

Identification of a T-cell epitope in the *Staphylococcus aureus* Panton-Valentine LukS-PV component*

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ABSTRACT

We previously observed the elicitation of a significant delayed-type hypersensitivity response to the Panton-Valentine leukocidin (PVL) LukS-PV subunit following subcutaneous immunization (Brown *et al.*, *Clinical Microbiology and Infection*, 2009, 156: 156-164). A LukS-PV-specific cell line (L_ST) was screened for proliferative responses against a panel of 25 amino acid-long peptides spanning the length of LukS-PV (amino acids 29 - 312). This analysis demonstrated that stimulation of L_ST with LukS-PV resulted in significant proliferative responses and adoptive transfer of L_ST into naïve mice conferred a LukS-PV-specific DTH response following challenge. Challenge of mice adoptively transferred with L_ST with peptides 7 (149 - 173), 8 (169 - 193) and 14 (289 - 312) also elicited a measurable DTH response suggesting that these peptides contained T cell epitopes.

Keywords: Staphylococcus; Cell Mediated Immunity; Panton-Valentine Leukocidin; T Cells

1. INTRODUCTION

Infections caused by *Staphylococcus aureus* have dramatically increased during the last decade, largely due to the emergence of strains that are resistant to numerous antibiotics and a significant change to the epidemiology

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of infections caused by this pathogen; namely the emergence of community-acquired (CA) infections [1]. Presentation of disease resulting from either CA or hospital acquired (HA) infections can range from minor skin infections to cellulitis, myositis, or deep bone and tissue infections [1,2].

2. RESULTS, MATERIALS AND DISCUSSION

Previously, we demonstrated that intranasal or subcutaneous immunization of Balb/c mice with LukS-PV (the S component of the Panton Valentine leukocidin; PVL) conferred immunity against lung or skin infection, respectively, with *S. aureus* USA300 [3]. Immunization with LukF-PV did not confer protection [3]. Surprisingly, both immunization routes elicited cellular immune responses to LukS-PV measured by the significant delayed-type hypersensitivity (DTH) responses elicited in response to LukS-PV following challenge [3]. The anti-LukS-PV DTH response was further examined here following infections with either *S. aureus* USA300 (LAC) or the PVL deletion mutant LAC Δ *pvl* (strains generously provided by Dr. Frank DeLeo, National Institute of Allergy and Infectious Diseases) [4] that were maintained and cultured as described [3] (**Figure 1(a)**). Infections with either strain elicited a significant response to inactive α -toxin_(H35L) which is expressed by both isolates, but elicitation of DTH was only observed in the mice infected with the LAC strain (**Figure 1(a)**).

To further define the role of cellular anti-LukS-PV-specific responses, a LukS-PV-specific T cell line (L_ST) was established and tested *in vitro* and *in vivo* for reactivity against a panel of peptides (**Table 1**) spanning the length of LukS-PV. Peptides (n = 14) 25 amino acids (aa) long with a 5 aa overlap spanning the entire length of the

secreted protein were synthesized by Peptide 2.0 (Chantilly, VA) and ranged in purity from 90.31% - 98.1%. The signal peptide (SP) sequence was synthesized by Anaspec (San Jose, CA) (Table 1).

The L_ST line was derived from immunized Balb/c

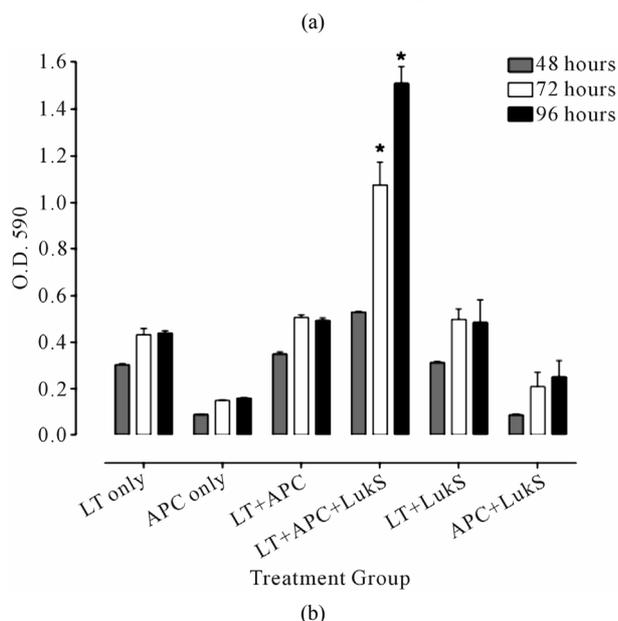
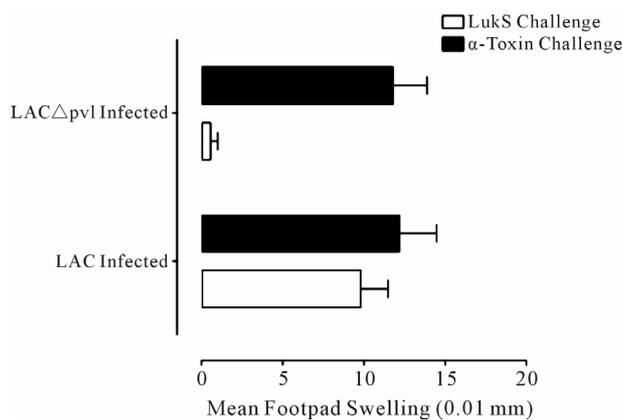


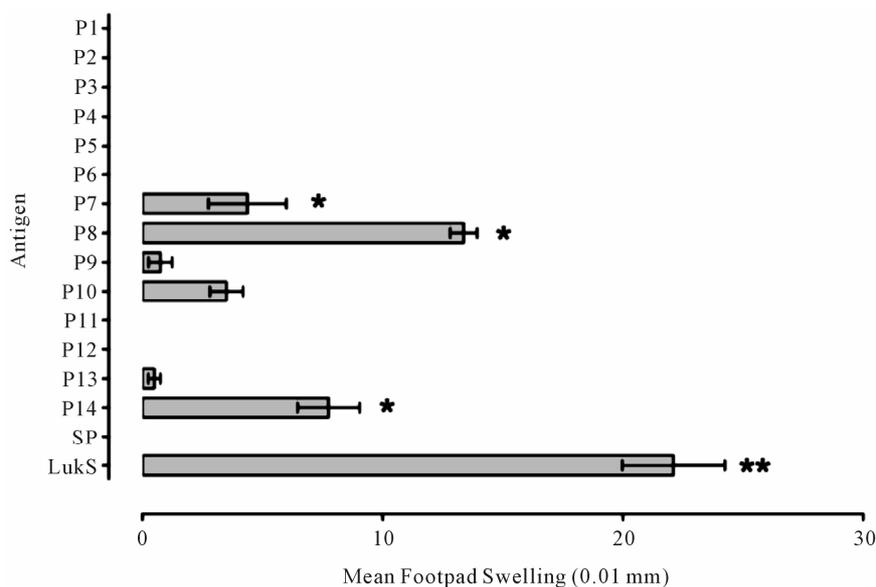
Figure 1. Proliferative response to LukS-PV. (a) DTH response to LukS-PV and α -toxin following infection. Mice were infected with 5×10^7 *S. aureus* USA300 (LAC) or the isogenic LACΔpvI. Two-weeks post infection mice were challenged in their right and left footpads with 2.5 μ g LukS-PV and α -toxin_(H35L), respectively. Footpads were measured at 0 and 24 h post challenge with a spring-loaded micrometer. The data are expressed as the mean \pm SEM of 10 mice per group. * $P < 0.005$ vs. the LukS-PV response measured in LACΔpvI mice using the unpaired *t*-test; (b) Characterization of the L_ST cell line. The L_ST cell line was obtained by purifying T cells from the popliteal lymph nodes of LukS-PV-immunized mice and maintained in culture in complete media containing IL-2. Proliferation of L_ST cells (1×10^4) in 96-well plates following stimulation with LukS-PV (1 μ g) and APCs (1×10^5) for 48, 72 and 96. The data are expressed as the mean O.D. 590 \pm SEM of triplicate wells for each treatment group. * $P < 0.03$ vs. all groups at the respective time points using the unpaired *t* test.

Table 1. LukS-PV peptides^a.

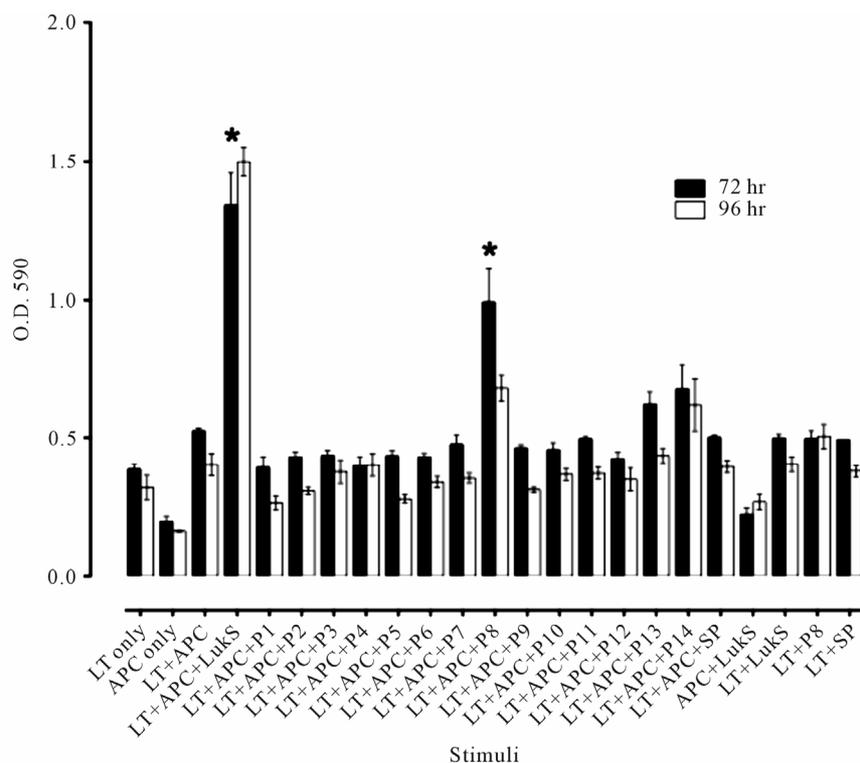
Peptide Number	Peptide Sequence
SP ^b	M ₁ VKKRLLAATLSLGIITPIATSFHESKA ₂₈
1	D ₂₉ NNIENIGDGAEVVVKRTEDTSSDKW ₅₃
2	<u>S</u> ₄₉ <u>SDKW</u> GVTQNIQDFVVKDKKYNKDA ₇₃
3	<u>Y</u> ₆₉ <u>NKDALILKMQGFINSKTTYYNYKN</u> ₉₃
4	<u>Y</u> ₈₉ <u>NYKNTDHIKAMRWPQYNI</u> GLKTN ₁₁₃
5	<u>G</u> ₁₀₉ <u>LKTNDPNVDL</u> INYLPKNKIDSVNV ₁₃₃
6	<u>D</u> ₁₂₉ <u>SVNVSQTLGYNIGGFNSG</u> PSTGG ₁₅₃
7	<u>P</u> ₁₄₉ <u>STGGNGSFNYSK</u> TISYNQQNYISE ₁₇₃
8	<u>N</u> ₁₆₉ <u>YISEVERQNSKSVQW</u> GIKANSFIT ₁₉₃
9	<u>N</u> ₁₈₉ <u>SFITS</u> LGKMSGHDPNLFVGYKPY ₂₁₃
10	<u>Y</u> ₂₀₉ <u>KPYSQNP</u> RDYFVPDNLPLVHSG ₂₃₃
11	<u>L</u> ₂₂₉ <u>VHSGFN</u> PSFIATVSHEKSGSDTSE ₂₅₃
12	<u>G</u> ₂₄₉ <u>DTSEFEITYGRNMDV</u> THATRRRTH ₂₇₃
13	<u>R</u> ₂₆₉ <u>RTTHYGNSYLEG</u> SRIHNAFVNRNY ₂₉₃
14	<u>V</u> ₂₈₉ <u>NRNYTVKYE</u> VNWKTHEIKVKGHN ₃₁₂

^aLukS-PV peptides (25 amino acids each) contain a 5 amino acid overlap with the preceding peptide (underlined). Position of respective amino acids indicated by the subscript number. ^bSignal peptide.

mice (Harlan, Indianapolis, IN) maintained *in vitro* as previously described [5]. All animal experiments were approved by the Institutional Animal Care Use Committee at the Texas A&M Health Science Center Institute of Biosciences and Technology. Briefly, L_ST were maintained in 24-well plates that contained 1×10^6 T cells and 5×10^6 mitomycin-treated APCs + 2 μ g LukS-PV in a total volume of 1.5 ml complete medium [5]. T cells were cultured for 5 days in 7% CO₂ - 93% air incubator at 37°C. Cells were then pooled, washed, resuspended in complete medium containing 3 U/ml murine recombinant IL-2 (rIL-2 Boehringer-Mannheim) and distributed into 24-well plates at a concentration of 5×10^5 cells/well in a total volume of 1.5 ml complete medium. The cultures were fed every 3 - 4 days with complete medium containing 5 U/ml rIL-2 and kept at a cell density of 5×10^5 - 1×10^6 cells/well. T cells were maintained in rest culture for 14 - 21 days, after which they were collected, washed and replated in 24-well cell culture plates with fresh mitomycin-treated APCs + 1 μ g LukS-PV in a total volume of 1.5 ml complete medium/well. Three days after *in vitro* stimulation, 3 U of rIL-2 were added to each well. The cultures were then incubated for 3 more days, after which they were put into a rest cycle for another 14 - 21 days in medium containing 5 U/ml of rIL-2. At the end of each rest cycle, the T cells were tested for specificity by testing the proliferation response as follows:



(a)



(b)

Figure 2. Reactivity of the $L_S T$ cell line to LukS-PV and LukS-PV peptides. (a) Mice ($n = 5$ per group) adoptively transferred with $L_S T$ cells (mice received 1×10^7 $L_S T$ or a whole spleen equivalent [control]) 7 days post challenge with recombinant LukS-PV (0.5 μ g) or peptides (2.5 μ g each peptide in 50 μ l PBS). Adoptively transferred $L_S T$ mice and naive mice challenged with LukS-PV served as positive and negative controls, respectively. The data are expressed as the mean \pm SEM. * $P < 0.05$, ** $P < 0.0008$ compared to the challenge only group. (b) Proliferation of $L_S T$ cells in response to LukS-PV peptides. $L_S T$ cells were stimulated with either LukS-PV (1 μ g) or LukS-PV peptides (2 μ g/well). Data are expressed as the mean O.D. 590 nm reading \pm SEM of triplicate wells. * $P < 0.007$ vs. APC only, $L_S T$ only, and $L_S T + APC$ groups. This experiment was repeated twice with similar results.

1×10^4 T cells were cultured in 96-well round-bottom plates along with 1×10^5 mitomycin-treated APCs in complete medium in a volume of 100 μ l/well in the presence of 0.5 μ g/well of LukS-PV. MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 125 μ g/well) was added to the wells and the plate incubated for another 2.5 h at 37°C. Extraction buffer (50% dimethyl formamide, 20% sodium dodecyl sulfate in water) was added to dissolve the insoluble purple formazan product and the absorbance quantified at 590 nm in a spectrophotometer [5]. Using this procedure, the L_ST cell line was generated (**Figure 1(b)**). Significant proliferation was observed at 72 and 96 h ($P < 0.003$, Student's *t* test) in response to LukS-PV compared to proliferation observed in the respective control groups (**Figure 1(b)**). Confirmation that the L_ST cell line was CD4⁺ was carried out by incubating L_ST cells with fluorescently-labeled antibodies specific to either CD3, CD4 or CD8. Two-color flow cytometric analysis demonstrated that the L_ST cell line was >98% CD3⁺CD4⁺ (data not shown).

LukS-PV peptides were used to challenge mice adoptively transferred with L_ST cells (**Figure 2(a)**). Mice challenged with peptides 7 (149 - 173), 8 (169 - 193) or 14 (289 - 312) elicited significant responses compared to footpad swelling measurements observed following challenge with the remaining peptides, however, responses observed following challenge with either peptide 7 (149 - 173), 8 (169 - 193) or 14 (289 - 312) were significantly below the response elicited following challenge with LukS-PV (**Figure 2(a)**). Interestingly, *in vitro* stimulation of the L_ST cell line with the peptide panel did not reveal as well defined a proliferative response compared to the DTH data described above. Only peptide 8 (169 - 193) elicited significantly different proliferation of L_ST cells *in vitro* (**Figure 2(b)**). Peptides 7 (149 - 173), 10 (209 - 233) and 14 (289 - 312) elicited slightly elevated proliferative responses over background proliferation *in vivo*, but these peptides did not significantly stimulate L_ST proliferation *in vitro* (**Figure 2(b)**). These data suggest that an epitope contained in the peptide 8 sequence (N₁₆₉YISEVERQNSKSVQWGIKANSFIT₁₉₃) induced the highest levels of L_ST proliferation *in vitro* and *in vivo* (in mice adoptively transferred with L_ST cells). Further characterization of the epitope present within peptide 8 may further improve the anti-LukS-PV response if used as part of a vaccine.

3. CONCLUDING REMARKS

Due to the lack of success in human trials utilizing immunogens targeted for their potential to elicit humoral responses, future *S. aureus* vaccine candidate searches should consider targets with the potential of eliciting protective cellular and humoral responses; a two-pronged

immune attack against this pathogen would most likely benefit containment and clearance of cutaneous disease [6]. To what end cellular immune responses may prevent infections or accelerate clearance of this pathogen following infections associated with blood or lung tissues remains to be examined, however, cellular responses, particularly those associated with elevated production of INF γ are beneficial in protection against various types of *S. aureus* infections [7-9]. It is likely, that vaccine-mediated induction of cellular immunity may bolster antibody responses generated following exposure (whether from infection, carriage, or contact) to *S. aureus*.

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