cells were found. However, an increase in CD44 fluorescence intensity (i.e., GMFI) was detected in all groups ($P \leq 0.0001$) (Figure 2). Similarly to our findings, Waters et al. (2003) reported that the expression of CD44 (i.e., GMFI) was higher in proliferative cells than nonproliferative ones upon stimulated by PPD stimulation in all T cell subsets evaluated investigated from *M. bovis*-infected cattle. In this regard, it should be noted that dairy cows *S. aureus* are constantly exposed to *S. aureus* as they are part of skin microbiota (Da Costa et al., 2014; Santos et al., 2020).

The percentage of $CD62L^+$ cells did not differ among groups in both overall PBMC ($P = 0.13$) and $WC1.1^+$ γδ T cells ($P = 0.60$; Supplemental Figure 2). However, we found an enhancement of CD62L GMFI by both PBMC ($P = 0.01$) and $WC1.1^+$ γδ T cells ($P = 0.01$) upon stimulation by *S. aureus* when compared to mitogen Con-A (Supplemental Figure 3). From other perspectives, we observed that both PBMCs and $WC1.1^+$ γδ T proliferative cells ($ki67^+$) had a markedly higher percentage of $CD62L^+$ than nonproliferative ones ($P \leq 0.0001$) (Figure 3). Nevertheless, the PBMC and $WC1.1^+$ γδ T cells had a distinct behavior, as proliferative overall PBMC cells ($ki67^+$) had higher GMFI of CD62L ($P = 0.0002$) (molecules per cell) than nonproliferative ones, while this phenomenon was observed in unstimulated $WC1.1^+$ γδ T cells, no significant difference on the GMFI of CD62L between proliferative and nonproliferative $WC1.1^+$ γδ T cells stimulated by *S. aureus*- and ConA was found (Figure 3). In this regard, CD62L is classically regarded an adhesion molecule, as well as a lymph node homing receptor that allow cells to migrate to secondary lymphoid tissues maintain the capacity to remain in or back into lymph nodes (Oehen and Brduscha-Riem, 1998; Jameson and Masoput; 2018).

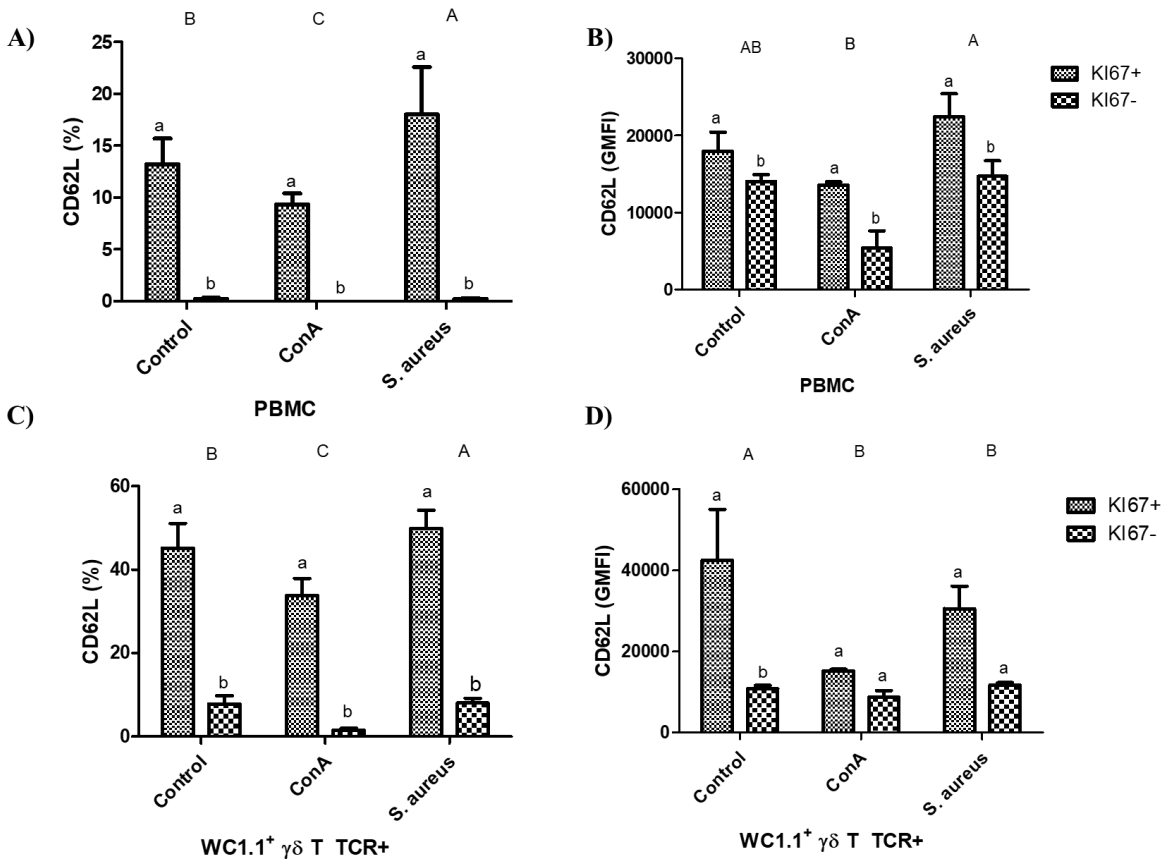

Figure 3. The percentage of CD62L^{+} was almost abrogated in nonproliferative (ki67^{-}) peripheral blood cells (PBMC; A) and WC1.1^{+} γδ T cells (C). The percentage of CD62L^{+} cells was higher upon stimulation with *S. aureus* in both PBMCs (A) and WC1.1^{+} γδ T cells (C). Furthermore, the CD62L geometric mean fluorescence intensity (GMFI) was lower in nonproliferative PBMCs in all groups (B), although this phenomenon was just observed in nonstimulated control WC1.1^{+} γδ T cells (D). The CD62L GMFI was highest upon stimulation with *S. aureus* in PBMCs (B), otherwise nonstimulated control WC1.1^{+} γδ T cells showed the highest levels of CD62L expression. Uppercase letters indicated statistical difference among unstimulated control, and upon stimulation with concanavalin-A (Con-A) and *S. aureus*. Lowercase indicated indicated statistical difference proliferative (ki67^{+}) and nonprolifeative (ki67^{-}) cells. Different letters indicated $P \leq 0.05$.

Regarding memory T cells, we used the CD44 surface marker as it has been considered as the most reliable marker that is expressed at high levels in all memory T cells irrespective of their activation status. The memory T cells was also segregated into two distinct populations: a CD44^{high} CD62L^{low} that exert a rapid effector function (so-called effector memory), which have poor proliferative capability, and a CD44^{high} CD62L^{high} population with no immediate effector function (central memory) that possess high proliferative potential (Van Faassen et al., 2005; Krishnan et al., 2007). In this regard, our study showed that bovine γδ T cells can differentiate

into effector and memory T cells subsets under *S. aureus* stimulation ($P = 0.003$), similarly to those previously described in *M. bovis* infection (Guerra-Maupone et al., 2019). In this sense, *S. aureus* could trigger the formation of central memory T cells (T_{CM}), especially WC1.1$^+$ γδ T cells (Figure 4). Central memory T cells do not produce any the prototypic cytokines of the effector cell lineage immediately after stimulation through TCR, although they secrete IL-2, IL-7 and IL-15 and proliferate extensively, but after proliferation they could efficiently differentiate to effector cells and produce large amounts of cytokines given them rapid effector function (Schluns et al., 2003).

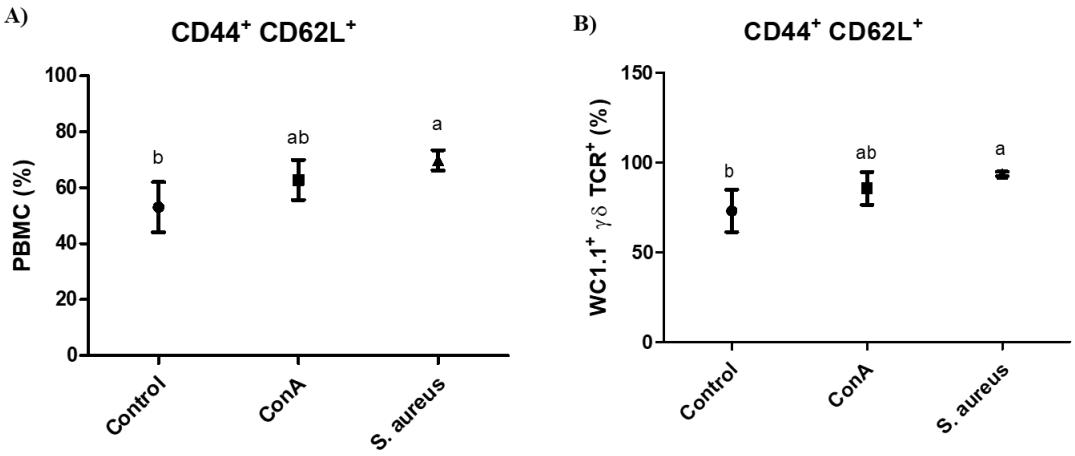

Figure 4. Increased percentage of CD44$^+$ CD62L$^+$ upon stimulation with *S. aureus* than unstimulated control in both overall peripheral blood mononuclear cells (PBMC; A) and WC1.1$^+$ γδ T cells (B). Different letters indicated $P \leq 0.05$. Con-A: concanavalin-A type III.

These cells circulate through the lymph nodes and mucosal lymphoid organs (Sallusto et al., 2004; Peppers and Jenkins, 2011). However, although it needs to be better elucidated in bovines, T CD8$^+$ cells stimulated *in vitro* in the presence of IL-15 acquired a central memory phenotype, whereas T CD8$^+$ cells activated with IL-2 resemble effector memory T cells, which overall regarding our results indicated that *S. aureus* may induce the production of IL-15 rather than IL-2, whereas in this respect little is known about T CD4 and γδ T cells (Schluns et al., 2003).

2.4 CONCLUSION

In summary, our study could identify that *S. aureus* was associated with high expression of CD44 in overall PBMC and WC1.1^{+} γδ T cells, and they could generate a memory WC1.1^{+} γδ T cells, preferably central memory cells.

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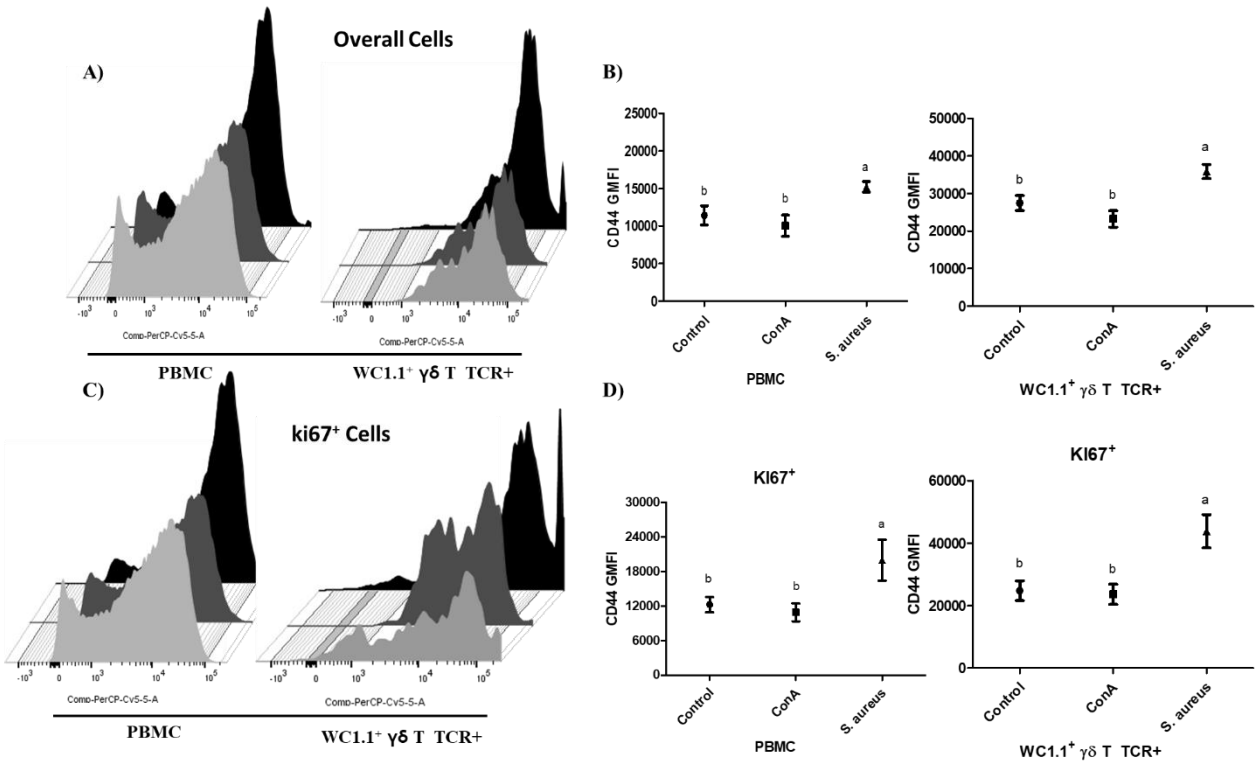
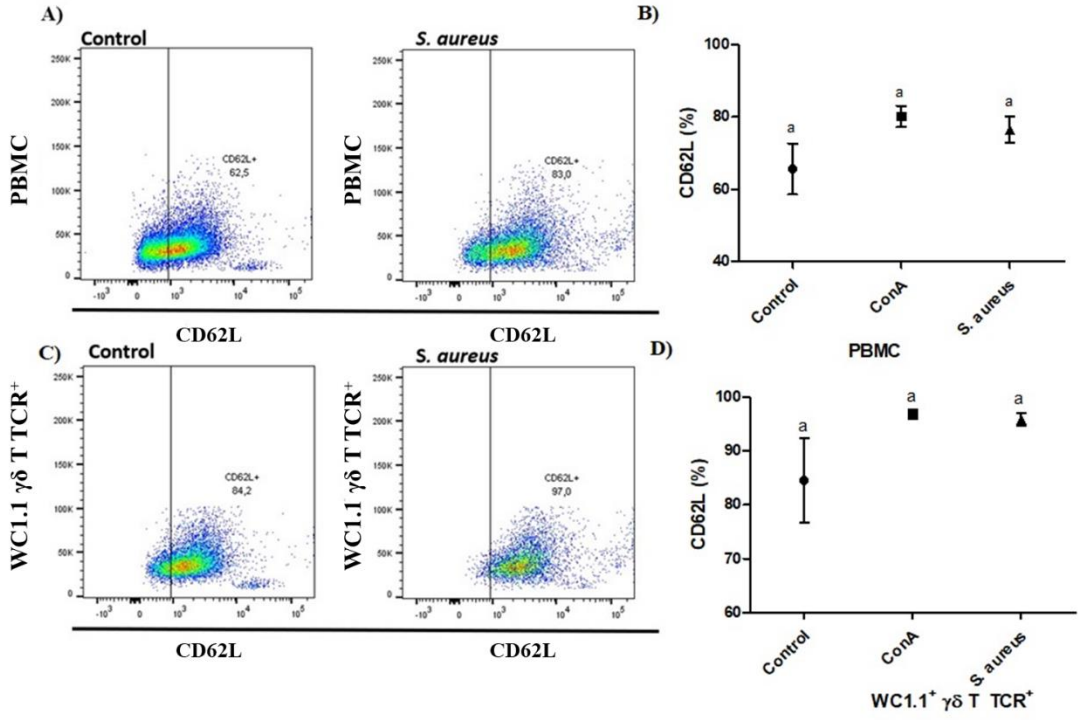
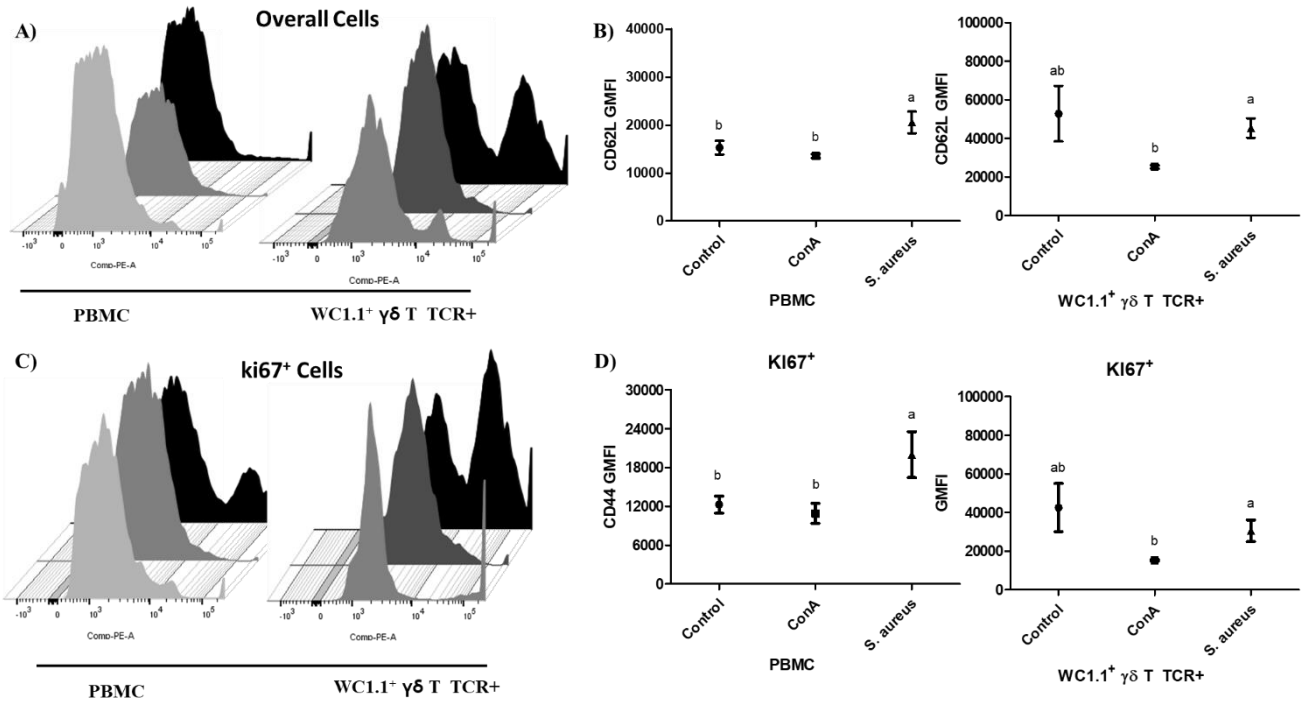
Supplementary Figures



Supplemental Figure 1. Representative histogram from flow cytometry analysis of the CD44 expression in peripheral blood mononuclear cells (PBMCs; A and C) and WC1.1$^+$ γδ T cells (A and C) in unstimulated control (light gray) and upon stimulation with concanavalin-A (Con-A; dark gray) and *S. aureus* (black). An increased in the CD44 geometric mean fluorescence intensity (GMFI) values upon stimulation with *S. aureus* in overall PBMCs and WC1.1$^+$ γδ T cells (B) and in proliferative (Ki67$^+$) PBMCs and WC1.1$^+$ γδ T cells (D) was found. Different letters indicated $P \leq 0.05$. Con-A: concanavalin-A type III.



Supplemental Figure 2. Representative dot plots from flow cytometry analysis demonstrating the CD62L population in peripheral blood mononuclear cells (PBMCs; A) and WC1.1$^+$ γδ T cells (C) in unstimulated control and upon stimulation with *S. aureus*. An increase in the percentage of CD62L$^+$ among WC1.1$^+$ γδ T cells (D) upon stimulation with *S. aureus* was observed, although no statistical difference was observed in PBMCs (C). Different letters indicated $P \leq 0.05$. Con-A: concanavalin-A type III.



Supplemental Figure 3. Representative histogram from flow cytometry analysis of the CD62L expression in peripheral blood mononuclear cells (PBMCs; A and C) and WC1.1^{+} γδ T cells (A and C) in unstimulated control (light gray) and upon stimulation with concanavalin-A (Con-A; dark gray) and *S. aureus* (black). An increased in the CD62L geometric mean fluorescence intensity (GMFI) values upon stimulation with *S. aureus* in overall PBMCs and proliferative (Ki67^{+}) (B and D) was found, although WC1.1^{+} γδ T cells (B and D) upon stimulation with *S. aureus* did not expressed higher levels of CD62L than unstimulated control. Different letters indicated $P \leq 0.05$.

CHAPTER 2

Type 3 cell-mediated response elicited by three-recombinant *S. aureus* proteins and GM-CSF DNA vaccine

Will be submitted to: Vaccines (mdpi)

3 CHAPTER 2 - TYPE 3 CELL-MEDIATED RESPONSE ELICITED BY THREE-RECOMBINANT *S. AUREUS* PROTEINS AND GM-CSF DNA VACCINE

ABSTRACT

Bovine mastitis caused by *Staphylococcus aureus* represents a big concern for dairy industry and public health, bringing great economic impacts and apprehensions related to food security and public health. Thus, the development of vaccines against bovine *S. aureus* mastitis have been unsuccessful so far. Although, there is an increasing evidence of the role of T cells in the protective immunity against *S. aureus*, T cell responses in development of an effective vaccine for bovine *S. aureus* mastitis have not so far received much attention. Here, as an initial approach, we assessed the immunogenicity of three vaccine candidate antigens, so-called: F0F1 ATP synthase subunit α (SAS), succinyl-diaminopimelate (SDD) and cysteinyl-tRNA synthetase (CTS). To determine the immunogenicity of these proteins we vaccinated twenty-four C57BL that were uniformly divided in four groups; G1: control; G2: animal that received GM-CSF DNA plasmid DNA vaccine; G3: animal that received the combination of SAS+SDD+CTS; and G4: animals that received the combinations of SAS+SDD+CTS proteins associated with GM-CSF DNA plasmid DNA vaccine. Animals were euthanized and the draining lymph nodes and spleen were removed to obtain the cells for further immunophenotyping assays and lymphocyte proliferation upon unstimulated and *S. aureus* stimulation conditions. Vaccination with *S. aureus* recombinant proteins generate memory cells in draining lymph nodes. Vaccination with the three recombinant proteins together with the GM-CSF DNA was associated with an increase in the percentage of IL-17A$^+$ cells among overall CD44$^+$ (memory) cells, T CD4$^+$ cells, CD4$^+$ T CD44$^+$ CD27$^-$ cells, cells, γδ TCR cells, γδ TCR$^+$ CD44$^+$ CD27$^+$ cells and TCRVγ4$^+$ cells were found. The GM-CSF DNA vaccination was essential for sustain IL-17A production by splenocytes culture upon *S. aureus* stimulation. Thus, our vaccination approach fosters type 3 immunity, a protective immune response against *S. aureus* was expected.

***Keywords**:* vaccine, *Staphylococcus aureus*, T cell response, mastitis, bovine.

CHAPTER 2

3.1 INTRODUCTION

Bovine mastitis is the disease with the greatest global impact on dairy farming, causing pronounced decreases in milk production and quality ~~and of derived dairy products~~. Several etiologic agents are implied in mastitis, but *Staphylococcus aureus* is considered one of most prevalent mastitis pathogens. It is an extremely critical pathogen due to its pathogenicity, contagiousness and refractoriness to antimicrobial treatment (Rainard et al., 2018; Cunha et al, 2020), beyond critical issues related to food security and public health (Richardsson et al., 2018). Therefore, to decrease the impact of *S. aureus* in dairy farming, alternative strategies to control mastitis should be evoked, such as the development of an effective vaccine.

Staphylococcus aureus pathogenicity is a complex issue, thus a single immunogen may not confer protective immunity (Zheng et al., 2020). Most studies have focused on *S. aureus* virulence factors for developing vaccines against *S. aureus* intramammary infections (IMIs) in dairy cows, but this initiative has been unsuccessful so far. Here we considered an innovative concept approaching in detail and characterizing the animals that developed successful pathogen-host immune responses to use in favor of the host (Montgomery et al., 2014; Rainard et al., 2018; Souza et al., 2019; Cunha et al., 2020). In a previous study, using sera from *S. aureus* -infected but non-diseased animals, we searched *S. aureus*-derived proteins likely related to protection (Cunha et al., 2020). Based on these antigenic proteins revealed by this immunoproteomic approach, we obtained three vaccine candidate proteins: F0F1 ATP synthase subunit α (SAS), succinyl-diaminopimelate (SDD) and cysteinyl-tRNA synthetase (CTS).

Despite observing induction of robust humoral immunity with some vaccine candidate antigens, the researches of vaccines against *S. aureus* infections have been failed so far (Armentrout et al., 2020; Rainard et al., 2020). Thus, there is growing evidences that *S. aureus* vaccine strategies aimed at eliciting appropriate long-lived B- and T-cell responses should be prioritized (Montgomery et al., 2014; Bekeredjian-Ding, 2017; Zhang et al., 2018). Thus, a

more comprehensive characterization of T-cell immunity (Zhang et al., 2018) is pivotal to characterize the immune response induced by the aforesaid proteins and to analyze in the context of protective immunity.

T-cell immunity has been increasingly reckoned as a central player in host–pathogen interaction, and T cell-derived interleukin (IL)-17 is a cornerstone of protection in *S. aureus* infections (Cua et al., 2010; Proctor et al., 2012a; Proctor et al., 2012b; Murphy et al., 2014; Bekeredjian-Ding, 2017). In this regard, the use a DNA vaccine containing GM-CSF appears as a powerful immunoadjuvant as it results in an increase of the lymphoproliferative response and IL-17 cytokine production (Mahdavi et al., 2017). Furthermore, the administration of a recombinant human GM-CSF alone leads to a reduction of 46.7% in the rate of new IMIs following challenge with *S. aureus* (Nickerson; Owens;Watts; 1989).

Aiming for an effective vaccine for *S. aureus* bovine IMIs, in the present study we propose as an initial approach the evaluation of the immune response profile triggered by three recombinant *S. aureus* vaccine candidate antigen (SAS, SDD and CTS) in association with GM-CSF DNA vaccine in a mouse model.

3.2 MATERIAL AND METHODS

3.2.1 Ethical statement

This study was approved by the Animal Research Ethics Committee of the Universidade de São Paulo - Brazil under the protocol number 6276100519.

3.2.2 Production of S. aureus recombinant proteins

The sequence of amino acid corresponding to the SAS (Genbank gi|446897629), SDD (Genbank gi|486621908) and CTS (Genbank gi|257275504) antigens were derived from *S. aureus* and were codon-optimized for *Escherichia coli* expression. Genes were commercially synthesized by Genscript, USA. The produced synthetic genes were cloned into pUC57 vector and then were sub-cloned into pET28a expression vector (Carneiro et al, 2006). Recombinant plasmids were used to transform the expression strain *E. coli* BL21-Star™(DE3) as previously described (Faria et al., 2015) with modifications. Briefly, transformed *E. coli* BL21-Star™(DE3) were selected on kanamycin plates. An overnight bacterial culture of three colonies containing the respective expression plasmid were grown in Luria-Bertani medium (LB) in the presence of kanamycin (0.05 mg mL^{-1}) until an optical density (OD) of 0.4 at 600 nm, on a rotary shaker at 37 °C. Then, isopropyl-ß-D-1-thiogalactopyranosida (IPTG, Sigma) was added to the culture to a final concentration of 0.4 mM and the induced cultures were grown for 4 h.

Cells were ruptured by ultrasound sonication on ice and debris were removed by centrifugation (20,000 x *g*, at 4 °C, for 30 minutes). The recombinant proteins were purified using columns Ni Sepharose High Performance immobilized metal ion affinity chromatography columns (HisTrap Hp, cat. n. GE17-5248-02, GE Healthcare, Logan, USA), attached to an ÄKTA Pure (GE Healthcare, USA), under denaturing conditions according to the manufacturer's instructions.

3.2.3 Production of GM-CSF DNA vaccine

The amino acid sequence of the GM-CSF (Genbank, GI: 145301581, was forwarded to FastBio (Ribeirão Preto, Brazil) for optimization for eukaryotic cells and to synthesize the respective gene. The produced synthetic genes were received cloned in pUC57 vector and then were sub-cloned into the pCI-neo mammalian expression vector insert (Promega Incorporation, USA).

E. coli DH5α was transformed with pCI-GM-CSF and immunization plasmids were purified using the ZR plasmid Gigaprep Kit (Zymo Research, USA) according to the manufacturer's instructions.

3.2.4 Liposome preparation and entrapment of plasmid DNA

We used a cationic liposome-based approach to efficiently delivery the GM-CSF DNA plasmid DNA, which has been associated to induce a strong humoral and cell-mediated responses (Fotoran et al., 2017). Briefly, liposome were prepared using dimethyl-di-octadecyl-ammonium (DDAB), cholesterol (molar ratio 1:4) and 1,2-distearoyl-sn-glycero-3-phosphoetanolamine-N-[amino(polyethylene glycol)-2000 (DSPE + PEG2000), 5% of total lipids used.

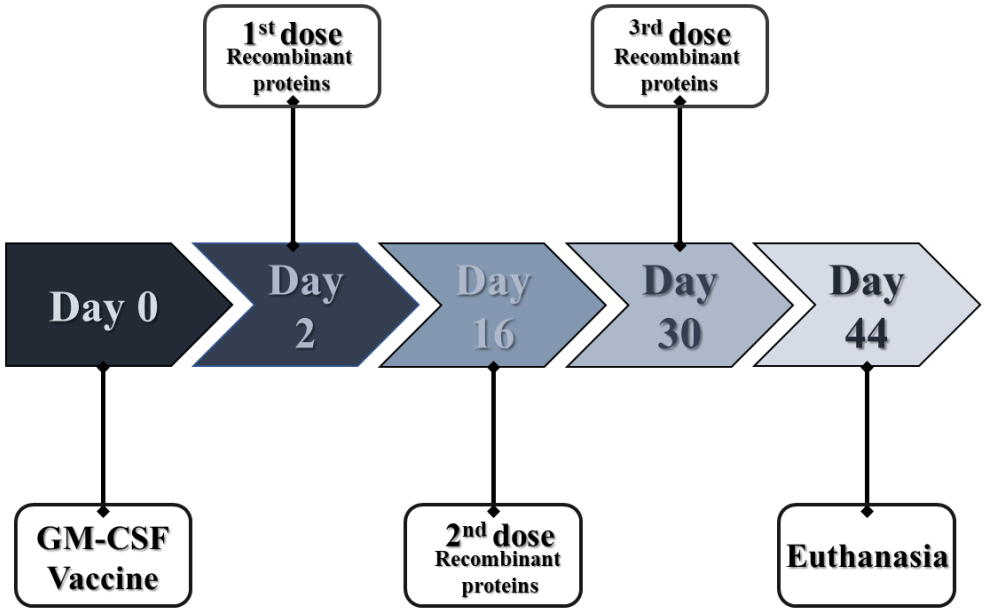
Firstly, 1 mL of chloroform of was used to dissolve this lipid solution, and then left under a constant N2 flow for evaporation of chloroform and to develop a phospholipid film on the tube walls. This film was then retained under vacuum for at least 1 h to eliminate any remaining chloroform. The film was rehydrated in 5 mM Tris-HCl (pH 7.5) at 60 ℃ for 1 h and vigorously stirred every 10 min. The obtained opaque solution was then exposed to sonication at high energy until the solution became almost fully transparent. Then, the solution was centrifuged for 1 h at 100,000 x g. Any residual pellet was removed, and the supernatant containing unilamellar lipid vesicles were used for formulation of the liposome. The genetic material was then added to the liposomes in a molar stoichiometry of 8nM DDAB for each 1 µg of nucleic acids, the molarity being approximately 0.2 pmol.

3.2.5 Animals and immunization

Twenty-four C57BL/6J mice (six-week-old, male) were purchased from the Centro de Bioterismo at Faculdade de Medicina - Universidade de São Paulo. After three weeks of adaptation, the animals (nine-week-old) were divided in four groups (six animals per group) and immunized as defined in Figure 1. Here, 50 µg of pCI-GM-CSF plasmid DNA resuspended

in 100 µL of sterile liposomal formulation were administered subcutaneously under the skin of the interscapular area near of the draining lymph nodes, and the recombinant SAS, SDD and CTS proteins (20 µg each protein) in 100 µL of saponin adjuvant (Quil-A®, cat. n. 8047-15-2, Invivogen, San Diego, California, USA) were administered intramuscularly in the deltoid muscles, as described in Figure 1.

A)



B)

GROUPS

VACCINE	G1	G2	G3	G4
SAS+SDD+CTS	-	-	+	+
GM-CSF	-	+	-	+

Figure 1. Scheme of the timeline of vaccination (A) and experimental groups (B). SAS: F0F1 ATP synthase subunit alpha recombinant *S. aureus* protein; SDD: succinyl-diaminopimelate desuccinylase recombinant *S. aureus* protein; CTS: cysteinyl-tRNA synthetase recombinant *S. aureus* protein; GM-SCF: pCI-granulocyte and macrophage colony stimulating factor plasmid DNA (GM-CSF DNA vaccine). The unvaccinated group just received the liposome or saponin adjuvant.

At day 44 [14 days after the last administration of the recombinant protein(s)] the animals were submitted to intramuscular administration of an anesthetic solution containing xylazine (10 mg kg^{-1}) and ketamine (50 mg kg^{-1}) and were euthanized by cervical dislocation. Then the draining lymph nodes (accessory axillary and proper axillary) and spleen were removed to obtain the cells for further immunophenotyping assays and lymphocyte proliferation, respectively. For each animal, the accessory axillary and proper axillary lymph nodes from the side of vaccination were combined.

3.2.6 Obtaining the spleen and lymph nodes cells

The spleens or lymph nodes of donor mice were aseptically removed, and mechanically disrupted and homogenized by syringe plunger into cell strainer (cat. n. Z742102, Sigma Aldrich, St. Louis, USA) on the top of 50 mL tube with 10 mL of proliferation medium containing RPMI 1640 medium (cat. n. R7638, Sigma Aldrich, St. Louis, USA) supplemented with 5% of heat-inactivated fetal bovine serum (Gibco, USA), and 100 U mL^{-1} penicillin, 100 µg mL^{-1} streptomycin, 0.25 µg mL^{-1} Fungizone (cat. n. 15240-096, Gibco).

The spleen and lymph node cells were centrifuged (380 x g, 10 min, 4 °C). Thereafter, the hypotonic lysis of erythrocytes was performed by adding 1,000 µL of 0.2% NaCl for 20 s; then isotonicity was restored by adding 1,000 µL of 1.6% NaCl. Cells were further centrifugated and placed in cell proliferation medium. An aliquot of the cells was suspended in trypan blue solution (cat. n. T8154-100ML, Sigma Aldrich, St. Louis, USA) to determine the number of viable cells. For lymphocyte proliferation of spleen's cells, 2 x 10^5 viable cells per well were cultured at 37 °C and 5% CO_2 for 96 h in 96-wells flat-bottom plates in the presence or not (media without bacteria – unstimulated control) of 10 µL of heat-inactivated *S. aureus* (2 x 10^8 CFU mL^{-1}).

3.2.7 Immunophenotyping of non-cultured lymph nodes

The lymph nodes cells were phenotyped using 0.5 µL of each of the fluorescent-conjugated monoclonal antibodies (mAbs; Table 1) for cell surface CD4 (clone GK1.5), CD8 (clone 53-6.7), CD19 (clone 1D3), γδ TCR (clone GL3), TCRVγ4 (clone Gl2), CD44 (clone IM7) and CD27 (clone LG.3A10) by incubating the cells for 30 min at room temperature in the dark. After the incubation period, the cells were washed with phosphate buffered saline (PBS) with pH 7.4 at 250 x g at 4ºC for 8 minutes. Furthermore, the intracellular production of IL-17A and interferon (IFN-γ) were determined. After centrifugation, the cells were fixed using 500 µL of the fixation solution (paraformoaldeide 2 %, 0.09 % azide and PBS) and incubated for 15 minutes at room temperature in the dark. Then the suspension was washed with 500 µL of permeabilization solution and centrifuged at 250 x *g* at 4 ºC for 8 minutes.

Afterwards, the supernatant was discarded, and 0.5 µL of fluorescent-dye anti-mouse IL-17 and anti-mouse IFN-γ antibodies (Table 1) was added and incubated for one hour at 4 ºC. After the incubation period, 500 µL of the permeabilization solution was added and the samples centrifuged at 250 x g at 4ºC for 8 minutes. After centrifugation, the samples were resuspended in 300 µL of PBS with 1% SFB and analyzed by flow cytometry (BD LSRFortessa™ X-20 flow cytometer, Becton Dickinson Immunocytometry System™, San Diego, USA). For this assay, 100,000 events were examined in each sample. Flow Jo Tree Star software (FlowJo - Treestar 10.5.3 for Windows, Tree Star Inc., Ashland, OR, USA) was used to analyze the data. Non-stained control and single-stained samples were prepared as compensation controls. Negative control samples were also stained with conjugated isotype control antibodies. In addition, cells were stained with fluorescence minus-one (FMO) controls. Doublets were excluded using forward scatter (FSC) area versus FSC height.

3.2.8 Preparation of *S. aureus* inoculum

An udder-adapted *S. aureus* isolate originated from a case of chronic subclinical IMI was used (Santos et al., 2020). *Staphylococcus aureus* (spa typing t605) inoculum was prepared as previously described (Souza et al., 2016b) with minor modifications. Afterwards, bacteria were resuspended in RPMI-1640 (cat. n. R7638, Sigma Aldrich, St. Louis, USA) with 10% heat-inactivated fetal bovine serum (Cultilab, Campinas, Brazil). To determine the bacteria concentration in the inoculum, an aliquot of the bacterial suspension was further cultured on BHI agar plates in dilution series and colonies numbers (colony forming units mL^{-1}) were determined. Then, the bacterial concentration was adjusted to the final inoculum dose (2×10^8 staphylococci mL^{-1}) to obtain a multiplicity of infection (MOI) = 10. The inoculum was heat-inactivated at 60 ºC for one hour, after which 100 µL of the solution were plated on a plate containing blood agar and incubated at 37 ºC for 24 h to confirm inactivation.

3.2.9 Immunophenotyping and lymphocyte proliferation of cultured spleen cells

After the incubation period, the cells were harvest from the 96-well plates and transferred to 5 mL tubes, round bottom, 12 x 75 mm, for flow cytometry, and centrifuged at 250 x *g* at 4 ºC for 8 minutes. Then, the supernatant was collected and stored for cytokines measurement, the cells were phenotyped using fluorescent-conjugated mAbs (Table 1) as abovementioned.

Furthermore, the intracellular production of interleukin (IL)-17A and interferon (IFN)-γ, beyond the antigen-specific *in vitro* lymphocyte proliferation (Soares et al., 2010), were determined. Ki67 is a nuclear protein that act in the regulation of cell division process. This protein is expressed during all active phases of cell division. thus, it has been widely used for the evaluation of cell proliferation (Soares et al., 2010). After centrifugation, the cells were fixed and further permeabilized, as aforesaid. Afterwards, 0.5 µL of fluorescent-dye anti-mouse IL-17A and IFN-γ (Table 1) and 10 µL of diluted (2 µL of ki67 diluted in 198 µL of

permeabilization solution) rabbit anti-Ki67 antibody solution (cat n. ab15580, abcam, Cambridge, UK) was added and incubated for one hour at 4 ºC.

After the incubation period, 500 µL of the permeabilization solution was added and the samples centrifuged at 250 x g at 4ºC for 8 minutes. The supernatant was discarded and 1 µL of fluorescein isothiocyanate (FITC) goat anti-rabbit IgG H&L secondary antibody (cat n. ab6717, Abcam, Cambridge, UK) was added to tubes and incubated again for 30 min at room temperature in a dark place. After incubation 500 µL of the permeabilization solution were added and the samples centrifuged at 250 x *g* at 4 ºC for 8 minutes. After centrifugation, the samples were resuspended in 300 µL of PBS with 1% SFB and analyzed by flow cytometry (BD LSRFortessa™ X-20 flow cytometer, Becton Dickinson Immunocytometry System™, San Diego, USA).

For this assay, 100,000 events were examined in each sample. Flow Jo Tree Star software (FlowJo - Treestar 10.5.3 for Windows, Tree Star Inc., Ashland, OR, USA) was used to analyze the data. Non-stained control and single-stained samples were also prepared as compensation controls. Negative control samples were also stained with conjugated isotype control antibodies. In addition, cells were stained with fluorescence minus-one (FMO) controls. Doublets were excluded using forward scatter (FSC) area versus FSC height.

Table 1. Monoclonal antibodies (mAbs) used for immunophenotyping of splenocytes and lymph nodes lymphocytes by flow cytometry

mAbs	Fluorescent probes	Target	Clone	Host	Concentration (mg mL^{-1})	Cat. n.
Anti-CD4[1]	APC-Cy7	Mouse	GK1.5	Rat	0.2	552051
Anti-CD8[1]	BV510	Mouse	53-6.7	Rat	0.2	563068
Anti-CD44[1]	BV421	Mouse	IM7	Rat	0.2	563970
Anti-CD27[1]	BV750	Mouse	LG.3A10	Hamster	0.2	747399
Anti-CD19[1]	PE-Cy7	Mouse	1D3	Rat	0.2	552854
Anti- γδ TCR[1]	BV650	Mouse	GL3	Hamster	0.2	563993
Anti-TCRVγ4[1]	FITC	Mouse	GL2	Hamster	0.2	552143
Anti-IL-17A[1]	Alexa 700	Mouse	TC11-18H10	Rat	0.2	560820
Anti-IFN-γ[1]	PE	Mouse	XMG1.2	Rat	0.2	554412

[1]BD Pharmingen™ (San Diego, EUA); IL-17A: interleukin-17A; IFN-γ: interferon-γ; FITC: Fluorescein isothiocyanate; APC-Cy7: Allophycocyanin-cyanine 7; PE: R-Phycoerythrin; PE-Cy7: R-Phycoerythrin-cyanine 7; BV510: Brilliant Violet 510; BV421: Brilliant Violet 421; BV650: Brilliant Violet 650; BV750: Brilliant Violet 750.

3.2.10 Cytokines measurement

The production of cytokines IL-2, IL-4, IL-6, IL-10, IFN-γ, IL-17A, and tumor necrosis factor (TNF)-α was determined from the supernatant of the cultured spleen cells under unstimulated control and upon *S. aureus* stimulation using the BD Cytometric Bead Array Mouse T_H1, T_H2 and T_H17 Cytokine (cat. n. 560485, BD Bioscience™, San Jose, USA) using a flow cytometer (BD LSRFortessa™ X-20 flow cytometer, Becton Dickinson Immunocytometry System™, San Diego, USA), as manufacturer's instructions. The FCAP Array™ v3.0 softaware (Softflow™, Pécs, Hungary) were used to analyze the data.

3.2.11 Statistical analysis

Statistical analysis was performed using GraphPad Prism 9 (GraphPad Software, Inc., San Diego, USA). To assess the percentage of each lymphocyte subpopulation, as well as the

percentage of proliferative cells (ki67^{+}), a stimulation index (SI) was calculated by dividing the percentage of positive cells upon *S. aureus* stimulation per the percentage of positive cells under unstimulated control condition (Giunchetti et al., 2007; Abdeladhim et al, 2011; Duz et al., 2014; Mackroth et al., 2016; Mann et al., 2020). Firstly, the data were tested for normality of the distribution using the Kolmogorov-Smirnov and Shapiro-Wilk tests. If the distribution were normal, the data of the experimental groups were subjected to a one-way ANOVA analysis followed by Tukey test. Variables with non-parametric distributions were analyzed using the Kruskal-Wallis test followed by the Dunn's test. Results are reported as mean $\pm$ standard error of the mean. $P \leq 0.05$ was considered significant, unless otherwise indicated.

3.3 RESULTS

3.3.1 Immunization with *S. aureus* recombinant proteins generate memory cells in draining lymph nodes

CD44 is a prominent activation marker which allow us to differentiate memory and effector T cells from their naïve counterparts (Schumann et al., 2015). In this regard, a higher percentage of CD44^{+} cells in draining lymph nodes was found in animals that just received *S. aureus* recombinant proteins (data not shown). Furthermore, the memory T cells was also segregated into two distinct populations: a CD44^{high} CD62L^{low} that exert a rapid effector function (so-called effector memory, T_{EM}), which have poor proliferative capability, and a CD44^{high} CD62L^{high} population with no immediate effector function (central memory, T_{CM}) that possess high proliferative potential (van Faassen et al., 2005; Krishnan et al., 2007).

In this concern, we observed that the percentage of T CD4^{+} CD44^{+} CD27^{+}, so-called T_{CM} T CD4^{+} cells, was enhanced in the draining lymph nodes from vaccinated animals that just received the combination of *S. aureus* recombinant proteins (Figure 2 A and B), while a

reduction in the percentage of T CD8$^+$ CD44$^+$ CD27$^-$, so-called effector T CD8$^+$ cells, in all groups that received the *S. aureus* recombinant proteins (Figure 21 B) was found.

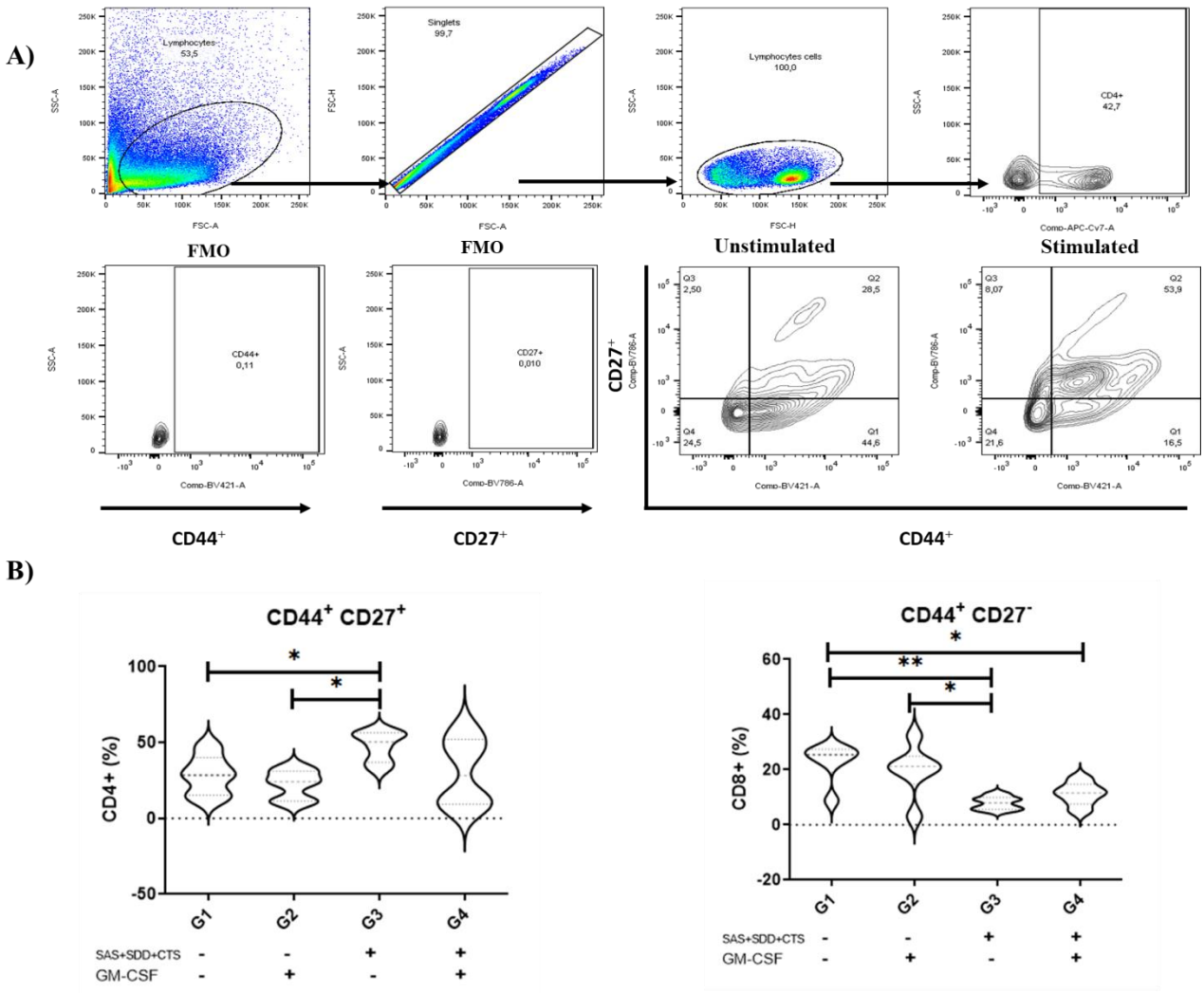


Figure 2. The antidromic trend of the percentage of T CD4$^+$ CD44$^+$ CD27$^+$ cells and T CD8$^+$ CD44$^+$ CD27$^-$ cells in draining lymph nodes from mice vaccinated with three-recombinant *S. aureus* proteins. A) A representative gating hierarchy (successive gating scheme as depicted by arrows): lymphocytes (gate 1), single cells are identified by plotting forward scatter area against forward scatter-height (gate 2), single lymphocyte cells (gate 3), T CD4$^+$ cells (gate 4), FMO controls for CD44 (gate 5) and CD27 (gate 6), and representative plots used to discriminate and identify two distinct T CD4$^+$ memory cell populations: a CD44^{high} CD27^{low} population, so-called T CD4$^+$ T_{EM}, and a CD44^{high} CD27^{high} population, so-called T CD4$^+$ T_{CM} cells. **B)** Percentage of T CD4$^+$ CD44$^+$ CD27$^+$ cells and T CD8$^+$ CD44$^+$ CD27$^-$ cells in draining lymph nodes among groups. FMO: fluorescence minus one; F0F1 ATP synthase subunit α (SAS), succinyl-diaminopimelate (SDD) and cysteinyl-tRNA synthetase (CTS).* indicate $P \leq 0.05$, ** indicate $P \leq 0.01$; *** indicate $P \leq 0.001$; **** indicate $P \leq 0.0001$

3.3.2 TCRVγ4$^+$ cells are responsible for the greatest contribution for type 1 and 3 cell-mediated immunities in the draining lymph nodes

There is an emerging concept that unconventional T cells, such as γδ T cells, could confer protective immunity against *S. aureus* (Murphy et al., 2014; Miller et al., 2020). Here, we observed that γδ TCR cells and its subpopulation TCRVγ4$^+$ cells in the studied draining lymph nodes were associated with the highest percentage of IL-17A$^+$ cells in the draining lymph nodes ($P \leq 0.0001$; Figure 3), highlighting the critical importance of this lymphocyte population in triggering type 3 cell-mediated immunity (Murphy et al., 2014; Marchitto et al., 2019). Furthermore, TCRVγ4$^+$ cell population exhibited a higher percentage of IFN-γ$^+$ cells than all αβ T lymphocytes subpopulations, while γδ TCR$^+$ cells just differ from T CD8$^+$ lymphocytes (Figure 3).

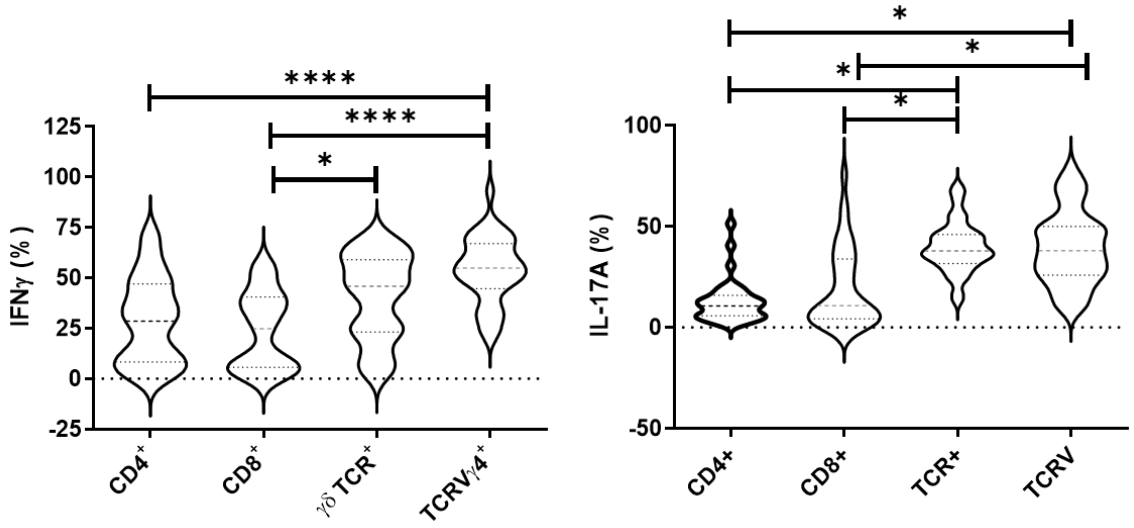


Figure 3. The γδ TCR cells and its subpopulation TCRVγ4$^+$ cells are the primary source of both IL-17A and IFN-γ in draining lymph nodes rather than αβ T cells. * indicate $P \leq 0.05$, ** indicate $P \leq 0.01$; *** indicate $P \leq 0.001$; **** indicate $P \leq 0.0001$

3.3.3 Vaccination with *S. aureus* recombinant proteins associated with GM-CSF DNA vaccine drives type 3 immunity that largely relies on γδ T and TCRVγ4$^+$ cells

There is an increasing evidence that type 3 immunity mediated by cells that produce IL17-A and IL-17F is crucial for mammary gland protective immunity (Rainard et al., 2020), and consequently a promising target for vaccine development. No effect of vaccination on B cell response was revealed (data not shown). To fairly quantify the intensity of response upon *S. aureus* stimulation, T cell responses are normalized by dividing the percentage of positive cells upon *S. aureus* stimulation per the percentage of positive cells under unstimulated control condition, thereby creating a stimulation index. Thus, when *in vitro* cultures of spleen cells were stimulated with *S. aureus* from vaccinated mice with the three recombinant proteins together with the GM-CSF DNA vaccine, an increase in the percentage of IL-17A$^+$ cells among overall CD44$^+$ (memory) cells (35.34 %), T CD4$^+$ cells (54.36 %), CD4$^+$ T_{EM} (CD44$^+$ CD27$^-$) cells (142.87 %), and TCRVγ4$^+$ cells (56.75 %) were found (Figure 4).

This effect was due almost to no significant impact on the IL-17A$^+$ among the distinct T cells subsets in spleen cell culture under *S. aureus* stimulated when compared with unstimulated conditions in animals from unvaccinated, GM-CSF DNA vaccine and those vaccinated with the *S. aureus* recombinant proteins alone ($P \leq 0.05$), while mice vaccinated with the three-antigens *S. aureus* recombinant proteins associated with the GM-CSF DNA vaccine generated a pronounced enhancement in the percentage of IL-17A$^+$ cells in all abovementioned T cell populations, primarily on γδ T cells and TCRVγ4$^+$ cells. Nevertheless, no effect of vaccination on type 1 (IFN-γ$^+$) immune cells were observed. Furthermore, no effect of vaccination on proliferative (ki67$^+$) cells was found.

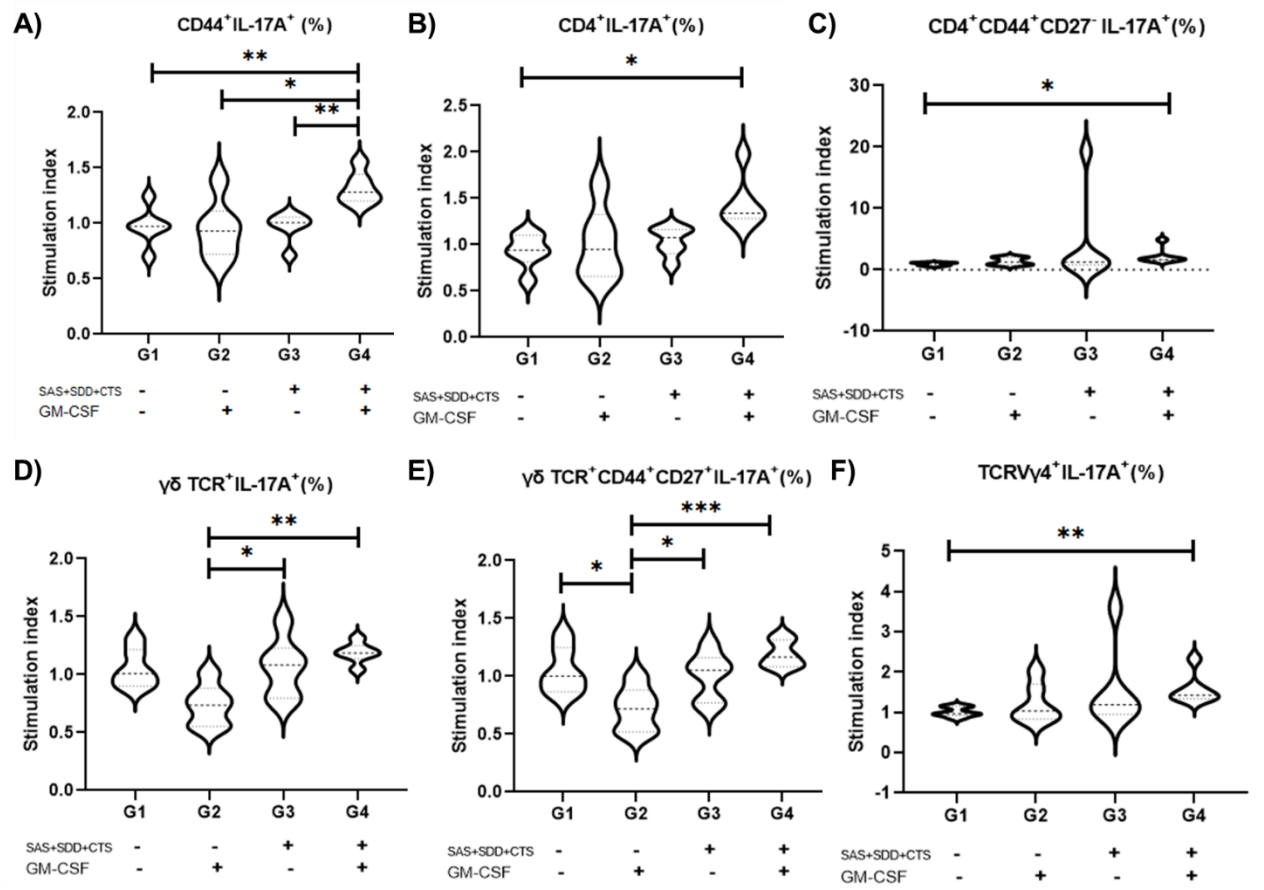


Figure 4. IL-17A$^+$ T cells in mice immunized with three-antigens *S. aureus* recombinant proteins associated with the GM-CSF DNA vaccine. T cell responses are normalized by dividing the percentage of IL-17A$^+$ cells upon *S. aureus* stimulation per the percentage of IL-17A$^+$ cells under unstimulated control condition, thereby creating a stimulation index. Here, we shown this stimulation index of: A) overall CD44$^+$ (memory) cells, B) T CD4$^+$ cells, C) T CD4$^+$ TEM (CD44$^+$ CD27$^-$) cells, D) γδ TCR cells, E) γδ TCR TCM, F) TCRVγ4$^+$ cells. * indicate $P \leq 0.05$, ** indicate $P \leq 0.01$; *** indicate $P \leq 0.001$.

3.3.4 TCRVγ4$^+$ cells and T CD8$^+$ cells are the major contributor for type 1 immunity, while γδ TCR cells mainly support type 3 immunity in splenocyte cell culture

Here, we observed both TCRVγ4$^+$ cells and T CD8$^+$ cells are the primary source of both IFN-γ, followed by γδ TCR cells (Figure 5). On the other hand, γδ TCR cells represent the major producer of IL-17A, followed by TCRVγ4$^+$ cells and T CD8$^+$ cells (Figure 4). The T CD4$^+$ cells marginally produce IFN-γ and IL-17A, although it is major population found splenocytes cell culture in both unstimulated and *S. aureus* stimulation conditions (Figure 5).

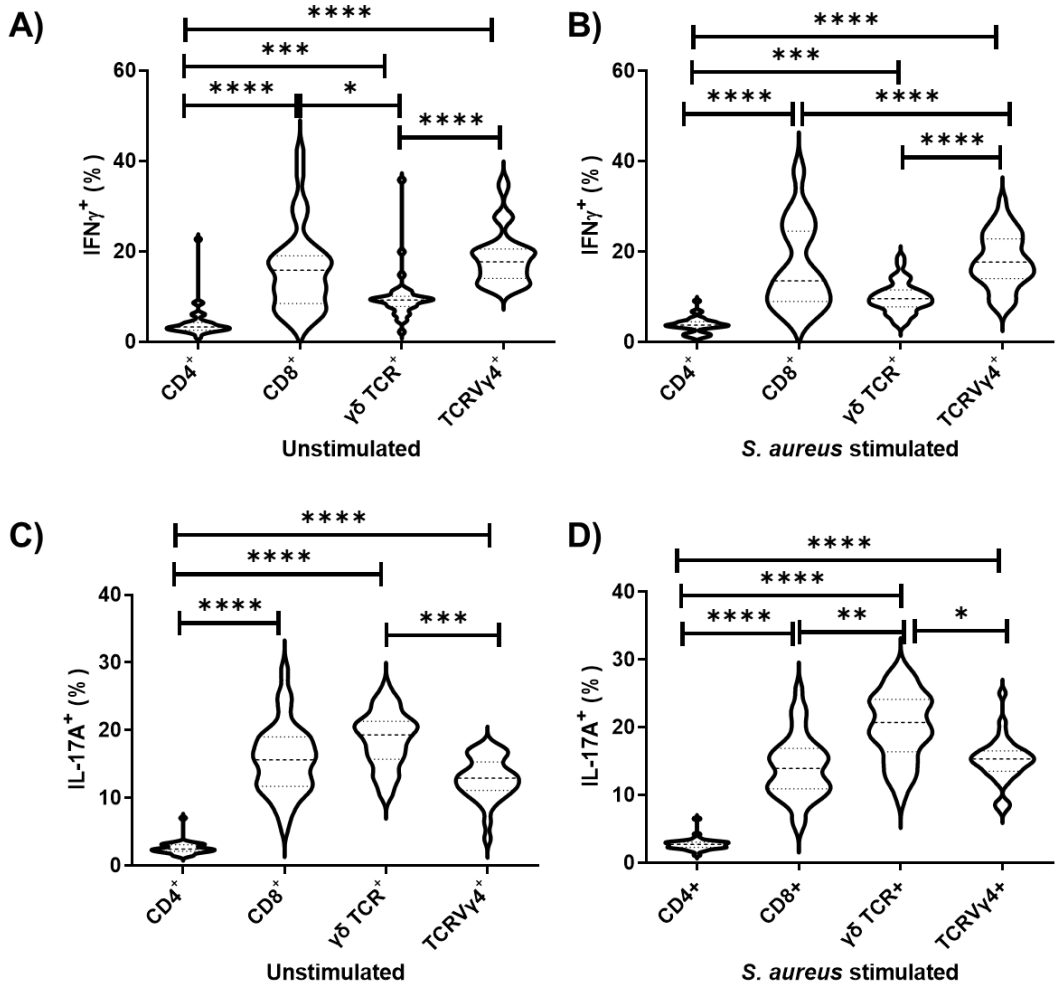


Figure 5. The TCRVγ4$^+$ cells and T CD8$^+$ cells are the primary source of both IFN-γ, while γδ TCR are the major source of IL-17A, under splenocyte cell culture in both unstimulated and *S. aureus* stimulated conditions. * indicate $P \leq 0.05$, ** indicate $P \leq 0.01$; *** indicate $P \leq 0.001$; **** indicate $P \leq 0.0001$

3.3.5 GM-CSF DNA vaccination did not allow *S. aureus* to modulate the production of IL-17A

DNA vaccination brought valuable inputs in the development of veterinary vaccines related to flexibility of design, speed, and simplicity for production, besides the possibility to incorporate immunomodulatory adjuvants into the plasmid that may trigger both humoral and cell-mediated immune responses (Zazayeri and Poh, 2019). Some DNA vaccines have been designed using the GM-CSF (Warren and Weiner, 2000; Shkreta et al, 2004; Shi et al., 2006) which affects

various cell types and promotes the survival, proliferation, activation, and differentiation of various hematopoietic cell lineages, especially macrophages and dendritic cells. In the present study, we noticed that IL-17A concentration from the supernatant of splenocytes culture upon *S. aureus* stimulation was lower than under unstimulated condition in unvaccinated control group ($P = 0.03$) and those animals vaccinated with the three-recombinant antigens ($P = 0.07$), but this phenomenon was not revealed in those animals vaccinated with GM-CSF DNA vaccinated mice ($P = 0.90$) and animals vaccinated with three recombinant proteins and the GM-CSF DNA vaccine ($P = 0.50$). Thus, GM-CSF DNA vaccination sustained IL-17A production by splenocytes culture upon *S. aureus* stimulation.

Otherwise, the IL-4 concentration in the supernatant of the splenocytes culture upon *S. aureus* stimulation was lower in animals vaccinated with the GM-CSF DNA vaccine ($P = 0.08$), those vaccinated with the three-recombinant antigens ($P = 0.03$) and vaccinated with the three-recombinant antigens together with the GM-CSF vaccine ($P = 0.06$), but this phenomenon was not revealed in unvaccinated control animals ($P = 0.50$). Moreover, the IL-4 concentration in the supernatant of the splenocytes culture upon *S. aureus* stimulated condition was higher in the unvaccinated control mice than in those animals that received the three *S. aureus* recombinant proteins alone ($P = 0.01$).

No effect in the concentration of TNF-α and IL-6 in the supernatant of the cell culture among groups and between unstimulated and *S. aureus* stimulated conditions were detected. Furthermore, the IFN-γ concentration in the supernatant of splenocyte culture upon *S. aureus* stimulation was higher in animals vaccinated with the three *S. aureus* recombinant proteins than the unvaccinated control ones ($P = 0.03$). In addition, *S. aureus* stimulation leads to a decrease in the concentration of IL-2 in the supernatant of splenocytes culture in mice that received the GM-CSF DNA vaccine together with the recombinant proteins ($P = 0.05$), while this fact was not found in the other groups. Finally, a higher level in the IL-10 in the supernatant of

splenocytes co-cultured with *S. aureus* was observed in animals vaccinated with the three recombinant proteins than the unvaccinated control ($P = 0.008$), but this phenomenon was not detected in both groups that received the GM-CSF vaccine.

3.4 DISCUSSION

Vaccination strategies could provide memory T cells, which are suited to combat pathogens as they persist for extended periods and respond quickly upon reencounter with the pathogen, which constitute a primary goal for cell-mediated vaccines (Krishnan et al., 2007; Sallusto et al., 2004; Kedzierska et al., 2012; Jameson and Masopust et al., 2018). In this regard, a hallmark finding of our study is the higher production of IL-17A by a diverse of T cell memory cells subsets using the combination of three *S. aureus* recombinant and GM-CSF DNA vaccination approach when stimulated with *S. aureus*. Beyond that, we must spotlight that TCRV$\gamma 4^{+}$ cell population also enlarge the production of IL-17A when stimulated with *S. aureus*. In the present study, we demonstrated for the first time that the combination of *S. aureus* CTS, SDD and SAS recombinant proteins associated with a GM-CSF DNA plasmid DNA vaccine foster type 3 cell-mediated immunity, which translate these impressive outcomes into a promising added value.

A noteworthy observation is that our strategy involved three recombinant proteins identified in our previous study using serum immunoproteomics approach (Cunha et al., 2020). In this latter study, we compared the antibodies produced by healthy dairy cows that were exposed to *S. aureus* but remained healthy, which allow us to support the concept that production of antibodies repertoire against these specific *S. aureus* proteins contribute to mastitis resistance. The first *S. aureus* protein so-called SAS protein plays a pivotal role in dictating biofilm growth and structure (Bosch et al., 2020), enhance tolerance of *S. aureus* towards some host antimicrobial peptides of the innate immunity (Liu et al., 2020), inhibited proinflammatory cytokines production by monocyte-derived macrophages and myeloid-

derived suppressor cells and renders *S. aureus* more resistant to bactericidal activity of monocyte-derived macrophages (Bosch et al., 2020).

The second *S. aureus* SDD protein is critical for bacteria survival and proliferation, and it is involved in protein synthesis and in the construction of the peptidoglycans of the bacterial wall (Nocek et al., 2010). Lastly, *S. aureus* CTS is an aminocyl-tRNA enzyme that catalyzes the binding of a specific amino acid to tRNA, but also attach the amino acid to other molecules, and therefore is involved in protein synthesis (Nagaev et al., 2001). Altogether, although molecular functions for these proteins are not exactly known, it is reasonable to speculate that stimulate the host immune response against these proteins may represent a sharp strategy to protect animals from *S. aureus* infections.

In this scenario, although the success of most of available vaccines clearly relies on antibody-mediated immunity, it is conceivable that humoral immunity alone may be insufficient to fully protect against *S. aureus* infections (Montgomery et al., 2014; Armentrout et al., 2020). For instance, protective immunity against recurrent *S. aureus* skin and soft tissue infection in human was associated with both antibody and T-cell mediated (i.e. IL-17) immunities, suggesting that multimechanistic approach targeting both humoral and T cell-mediated immunities may be a key strategy to provide protective immunity and prevent new *S. aureus* infections (Montgomery et al., 2014). Thus, to cover this idea, we identify that animals vaccinated with these *S. aureus* proteins associated with a GM-CSF DNA vaccine trigger type 3 cell-mediated immunity reinforcing their potential use in the prevention of new IMIs by this pathogen indicated by our previous serum immunoproteomic study.

It is notorious that defense against *S. aureus* relies first and foremost on the innate immunity by phagocytosis and killing of bacteria by phagocytes, especially neutrophils, that act in concert with the adaptive immune system. Extracellular *S. aureus* are engulfed and destroyed by phagocytes, and this process is greatly facilitated and enhanced by the binding of

specific antibodies. In this process of opsonophagocytosis, T lymphocytes have some crucial functions, such as: 1) T cells are important for the production of opsonizing antibodies, because T cells are required for antibody affinity maturation and class switch; and 2) T cells promote phagocytosis by recruitment neutrophils and macrophages to the site of infection. Furthermore, *S. aureus* is not exclusively an extracellular bacterium (Bharathan and Mullarky, 2011; Bröker et al., 2016; Souza et al., 2016b), and consequently intracellular *S. aureus* elimination is dependent on T cells.

Thus, there is an increasing consensus that memory T cells make an essential contribution toward *S. aureus* control (Bröker et al., 2016). In fact, T-cell deficiencies increase the susceptibility to *S. aureus* infections (Bröker et al., 2016; Armentrout et al., 2020). Astonishingly, the T cell responses in development of an effective vaccine for bovine *S. aureus* mastitis have not so far received much attention, especially unconventional T cells that has long been neglected. In this setting, γδ T cells represent a largely ignored targeting for vaccine design, although it comprises up to 60% of circulating lymphocytes in young cattle and up to 30% of blood mononuclear cells in adult animals (Baldwin et al., 2019).

The critical role of this T cell population in cattle could be illustrated by the fact that $WC1^+$ γδ T cells are the first to arrive at the sites of mycobacterial purified protein derivate (PPD) injection and in augment in blood frequency following mycobacterial vaccination (Buza et al., 2009; Baldwin et al., 2019). Actually, γδ T cells present cytotoxic activity in cows, and a decrease of this cell population is associated with periods of increased susceptibility to IMIs (Kabelitz, 2011).

Therefore, there is an increasing interest in studying the T-cell response to *S. aureus* infections. Different subpopulations of T cells are likely to contribute to anti-staphylococcal immune defense. Among them, recent evidences have highlighted the importance of type 3 immunity for protective immunity against infections by *S. aureus* (Cua et al., 2010; Proctor et

al., 2012a; Proctor et al., 2012b; Murphy et al., 2014), and also emphasize their importance in the development of new vaccines (Cua and Tato 2010; Spellberg and Daum, 2010; Proctor, 2012a; Proctor, 2012b). Type 3 immunity can be characterized by cells that encoded genes IL-17A, IL-17F and IL-22, and the transcription factors Rorγt and Rorα and their genes targets. The cells responsible for type 3 immunity are diverse, including γδ T cells, T $CD4^+$ helper (Th17), T $CD8^+$ (Tc17) and innate lymphoid cells 3 (Rainard et al., 2020).

Interleukin-17 is associated with neutrophil mobilization, immune response modulation and antigen-specific inflammation (Rainard et al., 2013; Murphy et al., 2014), and mediating the communication between the immune system and mammary epithelial cells (Bougarn et al., 2011). For example, epithelial cells may express receptors for IL-17 and IL-22, and exposure to IL-17 leads to the release of chemokines and granulocyte-macrophage colony-stimulating factor (GM-CSF), leading to neutrophil recruitment and activation, and culminating in the death of *S. aureus* (Cua and Tato 2010; Spellberg and Daum, 2010; Bougarn et al., 2011).

The effects of GM-CSF also include the activation of antigen-presenting cells, such as macrophages and dendritic cells, increasing major histocompatibility complex (MHC) expression, increasing its antigen-presenting capacity and amplifying the primary antibody response. GM-CSF also stimulates the maturation of dendritic cells and their consequent migration towards regional lymph nodes. GM-CSF can therefore induce differentiation, proliferation and activation of several types of cells, facilitating the development of both humoral and cellular immunity (Warren and Weiner, 2000).

It is therefore concluded that GM-CSF is an important mediator of the immune response in the mammary gland of dairy cows (Nickerson et al., 1989; Kehrli Jr et al.,1991; Stabel et al., 1991; Kimura, et al., 2014), corroborating the importance of our findings and the use of GM-CSF DNA vaccination to improve vaccine efficacy against *S. aureus*. Remarkably, *S. aureus* was associated with a decline in the concentration of IL-17A in the supernatant of the

splenocyte cell culture, but GM-CSF DNA vaccination sustained IL-17A production by splenocytes. Overall, this intriguing finding suggest that somehow the udder-adapted *S. aureus* strain used here dampen IL-17A signal in splenocytes cell culture, which is hindered by GM-CSF DNA vaccination by a mechanism that remains elusive. Although it remains unknown how GM-CSF DNA vaccination exactly works, a recent study shows that *S. aureus* activates the NLRC4 and dampen γδ T-cell derived IL-17A-dependent neutrophil recruitment during *S. aureus* infection (Paudel et al., 2018) that may explain, at least in part, our results in animals that did not received the GM-CSF DNA vaccine.

Among T-cells producing IL-17, the nontradicional γδ T cells have been identified as a potent source of innate IL-17 and implicated in host protection in murine models of *S. aureus* infection. These findings reveal that γδ T cells are an important source of IL-17 in adaptive immunity and indicate that targeting the induction of nontraditional lymphocytes such as specific subsets of γδ T cells that secrete IL-17 represent a potentially important and novel target for the rational design of future vaccines against *S. aureus* (Murphy et al., 2014, Armentrout et al., 2020). The γδ T cells are usually considered to substantially contribute to protective immunity against *S. aureus* infections bridging innate and adaptive immune responses, as they act as a first line of defense and control the innate response by neutrophil-mediated regulation (Kabelitz, 2011; Jing et al., 2012).

It has also been proposed that γδ T-lymphocytes play a crucial role in antibacterial immunity and may provide a unique barrier function for mucosal microenvironments against bacterial pathogens (Paape et al., 2000). Furthermore, it was recently shown that an increase in the subpopulation of γδ T cells (i.e. TCRVγ4^{+} cells) is essential for protection against subsequent infections by *S. aureus* (Cho et al., 2010; Maher et al., 2013; Murphy et al; 2014), and as a result this T γδ lymphocyte subpopulation represent an important property of a protective vaccine against *S. aureus* infections (Cho et al., 2010; Proctor, 2010b; Murphy et al.,

2014; Marchitto et al., 2019). In fact, a foremost finding of the present study is the higher percentage of IL-17A$^+$ TCRVγ4$^+$ cells upon stimulation with *S. aureus*, which strongly indicate a pivotal protective role of our vaccination approach against *S. aureus* subsequent infections, although further studies in vaccinated animals under intramammary challenge with *S. aureus* are needed to confirm this assumption.

The importance of γδ T cells is because they represent the primary source of interleukin-17 (IL-17), as other non-γδ T cells provide just a small contribution for the production of IL-17 during *S. aureus* infections (Cho et al., 2010; Maher et al., 2013; Sutton et al., 2009; Korn and Petermann,2011; Murphy et al., 2014; Miller et al., 2020). In a fashion analogous way, our outcomes demonstrated that γδ T cells, including its subpopulation TCRVγ4$^+$ cells, have critical role in IL-17A production, while αβ T cells just marginally produce IL-17A in the studied draining lymph nodes.

Nonetheless, although γδ T cells have a pivotal contribution for IL-17A by splenocytes under unstimulated and *S. aureus* cell culture conditions, Tc17 also contributed to type 3 cell-mediated immunity. Furthermore, γδ T cells, particularly TCRVγ4$^+$ cells, robustly support IFN-γ production in the studies draining lymph nodes, while both γδ T cells and Tc1 (T CD8$^+$) cells markedly contribute to the IFN-γ in the cell culture splenocytes in both unstimulated and *S. aureus* stimulated conditions. The production of IFN-γ by γδ T cells, including TCRVγ4$^+$ cells, has been previously demonstrated (Dillen et al., 2018; Marchitto et al., 2019, Cooper et al., 2020). Overall, these findings support the substantial and broad role of this nonconventional T cell population in immunity against *S. aureus*.

Furthermore, we observed that the vaccination with the three recombinant proteins alone favor at a certain level type 1 immunity, as IFN-γ concentration was higher in the supernatant of splenocytes cell culture upon *S. aureus* stimulation, while the IL-4 decreased (type 2

immunity). With this in mind, type 2 immunity environment can promote *S. aureus* colonization and superinfection (Miller et al., 2020).

Besides this, we found that lower levels of IL-2 in the supernatant of splenocytes cell culture in animals that received the combination of *S. aureus* proteins together with the GM-CSF. Although it remains speculative, as IL-15 was not determined here, the T cells activated with IL-2 resemble T_{EM} cells, while T cells stimulated *in vitro with* IL-15 acquire a T_{CM} phenotype (Schluns et al., 2003), which corroborating with the formation of the T_{CM} phenotype found here. However, although the mechanism is still under debate, it should be noted immediately after pathogen clearance, the antigen-specific memory T-cell pool is comprised mainly of T_{EM} cells, which gradually convert to a T_{CM} cell phenotype over time (Woodland and Kohlmeier, 2009). To this end, T_{CM} cells are regarded a renewable source of T effector cells responsible for protection from acute infections and are primed for a rapid effect response (Ahlers and Belyakov, 2010).

3.5 CONCLUSION

In the present study, the vaccination with the three recombinant proteins associated with GM-CSF DNA led to an improve of T-cell memory response, especially γδ TCR^+ and its subpopulation $TCRV\gamma4^+$, which are the major responsible for production of IL-17A. Thus, as our vaccination approach foster type 3 immunity, a protective immune response against *S. aureus* was expected. Therefore, although our promising outcomes, further studies in *S. aureus* challenged mice model and in ruminants will be necessary to validate our findings regarding the protective immunity against *S. aureus*.

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CHAPTER 3

Staphylococcus aureus-associated cure candidate antigens elicit type 3 immune memory t cells

Will be submitted to: Vaccine

4 CHAPTER 3 - *STAPHYLOCOCCUS AUREUS*-ASSOCIATED CURE CANDIDATE ANTIGENS ELICIT TYPE 3 IMMUNE MEMORY T CELLS

ABSTRACT

Background

Stphylococcus aureus antimicrobial treatment of bovine intramammary infection is usually unsatisfactory. Thus, it would be valuable an alternative approach, instead of antibiotic treatment to ensure cure, reducing the transmission rates and the prevalence of this pathogen in the herd. Aiming vaccine candidates, we previously searched *S. aureus*-derived antigens through immunoproteomic approach using sera from mastitis-cured animals. In the present study, we propose the analysis of immune response induced by these recombinant proteins in association or not with the granulocyte macrophage colony stimulating factor (GM-CSF) DNA vaccine in a murine model.

Methods

We generated three recombinant *S. aureus*-cure associated proteins: elongation factor-G (EF-G), enolase (ENO) and phosphoglycerate kinase (PGK), and a GM-CSF DNA vaccine. To evaluate the immunogenicity of these proteins and immune response, twenty-three isogenic mice of the lineage C57BL/6 were divided in four groups were immunized with: G1: none (control); G2: GM-CSF DNA plasmid DNA vaccine; G3: the combination of EF-G+ENO+PGK; and G4: the combinations of EF-G+ENO+PGK proteins associated with GM-CSF DNA plasmid DNA vaccine. After 44 days spleen cells were collected for immunophenotyping and lymphocyte proliferation by flow cytometry under unstimulated and upon *S. aureus* stimulus conditions.

Results

In the present study, the immunization with the three recombinant *S. aureus*-cure associated proteins, independently, lead to a highest increase in the stimulation index of the percentage of IL-17A$^+$ cells among γδ T cells, γδ T central memory (TCM) cells, CD4$^+$ TCM cells and CD8$^+$ TCM cells, sustained the type 3 immunity in splenocytes upon *S. aureus* stimulus. In addition, we also observed the same behavior among CD44$^+$ and TCRVγ4$^+$ proliferative (Ki67$^+$) populations. The combination of three *S. aureus* recombinant proteins associated with GM-CSF DNA vaccine had a lower percentage T CD4$^+$. The levels of cytokines such as IFN-γ, IL-6,

TNF-α and IL-17A tend to be lower in vaccinated animals with the three recombinant proteins and the GM-CSF DNA vaccine upon stimulation with *S. aureus*.

Conclusions

Immunization using the proposed recombinant *S. aureus*-cure associated proteins elicited immune response related to likely protection. Thus, the present data open new paths to tackle the intramammary infections by *S. aureus* focusing the prevention, especially regarding the refractoriness of this pathogen to the current antimicrobial therapies.

Keywords: Vaccine. *Staphylococcus aureus*. T cell response. Mastitis. Bovine. Recombinant antigens.

4.1 INTRODUCTION

Bovine mastitis is defined as the inflammation of the mammary gland usually caused by bacteria. Mastitis critically impacts economy of dairy sector, public health, and subsequently the image of dairy sector and animal welfare. *Staphylococcus aureus* is one of the most frequently isolated mastitis causing bacteria worldwide (Moura et al., 2018; Santos et al., 2020) that causes both clinical and subclinical mastitis. Limited number of species causing mastitis are prevalent and constitute major problem to the dairy industry worldwide because of its pathogenicity, contagiousness, likely persistence of the intramammary infections (IMIs) and refractoriness to antimicrobial therapy (Rainard et al., 2020).

S. aureus is a multi-host bacterial pathogen and may infect distinct host-species population, constituting a major threat to public health and food security (Richardsson et al., 2018). In this context, *S. aureus* mastitis appoints to an urgent need for innovation and search for effective alternative approaches to its treatment and prevention. Presently, mastitis implies on antimicrobial usage on dairy farms that reach approximately 70%) (Normanno et al., 2007).

There is a consensus that *S. aureus* antimicrobial treatment is usually unsatisfactory (Barkema et al., 2006). Thus, it would be valuable if we provide a non-antibiotic approach that

increase the likelihood of cure of an existing *S. aureus*-caused IMI. We would emphasize that, considering the contagious behavior of this pathogen (Santos et al., 2020), any alternative measure that leads to quarter cure an IMI may result in lower exposure, decreasing the likelihood of a healthy quarter to become infected, and consequently reducing the transmission rates and the prevalence of this pathogen in the herd (Barkema et al., 2006). In a previous study, using sera from *S. aureus* IMI-cured animals, we searched *S. aureus*-derived proteins (Cunha et al., 2010) aiming selection of proteins likely related to cure. From antigenic proteins revealed by immunoproteomic approach, we obtained three recombinant staphylococcal proteins: elongation factor-G (EF-G), enolase (ENO) and phosphoglycerate kinase (PGK). Besides the immunogens, some studies have encouraged the use DNA vaccine containing granulocyte-macrophage colony-stimulating factor (GM-CSF) as a stimulation strategy for the development of an effective vaccine for bovine *S. aureus* mastitis as it promotes a robust humoral and cellular immunity (Montgomery et al., 2014; Bekeredjian-Ding, 2017; Zhang et al., 2018). Thus, in the present study we propose an initial approach the evaluation of the profile immune response induced by three recombinant proteins of *S. aureus*, EF-G, ENO and PGK in association of GM-CSF DNA vaccine in mouse.

4.2 MATERIAL AND METHODS

4.2.1 Ethical statement

This study was approved by the Animal Research Ethics Committee of the Universidade de São Paulo - Brazil under the protocol number 6276100519.

4.2.2 Production of *S. aureus* recombinant proteins

The sequence of amino acid corresponding to the EF-G (Genbank gi|395759321), PGK (Genbank gi|446997488) and ENO; (Genbank gi|447044500) antigens were derived from S. aureus and were codon-optimized for *Escherichia coli* expression. Genes were commercially synthesized by Genscript, USA. The produced synthetic genes were cloned into pUC57 vector and then were sub-cloned into pET28a expression vector (Carneiro et al., 2006). Recombinant plasmids were used to transform the expression strain *E. coli* BL21-Star™(DE3) as previously described (Faria et al., 2015) with modifications. Briefly, transformed *E. coli* BL21-Star™(DE3) were selected on kanamycin plates. An overnight bacterial culture of three colonies containing the respective expression plasmid were grown in Luria-Bertani medium (LB) in the presence of kanamycin (0.05 mg/mL) until an optical density (OD) of 0.4 at 600 nm, on a rotary shaker at 37 °C. Then, isopropyl-ß-D-1-thiogalactopyranosida (IPTG, Sigma) was added to the culture to a final concentration of 0.4 mM and the induced cultures were grown for 4 h. Cells were ruptured by ultrasound sonication on ice and debris were removed by centrifugation (20,000 x g, at 4°C, for 30 minutes). The recombinant proteins were purified using Ni Sepharose High Performance immobilized metal ion affinity chromatography columns (HisTrap Hp, cat. n. GE17-5248-02, GE Healthcare, Logan, USA), attached to an ÄKTA Pure (GE Healthcare, USA), under denaturing conditions according to the manufacturer's instructions.

4.2.3 GM-CSF based DNA vaccine

The amino acid sequence of the GM-CSF (Genbank, GI: 145301581) was forwarded to FastBio (Ribeirão Preto, Brazil) for optimization for eukaryotic cells and to synthesize the respective gene. The produced synthetic genes were received cloned in pUC57 vector and then were sub-cloned into the pCI-neo mammalian expression vector insert (Promega Incorporation, USA). *E. coli* DH5α was transformed with pCI-GM-CSF and immunization plasmids were purified

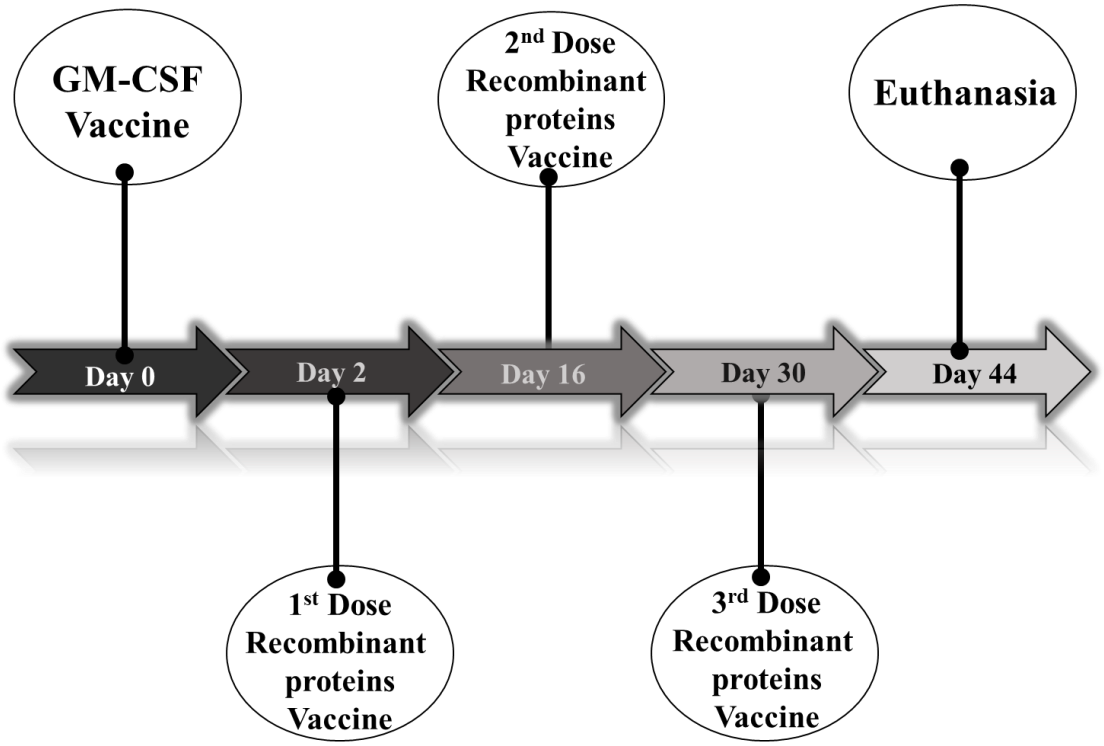
using the ZR plasmid Gigaprep Kit (Zymo Research, USA) according to the manufacturer's instructions.

4.2.4 Liposome preparation and entrapment of plasmid DNA

In the present study, we used a cationic liposome-based approach to efficiently delivery the GM-CSF DNA plasmid DNA, which has been associated to induce a strong humoral and cell-mediated responses (Fotoran et al., 2017) with minor modifications described by Santos et al. (2021).

4.2.5 *S. aureus* antigens candidates: immunological evaluation in mice

The test was conducted as previously demonstrated by Santos et al. (2021) with some modifications. Briefly twenty-three isogenic mice of the lineage C57BL/6J mice (six-week-old, female) were purchased from the Centro de Bioterismo, of the Faculdade de Medicina, Universidade de São Paulo. After three weeks of adaptation, the animals were divided in four groups and immunized as defined in Figure 1. Here, 50 µg of pCI-GM-CSF plasmid DNA resuspended in 100 µL of sterile liposomal formulation were administered subcutaneously under the skin of the interscapular area near of the draining lymph nodes, and the recombinant EF-G, PGK and ENO proteins (20 µg each protein) in 100 µL of saponin adjuvant (Quil-A®, cat. n. 8047-15-2, Invivogen, San Diego, California, USA) were administered intramuscularly in the deltoid muscles, as described in Figure 1.



B)

	GROUPS			
VACCINE	**G1 (n = 6)**	**G2 (n = 6)**	**G3 (n = 5)**	**G4 (n = 6)**
EF-G+PGK+ENO	-	-	+	+
GM-CSF	-	+	-	+

Figure 1. Scheme of the timeline of vaccination (A) and experimental groups (B). EF-G: elongation factor-G recombinant *S. aureus* protein; PGK: transmembrane protein phosphoglycerate kinase recombinant *S. aureus* protein; ENO: enolase recombinant *S. aureus* protein; GM-CSF: pCI-granulocyte and macrophage colony stimulating factor plasmid DNA. The unvaccinated group just received the liposome or saponin adjuvant.

At day 44 [14 days after the last administration of the recombinant protein(s)], the animals were submitted to deltoid intramuscular administration of an anesthetic solution containing xylazine (10 mg kg^{-1}) and ketamine (50 mg kg^{-1}) and were euthanized by cervical dislocation. Then the spleen was removed to obtain the cells for further immunophenotyping and lymphocyte proliferation assays.

4.2.6 Spleen and cell recovery

The spleens were aseptically removed, and mechanically disrupted and homogenized by syringe plunger into cell strainer (cat. n. Z742102, Sigma Aldrich, St. Louis, USA) on the top of 50 mL tube with 10 mL of proliferation medium containing RPMI 1640 medium (cat. n. R7638, Sigma Aldrich, St. Louis, USA) supplemented with 5% of heat-inactivated fetal bovine serum (Gibco, USA), and 100 U/mL penicillin, 100 µg/mL streptomycin, 0.25 µg/mL Fungizone (cat. n. 15240-096, Gibco).

The spleen cells were centrifuged (380 x *g*, 10 min, 4 °C). Thereafter, the lysis of erythrocytes was performed by adding 1,000 µL of 0.2% NaCl for 20 sec; then isotonicity was restored by adding 1,000 µL of 1.6% NaCl. Cells were further centrifugated and placed in cell proliferation medium. An aliquot of the cells was suspended in trypan blue solution (cat. n. T8154-100ML, Sigma Aldrich, St. Louis, USA) to determine the number of viable cells. For lymphocyte proliferation of spleen's cells, 2 x 10^5 viable cells per well were cultured at 37 °C and 5% CO_2 for 96 h in 96-wells flat-bottom plates in the presence or not (media without bacteria – unstimulated control) of 10 µL of heat-inactivated *S. aureus* (2 x 10^8 CFU mL^{-1}).

4.2.7 Preparation of *S. aureus* inoculum

An udder-adapted *S. aureus* isolate originated from a case of persistence subclinical IMI was used (Santos et al., 2020). *Staphylococcus aureus* (spa typing t605) inoculum was prepared as previously described (Souza et al., 2016) with minor modifications (Santos et al., 2021). The bacterial concentration was adjusted to the final inoculum dose (2 x 10^8 staphylococci mL^{-1}) to obtain a multiplicity of infection (MOI) = 10.

4.2.8 Immunophenotyping and lymphocyte proliferation of cultured spleen cells

The immunophenotyping and lymphocyte proliferation were performed as previously described by Santos et al. (2021). Briefly, after the incubation the cells were harvest from the 96-well plates and transferred to 5 mL tubes for flow cytometry and centrifuged at 250 x *g* at 4 ºC for 8 minutes. After that, the supernatant was collected and stored for cytokines measurement. The spleen cells were phenotyped using 0.5 µL of each of the fluorescent-conjugated monoclonal antibodies (mAbs; Table 1) by incubating the cells for 30 min at room temperature in the dark. Then, the cells were fixed and permeabilized for assessment of intracellular production of interleukin (IL)-17A and interferon (IFN)-γ, beyond the antigen-specific *in vitro* lymphocyte proliferation using a nuclear protein that act in the regulation of cell division process so-called ki67 (Soares et al., 2010). The samples were resuspended in 300 µL of PBS with 1% SFB and analyzed by flow cytometry (BD LSRFortessa^{TM} X-20 flow cytometer, Becton Dickinson Immunocytometry System^{TM}, San Diego, USA). For this assay, 100,000 events were examined in each sample. Flow Jo Tree Star software (FlowJo - Treestar 10.5.3 for Windows, Tree Star Inc., Ashland, OR, USA) was used to analyze the data. An unstained control and single-stained samples were also prepared as compensation controls. Negative control samples were also stained with conjugated isotype control antibodies. In addition, cells were stained with fluorescence minus-one (FMO) controls. Doublets were excluded using forward scatter (FSC) area versus FSC height.

Table 1. Monoclonal antibodies (mAbs) used for immunophenotyping of splenocytes and lymph nodes lymphocytes by flow cytometry

mAbs	Fluorescent probes	Target	Clone	Host	Concentration (mg mL^{-1})	Cat. n.
Anti-CD4[1]	APC-Cy7	Mouse	GK1.5	Rat	0.2	552051
Anti-CD8[1]	BV510	Mouse	53-6.7	Rat	0.2	563068
Anti-CD44[1]	BV421	Mouse	IM7	Rat	0.2	563970
Anti-CD27[1]	BV750	Mouse	LG.3A10	Hamster	0.2	747399
Anti-CD19[1]	PE-Cy7	Mouse	1D3	Rat	0.2	552854
Anti- γδ TCR[1]	BV650	Mouse	GL3	Hamster	0.2	563993
Anti-TCRVγ4[1]	FITC	Mouse	GL2	Hamster	0.2	552143
Anti-IL-17A[1]	Alexa 700	Mouse	TC11-18H10	Rat	0.2	560820
Anti-IFN-γ[1]	PE	Mouse	XMG1.2	Rat	0.2	554412

[1]BD Pharmingen™ (San Diego, EUA); IL-17A: interleukin-17A; IFN-γ: interferon-γ; FITC: Fluorescein isothiocyanate; APC-Cy7: Allophycocyanin-cyanine 7; PE: R-Phycoerythrin; PE-Cy7: R-Phycoerythrin-cyanine 7; BV510: Brilliant Violet 510; BV421: Brilliant Violet 421; BV650: Brilliant Violet 650; BV750: Brilliant Violet 750.

4.2.9 Cytokine measurement

The production of cytokines IL-2, IL-4, IL-6, IL-10, IFN-γ, IL-17A, and tumor necrosis factor (TNF)-α was determined from the supernatant of the cultured spleen cells under unstimulated control and upon *S. aureus* stimulation using the BD Cytometric Bead Array Mouse T_H1, T_H2 and T_H17 Cytokine (cat. n. 560485, BD Bioscience™, San Jose, USA) using a flow cytometer (BD LSRFortessa™ X-20 flow cytometer, Becton Dickinson Immunocytometry System™, San Diego, USA), as manufacturer's instructions. The FCAP Array™ v3.0 softaware (Softflow™, Pécs, Hungary) were used to analyze the data.

4.2.10 Statistical analysis

Statistical analysis was performed using GraphPad Prism 9 (GraphPad Software, Inc., San Diego, USA). To assess the percentage of each lymphocyte subpopulation, as well as the percentage of proliferative cells (ki67$^+$), a stimulation index (SI) was calculated by dividing the percentage of positive cells upon *S. aureus* stimulation per the percentage of positive cells under unstimulated control condition (Giunchetti et al., 2007; Abdeladhim et al, 2011; Duz et al., 2014; Mackroth et al., 2016; Mann et al., 2020). Firstly, the data were tested for normality of the distribution using the Kolmogorov-Smirnov and Shapiro-Wilk tests. If the distribution were normal, the data of the experimental groups were subjected to a one-way ANOVA analysis followed by Tukey test. Variables with non-parametric distributions were analyzed using the Kruskal-Wallis test followed by the Dunn's test. Results are reported as mean ± standard error of the mean. $P \leq 0.05$ was considered significant, unless otherwise indicated.

4.3 RESULTS

4.3.1 Type 3 immunity is triggered by both αβ and γδ T memory cells in immunized animals with the three recombinant proteins

There is a growing evidence that type 3 immunity mediated by cells that produce IL17-A and IL-17F is critical for mammary gland protective immunity (Rainard et al., 2020). No effect of vaccination on B cell response was revealed (data not shown). To fairly quantify the intensity of response upon *S. aureus* stimulation, T cell responses are normalized by dividing the percentage of positive cells upon *S. aureus* stimulation per the percentage of positive cells under unstimulated control condition, thereby creating a stimulation index. Thus, the highest increase in the stimulation index of the percentage of IL-17A$^+$ cells among γδ T cells ($P = 0.005$), γδ central memory T cells (T_{CM}) ($P = 0.005$), CD4$^+$ T_{CM} cells ($P = 0.04$) and CD8$^+$ T_{CM} cells (P =

0.04) was found in animals vaccinated with three recombinant proteins (Figure 2). No effect of vaccination on type 1 (IFN-γ^+) immune cells were observed. Thus, we speculate that the overall contribution to IL-17A production by both αβ and γδ T memory cells induced by these recombinant proteins may contribute to disease clearance/resistance.

Furthermore, we observed a higher SI in the percentage of IL-17A$^+$ cells among proliferative T memory (CD44$^+$; P = 0.02) cells and TCRVγ4$^+$ (P = 0.01) lymphocytes populations in animals that received the three recombinant proteins alone than those that vaccinated with the GM-CSF DNA vaccine (Figure 2).

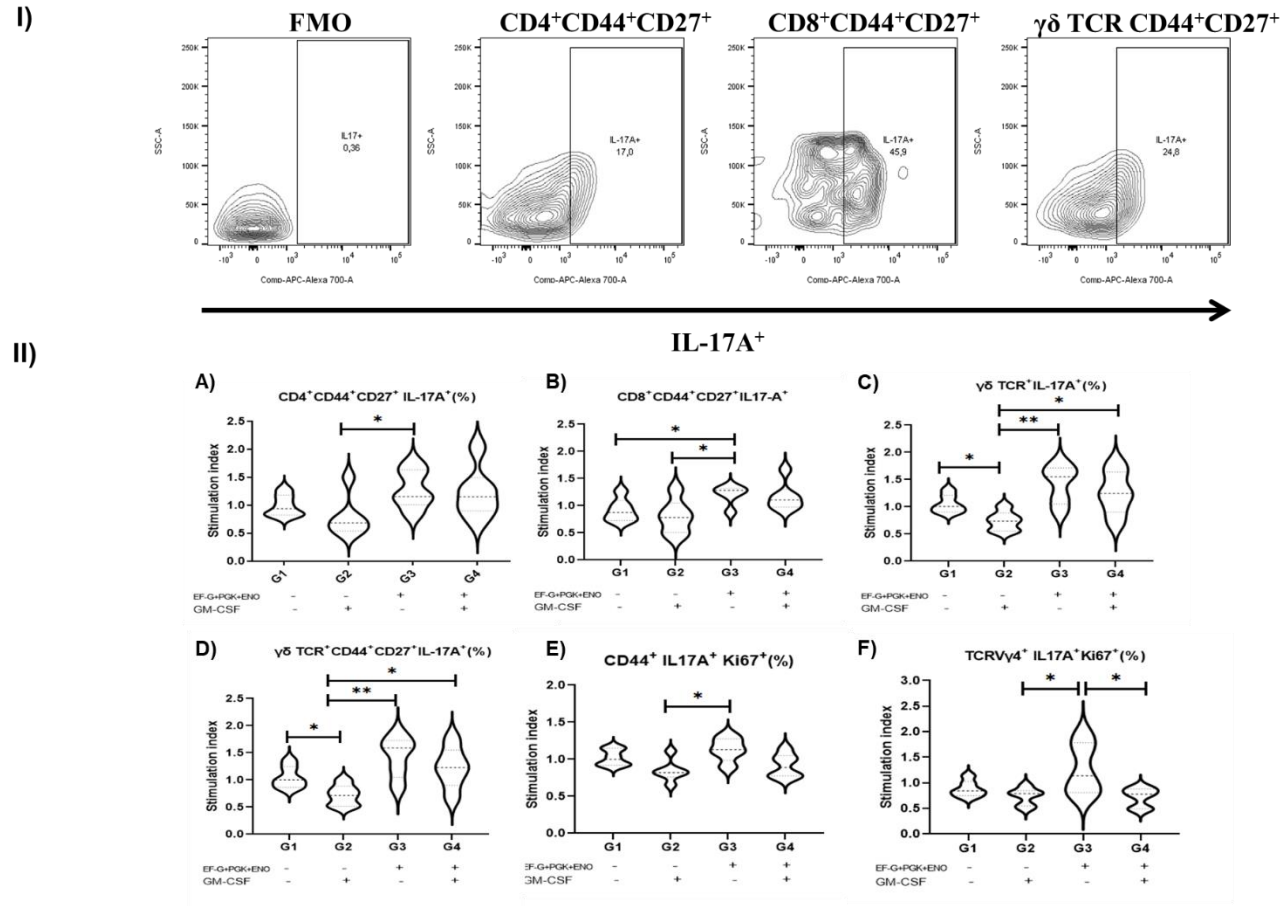


Figure 2. IL-17A$^+$ T cells in mice vaccinated with three-antigens *S. aureus* cure-associated recombinant proteins. T cell responses are normalized by dividing the percentage of IL-17A+ cells upon S. aureus stimulation per the percentage of IL-17A$^+$ cells under unstimulated control condition, thereby creating a stimulation index. Superior image shows a representative gating of IL-17A production by different lymphocyte populations. Here, we shown this stimulation index of: A) T CD4$^+$ CD44$^+$ CD27$^+$ (central memory) cells, B) T CD8+ CD44$^+$ CD27$^-$ (effector

memory) cells, C) overall γδ TCR$^+$ cells, D) γδ TCR$^+$ CD44$^+$ CD27$^+$ (central memory) cells, E) CD44$^+$ Ki67$^+$ cells and F) TCRVγ4$^+$ Ki67$^+$ cells. Granulocyte-macrophage colony-stimulating factor; EF-G: elongation factor-G, ENO: enolase; and PGK: phosphoglycerate kinase. * indicate $P \leq 0.05$, ** indicate $P \leq 0.01$.

The animals that received the combination of three *S. aureus* recombinant proteins and the GM-CSF DNA vaccine had a lower percentage T CD4$^+$ cells than the other vaccinated groups, although it did not significantly differ from the unvaccinated control animals ($P = 0.03$; Figure 3). The GM-CSF DNA vaccination favor an increase in the percentage of TCRVγ4$^+$ effector memory cells ($P = 0.06$), while dampen TCRVγ4$^+$ central memory cells ($P = 0.001$; Figure 3).

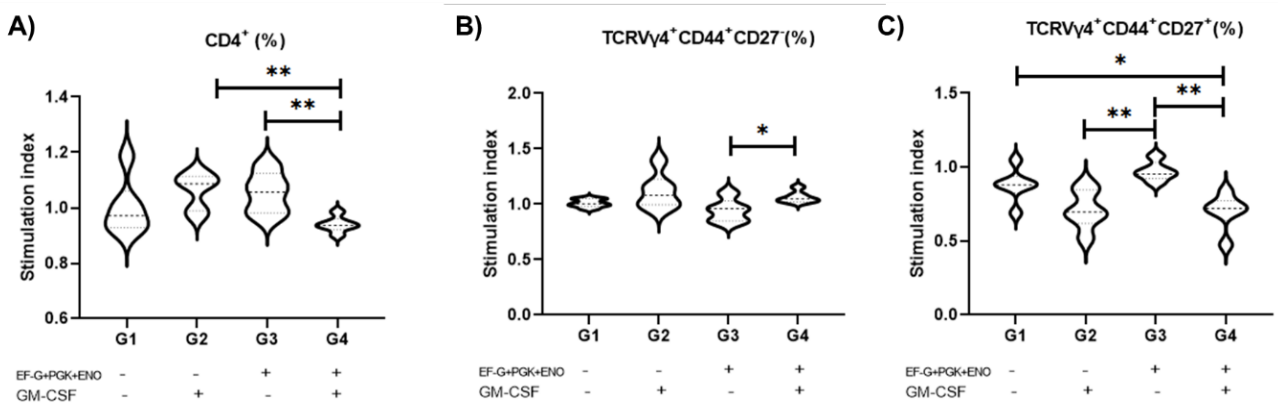


Figure 3. **Percentage of T CD4$^+$ central memory cells in vaccinated animals, and the antidromic trend of the percentage TCRVγ4$^+$ effector and central memory cells in mice vaccinated with GM-CSF DNA vaccine.** A) Overall T CD4$^+$, B) TCRVγ4$^+$ CD44$^+$ CD27$^-$ (effector memory cells), and C) TCRVγ4$^+$ CD44$^+$ CD27$^+$ (central memory). GM-CSF: Granulocyte-macrophage colony-stimulating factor; EF-G: elongation factor-G, ENO: enolase; and PGK: phosphoglycerate kinase. * indicate $P \leq 0.05$, ** indicate $P \leq 0.01$.

4.3.2 Animals vaccinated with three recombinant proteins was associated with the GM-CSF DNA vaccine was associated an anti-inflammatory cytokines production

No effect of vaccination on the levels of IL-2 and IL-4 cytokines was observed. The levels of IFN-γ ($P = 0.01$), IL-6 ($P = 0.06$), TNF-α ($P = 0.04$) and IL-17A ($P = 0.06$) tend to be lower in vaccinated animals with the three recombinant proteins and the GM-CSF DNA vaccine upon

stimulation with *S. aureus* than unstimulated control, while the IL-10 increased (P = 0.04). Apart from IL-10 and TNF-α, this latter phenomenon was observed in the other groups. The same behavior in the IL-10 (P = 0.03) and TNF-α (P = 0.06) production was observed in unvaccinated animals.

4.4 DISCUSSION

Here, we explored the profile of immune response induced by the combination of three recombinant *S. aureus* cure-associated proteins (i.e., ENO, PGK, EF-G) derived from a previous serum immunoproteomic study (Cunha et al., 2020). In this latter study, we assumed that antibodies recognized exclusively *S. aureus* proteins from chronically infected dairy cows that clinically and bacteriologically cured IMIs by *S. aureus* after vaccination with a commercial inactivated vaccine could translate with a therapeutic vaccine with a promising clinical relevance.

The first *S. aureus* protein, so-called ENO, is a conserved moonlighting protein that is expressed during times when critical nutrient (e.g., iron) is available in extremely narrow quantity (Mcneely et al., 2012), which is reflective of mammary gland environment (Le Maréchal et al., 2009). This protein is essential for bacteria growth and play an important role in several steps of the infection cycle that is likely assigned to its major role in the glycolysis, which is a critical pathway of bacterial energy metabolism (Bergmann et al., 2001; Yu et al., 2010).

Furthermore, ENO mediate the binding of *S. aureus* to laminin and collagen I that may act as a guidance mechanism, first allowing *S. aureus* adherence to extracellular matrix, initiating tissue colonization, followed by plasminogen activation and laminin degradation, which induce destruction of the extracellular matrix, favoring their invasion and dissemination (Carneiro et al., 2004; Adamczyk-Poplawska et al., 2011; Hemmadi and Biswas, 2020). Finally,

ENO is involved in cell wall biosynthesis and resistance to antimicrobials and had a critical role in biofilm formation (Yu et al., 2010; Foulston et al., 2014; Hemmadi and Biswas, 2020).

Similarly, PGK is also moonlighting protein that plays an important role in *S. aureus* energy metabolism, and its inhibition negatively impact bacterial population during biofilm formation. In addition, this staphylococcal protein may be involved in antimicrobial resistance, act as adhesion protein (Becker et al., 2001) and a potent neutrophil activator with the ability to trigger degranulation and dampen the classical and alternative complement pathway (Uhlmann, 2017).

Lastly, the EF-G belongs to GTPase superfamily that catalyze two different steps of protein synthesis, and is used as an energy source, protein synthesis and cell growth (Chen et al., 2010; Guo et al., 2012; Koripella et al., 2012; Nyfeler et al., 2012). Altogether, even though molecular functions for these proteins are not precisely understood, it is reasonable to hypothesize that stimulation the animals' immune response against these proteins may represent an exciting approach for vaccine development. Thus, although animals that have successfully eliminated the chronic IMIs by *S. aureus* produce specific antibodies against the abovementioned *S. aureus* proteins, which support adaptive humoral responses, it would be a good path to follow their effect on T cell-mediated immunity.

There is a consensus that defense against *S. aureus* heavily relies on the innate immune response by phagocytosis and killing of bacteria by phagocytes, especially neutrophils, that are orchestrated by the adaptive immune system. Extracellular *S. aureus* are engulfed and destroyed by phagocytes, which is deeply amplified by the binding of specific antibodies. For instance, T lymphocytes have some key functions in opsonophagocytosis, such as: 1) T cells are imperative to produce opsonizing antibodies, as T cells are essential for antibody class switch and affinity maturation; and 2) T cells boost phagocytosis by supporting freshly migrated macrophages and neutrophils to the site of infection. Additionally, *S. aureus* is not exclusively an extracellular

bacterium (Bharathan and Mullarky, 2011; Souza et al., 2016b; Bröker et al., 2016), and as a result intracellular elimination of *S. aureus* rely on T cells.

Thus, there is an emerging knowledge that memory T cells make a critical contribution toward *S. aureus* control (Bröker et al., 2016). Puzzling, the T cell-mediated immunity did not receive much attention so far. In the present study, both αβ and γδ T cells contributed to type 3 immunity, which has been regarded the major mechanism of mammary gland defense (Rainard et al., 2020). Type 3 immunity can be characterized by cells that encoded genes IL-17A, IL-17F and IL-22, and the transcription factors Rorγt and Rorα and their genes targets.

The cells responsible for type 3 immunity are diverse, including γδ T cells, T $CD4^+$ helper (Th17), T $CD8^+$ (Tc17) and innate lymphoid cells 3 (Rainard et al., 2020). A population of a highly IL-17A producing memory TCRV$\gamma4^+$ cells have recently been recognized as the most important population that confer protective immunity against subsequent infections by *S. aureus* (Murphy et al., 2014, Marchitto et al., 2019), however no disturbance of this subpopulation was detected in the present study. Nonetheless, in a similar study, using potential candidates of *S. aureus* for preventing new IMI by this pathogen identified in the same serum immunoproteomic study (Cunha et al., 2020), a robust increase in this subpopulation of γδ T cells was identified. Therefore, we assumed that both IL-17A highly producing αβ and γδ T cells are required to spontaneous recovery from IMI caused by *S. aureus*, rather than a specific γδ T cell subpopulation.

Furthermore, although GM-CSF is an important mediator of the immune response (Warren and Weiner, 2000), our outcomes did not show robust improvement in T-cell mediated immunity of the three *S. aureus* cure-associated antigens boosted by GM-CSF DNA vaccine. In this concern, animals that was vaccinated with the GM-CSF DNA vaccine had a lower SI in the percentage of IL-$17A^+$ cells among overall memory ($CD44^+$) proliferative lymphocytes and TCRV$\gamma4^+$ proliferative cells, which could have a great implication for vaccine efficacy.

Besides that, animals that were vaccinated with both the GM-CSF DNA vaccine and the three recombinant proteins are prone to have an anti-inflammatory cytokine profile in contact with *S. aureus*, as a decrease in the proinflammatory mediators (i.e., IFN-γ, IL-6, TNF-α and IL-17A) and an augment in IL-10 (anti-inflammatory cytokine) tend to occur when splenocytes were co-cultured with this pathogen. Altogether, we postulated that GM-CSF DNA vaccination has a complex versatility and could drive the immune response toward a pro- or anti-inflammatory cytokines profile. Although, we do not have a clear picture of this paradox, it has been postulated that it depends to numerous factors, including the immunogen(s), the dose and timing of GM-CSF administration (Chen et al., 2010).

In this regard, it has been proposed that great amounts of GM-CSF can expand myeloid cells in secondary lymphoid organs, which in turn can recruit T regulatory cells leading to an immunosuppressive effect (Chen et al., 2010). One factor that could raise this effect is the use of a cationic liposome-based approach to efficiently delivery the GM-CSF DNA plasmid DNA. In addition, *S. aureus* manipulate the production of IL-10 in its favor, as a mechanism of invasion (Leech et al., 2017), which in turn inhibits IL-17A secretion by T cells (Gu et al., 2008).

4.5 CONCLUSIONS

In the general context, the immunization with three *S. aureus* cure-associated recombinant proteins triggers the type 3 immune response in splenocytes when in contact with *S. aureus* by both αβ and γδ TCR^+ cells, which open new avenues to deal with intramammary infections by *S. aureus* using a possible non-antibiotic approach, especially regarding the refractoriness of this pathogen to the current antimicrobial therapies. The GM-CSF did not improve the immunogenicity of the abovesaid proteins, conversely it favors type 2 immune response.

Therefore, although its clinical relevance regarding our promising results, further studies in *S. aureus*-infected animals will be necessary to validate our findings.

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