Communication

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

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Simple Summary: *Staphylococcus aureus* is multi-host pathogen that threatens human and animal health. In this scenario, this bacterium has critical negative implications to the cow`s udder health and the dairy food chain. Therefore, a better understanding of how *S. aureus* modulates the expression of essential molecules in life is valuable. Thus, in the present study, we assessed how an udder-adapted *S. aureus* strain modulate the expression of two important adhesion molecules, so-called CD44 and CD62L in critical immune cells (e.g., WC1.1$^+$ γδ T cells). WC1.1$^+$ γδ T cells have a critical role in the protective immunity against *S. aureus*. We should also highlight that the CD44 and CD62L molecules are also used as a cell surface marker of memory cells. In this concern, we observed that *S. aureus* modulate the expression of (CD44, but it is not true for CD62L. Thus, *S. aureus* induce the preferably formation of central memory cells, which has high proliferative potential. Furthermore, our study indicated that memory cells in bovines are much shorter lived than their naïve counterparts (CD44$^-$ cells), and memory cells are probably mainly maintained by long-lived clones in bovines rather than individual cells with long lifespans.

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 *Staphylococcus aureus* cell culture stimulation. In this study, peripheral blood cells were isolated from ten dairy cows and cocultured with *S. aureus*. Afterward, the γδ T cell subpopulation and the expression of CD44, CD62L and proliferative (Ki67$^+$) cells were evaluated by flow cytometry. Our results showed that the percentages 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 did the CD44 geometric mean fluorescence intensity (GMFI). The rate of CD62L cells did not differ among groups for either PBMCs or WC1.1$^+$ γδ T cells. A higher GMFI of CD62L in proliferative PBMCs than nonproliferative PBMCs upon stimulation with *S. aureus* was detected, whereas no impact on the GMFI of CD62L was observed in WC1.1$^+$ cells. In summary, our study identified that *S. aureus* was associated with high expression of CD44 in overall PBMCs and WC1.1$^+$ γδ T cells, and they could generate memory WC1.1$^+$ γδ T cells, preferably central memory cells.

Keywords: mastitis, staphylococci, inflammation, memory cells, dairy cattle.

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1. Introduction

Bovine mastitis is the costliest disease affecting dairy farming and afflicts a high proportion of dairy cows. One of the most common bovine mastitis pathogens is *Staphylococcus aureus*, which is a notable issue for dairy farming due to its pathogenicity, contagiousness, the likelihood of persistence in the form of intramammary infections, and poor cure rates associated with current antimicrobial therapies; this pathogen has unquestionable importance in veterinary and human medicine [1–3]. This bacterium is also known to be part of the skin microbiota [4,5].

Extravasation and subsequent trafficking of lymphocytes to the site of inflammation represent a hallmark of immunity against infections [6,7]. Thus, there is a consensus that T cells are imperative for protection against *S. aureus* infections; otherwise, *S. aureus* can affect the T cell response [8]. Among T cells, it has been shown that a subpopulation of WC1.1$^+$ γδ T cells is the primary source of secreted IFN-γ [9] and IL-17 cytokines [10], which are essential for protection against subsequent infections by *S. aureus* [11–14]. However, almost nothing is known about this subpopulation of γδ T cells under *S. aureus* infection in dairy cattle.

CD44 is an adhesion molecule also known to participate in many cellular processes, including the regulation of growth, survival, proliferation, differentiation, and motility. CD44 is an adhesion molecule that is expressed by most cells and mediates binding to the extracellular matrix. This molecule is upregulated in naïve T cells after activation via the T cell receptor, and high levels are maintained on memory cells [15,16]. Thus, CD44 proteins have essential functions in life, and their absence or dysfunction causes pathogenic phenotypes [15].

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 of memory cells and is highly expressed in naïve T cells. These cells are responsible for vigilance in the bloodstream, and as soon as a pathogen enters the circulation, they migrate quickly to the lymph nodes [17]. The differentiation of naïve 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 on the cell surface [18]. Upon contact with the antigen, T cells shift to express the CD44 molecule on its surface, and this molecule is known to be expressed in effector memory cells; furthermore, the expression of CD62L is linked to central memory cells. However, almost nothing is known about bovine γδ T cell differentiation during *S. aureus* infection, especially the WC1.1$^+$ γδ T cell subpopulation.

Although the expression of CD62L and CD44 on bovine T cells has been studied in *Mycobacterium bovis* infection in cattle [7] and has been related to memory cell strategies. These markers have been extensively investigated in human and murine species, 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, in this pilot study, we explored the expression of CD62L and CD44 by bovine PBMCs and WC1.1$^+$ γδ T cells under *S. aureus* cell culture stimulation conditions.

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2. Materials and Methods

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 protocol n. 6380210218. Here, we used ten clinically healthy mid lactating Holstein dairy cattle (days in milk = 171.3 $\pm$ 13.96; milk production = 29.4 $\pm$ 1.71), from which 50 mL of peripheral blood was aseptically collected into a heparin tube for the isolation of peripheral blood mononuclear cells (PBMCs).

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% fetal bovine serum (FBS, 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 L-glutamine (cat. n. 21051- 024, GIBCO™, Invitrogen, Grand Island, USA) and 100 X antibiotic-antimycotic solution (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) and 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}.

2.3. Preparation of *S. aureus* inoculum

An udder-adapted *S. aureus* (spa typing t605) strain originating from a case of persistent subclinical mastitis [4] was used. The staphylococci inoculum was prepared as previously described by Souza et al. [18]. 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°C for a maximum of 7 d. The bacterial concentration was adjusted to the final inoculum dose (2 x 10^8 staphylococci mL^{-1}) to obtain the ratio of 10 bacteria per cell. After adjusting the bacterial concentration, the inoculum was heat-inactivated at 60°C for 1 h, after which 100 µL of the solution was plated on a plate containing blood agar and incubated at 37°C for 24 h to confirm inactivation.

2.4. Cell culture

The cells were washed twice in a cell culture medium, placed in plates with 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 cultured under unstimulated (basal, 10 µL of cell culture medium) and stimulated conditions with 10 µL of heat-inactivated *S. aureus* (10 bacteria per cell) 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} [19,20].

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

After the incubation period, the cells were harvested from the 96-well plates and transferred to 5-mL tubes, round bottom, 12 x 75 mm, suitable for flow cytometry, then centrifuged at 250 x g at 4°C for 8 min. After that step, the supernatant was discarded, and 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): 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 recognizing WC1.1$^+$ cells (cat n. BOV2119, WSU, Pullman, WA, USA) and γδ T cells. After the incubation period, the cells were washed with PBS at 250 x g at 4°C for 8 min. 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).

2.6. Lymphocyte proliferation

To determine whether proliferation affects CD62L and CD44 expression, Ki67-stained PBMCs were identified. Ki67 is a nuclear protein that plays a role in the regulation of cell division [21]. 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% FBS) and centrifuged at 250 x g at 4°C for 8 min. After centrifugation, the cells were fixed using 500 µL of the fixation solution (paraformaldehyde 4%, 0.09% azide and PBS) and incubated for 15 min 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°C for 8 min. Afterward, 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°C. After the incubation period, 500 µL of the permeabilization solution was added, and the samples were centrifuged at 250 x g at 4°C for 8 min. 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 the tubes and incubated again for 30 min at room temperature in the dark. After incubation, 500 µL of the permeabilization solution was added, and the samples were centrifuged at 250 x g at 4°C for 8 min. After centrifugation, the samples were resuspended in 300 µL of PBS with 1% FBS and analyzed by flow cytometry (FACSCantoII™ flow cytometer, Becton Dickinson Immunocytometry SystemTM, San Diego, USA). For this assay, 30,000 cells were examined in each sample. FlowJo Tree Star software (FlowJo - Treestar 10.5.3 for Windows, Tree Star Inc., Ashland, OR, USA) was used to analyze the data. Unstained control and single-stained samples were also prepared as compensation controls. Negative control samples were stained with conjugated isotype control antibodies. In addition, cells were stained with fluorescence minus one control. Doublets were excluded using forward scatter (FSC) area versus FSC height.

2.7. Statistical analysis

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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 subjected to repeated-measures ANOVA, and the means were compared by 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 comparisons of CD44 and CD62L expression between proliferative and nonproliferative cells and among groups, two-way ANOVA followed by the Bonferroni post hoc test was applied. The results are reported as the mean ± SEM. Significance was set at $P \leq 0.05$ unless otherwise indicated.

3. Results

3.1. Proliferate response in overall PBMCs and WC1.1⁺ γδ T cells

In the present study, we note that the percentage of proliferative (Ki67⁺) cells was higher when stimulated with *S. aureus* ($P = 0.03$) than in the unstimulated control. In addition, a trend toward more increased 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 stimulation with *S. aureus* ($P = 0.0004$) and Con-A ($P = 0.01$) than unstimulated control cells was found.

3.2. *S. aureus induced the CD44 expression in bovine PBMCs and WC1.1⁺ γδ T cells*

Here, the percentage of CD44⁺ cells increased in *S. aureus*-stimulated cultured PMBCs ($P = 0.02$) and WC1.1⁺ γδ T cells ($P = 0.002$) compared to nonstimulated control cultures (Figure 1). The CD44 fluorescence intensity (i.e., GMFI) was also enhanced in overall PBMCs when stimulated with *S. aureus* ($P = 0.0009$) (Supplemental Figure 1).

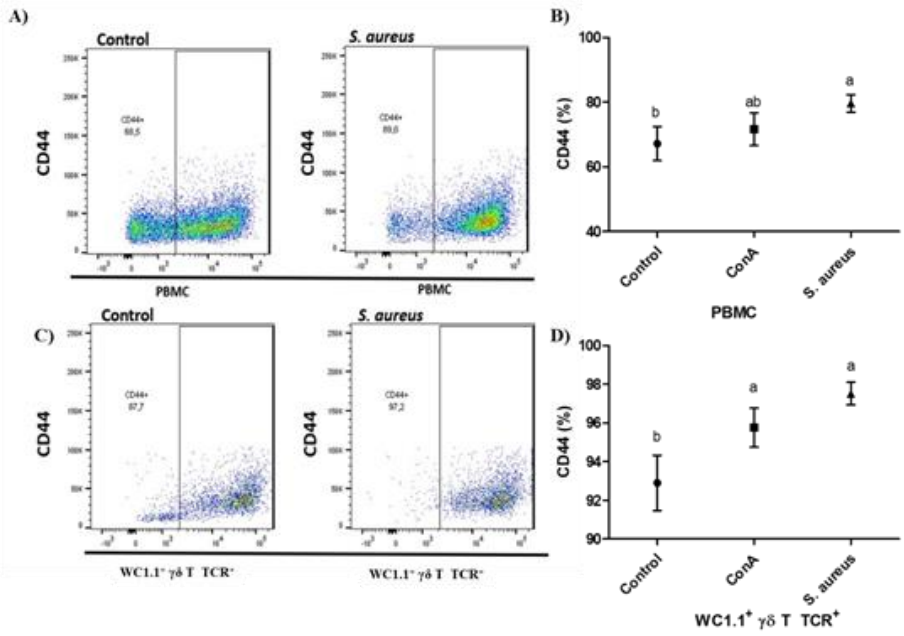

Figure 1. Representative dot plots from flow cytometry analysis demonstrating the CD44 population in peripheral blood mononuclear cells (PBMCs; A) and WC1.1⁺ γδ T cells (C) in unstimulated controls and upon stimulation with *S. aureus*. An increase in the percentage of CD44⁺ in PBMCs (B) and WC1.1⁺ γδ T cells (D) upon

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stimulation with *S. aureus* was found. Different letters indicate $P < 0.05$. Con-A: concanavalin-A type.

We also detected a higher percentage of CD44$^+$ cells among proliferative (Ki67$^+$) overall PBMCs than nonproliferative PBMCs (Ki67$^-$ cells) in all groups ($P = 0.02$) (Figure 2). Curiously, a significant enhancement in CD44 GMFI in proliferative of overall PBMCs (Ki67$^+$ cells) was detected only when stimulated with *S. aureus* compared to nonproliferative cells (Ki67$^-$ cells) ($P = 0.02$) (Figure 2). This finding indicated that CD44 does occur upon proliferation under *S. aureus* stimulation. On the other hand, no significant difference in the percentage of CD44$^+$ cells among proliferative (Ki67$^+$) and nonproliferative (Ki67$^-$ cells) WC1.1$^+$ γδ T cells was found. However, an increase in CD44 fluorescence intensity (i.e., GMFI) was detected in all groups ($P \leq 0.0001$) (Figure 2). Furthermore, *Staphylococcus aureus* could preferably trigger the formation of central memory T cells (TCMs), especially in WC1.1$^+$ γδ T cells (Figure 3).

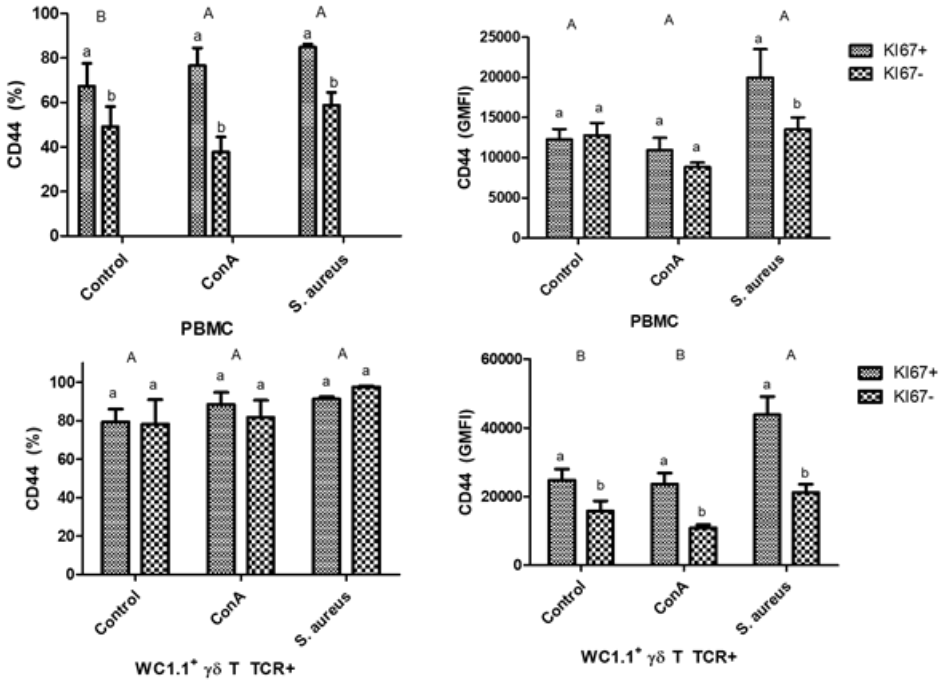

Figure 2. The percentage of CD44$^+$ PBMCs was higher in proliferative (Ki67$^+$) peripheral blood mononuclear cells (PBMCs; A) than in nonproliferative cells (Ki67$^-$), although no significant difference was observed in the percentage of CD44$^+$ WC1.1$^+$ γδ T cells between proliferative and nonproliferative cells (C). Here, the geometric mean fluorescence intensity (GMFI) was 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 indicate significant differences among unstimulated controls and upon stimulation with concanavalin-A (Con-A) and *S. aureus*. Lowercase letters indicate a significant difference in proliferative (Ki67$^+$) and nonproliferative (Ki67$^-$) cells. Different letters indicate $P < 0.05$.

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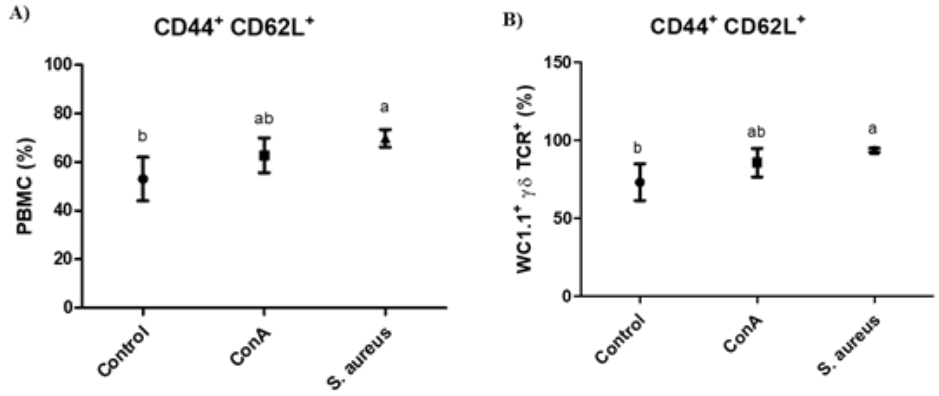

Figure 3 Increased percentage of CD44+ CD62L+ cells upon stimulation with *S. aureus* compared with the unstimulated control in both overall peripheral blood mononuclear cells (PBMCs; A) and WC1.1+ γδ T cells (B). Different letters indicate $P < 0.05$. Con-A: concanavalin-A type III.

3.3. *S. aureus did not elicit the expression of CD62L in PBMCs and WC1.1+ γδ T cells*

The percentage of CD62L+ cells did not differ among the groups in either overall PBMCs ($P = 0.13$) or WC1.1+ γδ T cells ($P = 0.60$; Supplemental Figure 2). However, we found an enhancement of CD62L GMFI by both PBMCs ($P = 0.01$) 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+ cells than nonproliferative cells ($P \leq 0.0001$) (Figure 4). Nevertheless, the PBMC and WC1.1+ γδ T cells exhibited distinct behaviors, as proliferative overall PBMC cells (Ki67+) had higher GMFI of CD62L levels ($P = 0.0002$) (molecules per cell) than nonproliferative cells; however, while this phenomenon was observed in unstimulated WC1.1+ γδ T cells, no significant difference in the GMFI of CD62L between proliferative and nonproliferative WC1.1+ γδ T cells stimulated by *S. aureus* and ConA was found (Figure 4).

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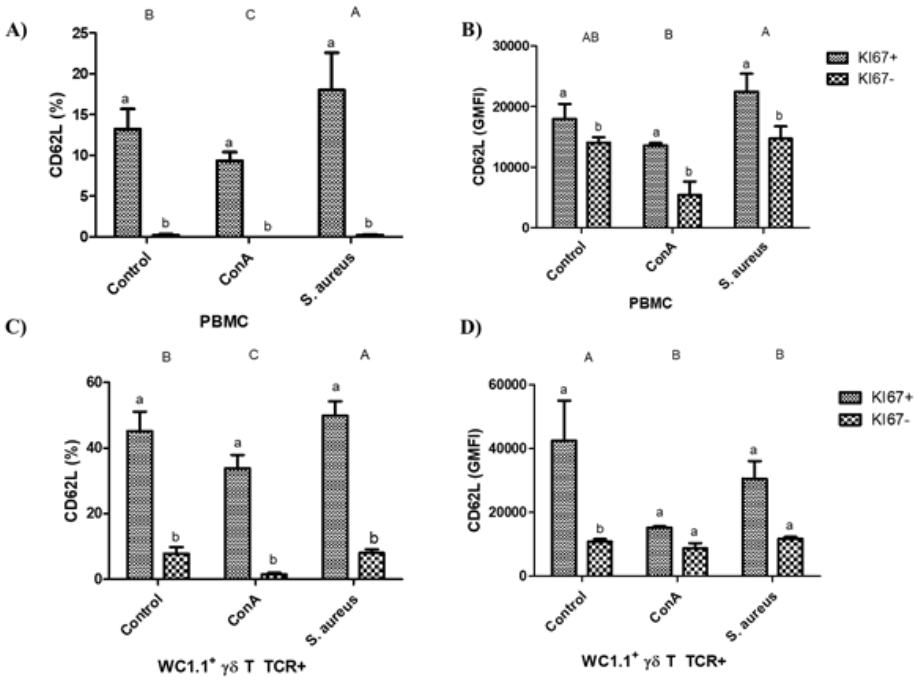

Figure 4. The percentage of CD62L$^+$ cells was almost abrogated in nonproliferative (Ki67$^-$) peripheral blood cells (PBMCs; A) and WC1.1$^+$ γδ T cells (C). The percentage of CD62L$^+$ cells was higher upon stimulation with *S. aureus* in both PBMCs (A) and WC1.1$^+$ γδ T cells (C). Furthermore, the CD62L geometric mean fluorescence intensity (GMFI) was lower in nonproliferative PBMCs in all groups (B), although this phenomenon was observed in nonstimulated control WC1.1$^+$ γδ T cells (D). 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 indicate significant differences among unstimulated controls and upon stimulation with concanavalin-A (Con-A) and *S. aureus*. Lowercase letters indicate a significant difference in proliferative (Ki67$^+$) and nonproliferative (Ki67$^-$) cells. Different letters indicate $P < 0.05$.

4. Discussion

CD44 is one of the universal activation markers of T cells. After antigen encounter, T cells quickly upregulate CD44, and its expression is also maintained in memory T cells. Beyond its use as an activation and memory marker, CD44 mediates numerous other functions [22]. A noteworthy observation of our findings is related to the fact that high CD44 expression is important for IL-17 production [22], which is crucial for protective immunity against *S. aureus* [11,12]. Furthermore, [9] showed that WC1.1$^+$ γδ T cells demonstrated a major contribution to IL-17 production that was even higher than that of CD4$^+$ T cells in response to the bovine respiratory syncytial virus. However, it is still unclear how specific populations of memory γδ T cells are generated, and in response to what antigens, cytokines, or other factors remain to be determined [23]. Furthermore, it is unclear whether γδ T cell expansion simply mirrors the dynamics of the total lymphocyte response [23]. In contrast to our findings, [6] reported that the percentage of cells in culture expressing CD44, regardless of the T cell subset, was not affected by *M. bovis* purified protein derivate (PPD) stimulation. Indeed, these authors showed that CD44 increased on CD4$^+$ T cells upon PPD stimulation in *M. bovis*-infected dairy cattle, but this effect was not observed in non-infected cattle. Furthermore, PPD stimulation did not affect the expression of CD44 in CD8$^+$ and γδ TCR$^+$ T cells.

In agreement with our results, previous reports in humans have shown that memory T cells have higher proliferation rates than naïve T cells [24]. Thus, our study indicated that memory cells in bovines are much shorter-lived than their naïve counterparts ($CD44^-$ cells) [25], and memory cells are mainly maintained by long-lived clones in bovines rather than individual cells with long lifespans, as has previously been demonstrated in mice and humans [24]. Similar to our findings, [6] reported that the expression of CD44 (i.e., GMFI) was higher in proliferative cells than in nonproliferative cells upon stimulation by PPD in all T cell subsets evaluated in *M. bovis*-infected cattle. In this regard, it should be noted that dairy cows are constantly exposed to *S. aureus*, as they are part of the skin microbiota [3,4].

CD62L is classically regarded as an adhesion molecule, as well as a lymph node homing receptor that allows cells to migrate to secondary lymphoid tissues to maintain the capacity to remain in or return to lymph nodes [26,27]. To investigate memory T cells, we used the CD44 surface marker, as it is considered the most reliable marker expressed at high levels in all memory T cells irrespective of their activation status. Memory T cells were also segregated into two distinct populations: a $CD44^{high}$ $CD62L^{low}$ population that exerts a rapid effector function (so-called effector memory), which has a poor proliferative capability, and a $CD44^{high}$ $CD62L^{high}$ population with no immediate effector function (central memory), which possesses high proliferative potential [28,29].

Central memory T cells do not produce any 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, after proliferation, they can efficiently differentiate into effector cells and produce large amounts of cytokines given their rapid effector function [31]. These cells circulate through the lymph nodes and mucosal lymphoid organs [32,33]. However, although the process needs to be better elucidated in bovines, $CD8^+$ T cells stimulated in vitro in the presence of IL-15 acquired a central memory phenotype, whereas $CD8^+$ T cells activated with IL-2 resemble effector memory T cells, which, together with our other results, indicated that *S. aureus* might induce the production of IL-15 rather than IL-2, although little is known about CD4 and γδ T cells [31].

5. Conclusions

In summary, our study identified that our udder-strain *S. aureus* was associated with high expression of CD44 in overall PBMCs and $WC1.1^+$ γδ T cells, and these cells could generate memory $WC1.1^+$ γδ T cells, preferably central memory cells.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, **Supplemental Figure 1.** Representative histogram from flow cytometry analysis of CD44 expression in peripheral blood mononuclear cells (PBMCs; A and C) and WC1.1+ γδ T cells (A and C) in unstimulated controls (light gray) and upon stimulation with concanavalin-A (Con-A; dark gray) and S. aureus (black). An increase 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 indicate $P < 0.05$. 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 controls and upon stimulation with S. aureus. An increase in the percentage of CD62L+ cells amongWC1.1+ γδ T cells (D) upon stimulation with S. aureus was observed, although no significant difference was observed in PBMCs (C). Different letters indicate $P < 0.05$. Con-A: concanavalin-A type III. **Supplemental Figure 3**. Representative histogram of flow cytometry analysis of CD62L expression in peripheral blood mononuclear cells (PBMCs; A and C) and WC1.1+ γδ T cells (A and C) in unstimulated controls (light gray) and upon stimulation with concanavalin-A (Con-A; dark gray) and S. aureus (black). An increase in CD62L geometric mean fluorescence intensity (GMFI) values upon stimulation with S. aureus in overall PBMCs and proliferative (Ki67+) (B and D) cells was found, although WC1.1+ γδ T cells (B and D) upon stimulation with S. aureus did not express higher levels of CD62L than unstimulated controls. Different letters indicated $P < 0.05$.

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Institutional Review Board Statement: "The study was conducted according to the guidelines of the ethics committee on animal use of the School of Veterinary Medicine and Animal Sciences of the University of São Paulo, under protocol n. 6380210218.

Data Availability Statement: Not applicable.

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Conflicts of Interest: The authors declare no conflict of interest.

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