

# CD4 T Cell Antigens from *Staphylococcus aureus* Newman Strain Identified following Immunization with Heat-Killed Bacteria

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*Staphylococcus aureus* is a commensal bacterium associated with the skin and mucosal surfaces of humans and animals that can also cause chronic infection. The emergence of antibiotic-resistant strains such as methicillin-resistant *S. aureus* (MRSA) and strains causing chronic intramammary infections (IMI) in cows results in severe human and livestock infections. Conventional approaches to vaccine development have yielded only a few noneffective vaccines against MRSA or IMI strains, so there is a need for improved vaccine development. CD4 T lymphocytes are required for promoting gamma interferon (IFN- $\gamma$ ) mediated immunoglobulin isotype switching in B lymphocytes to produce high-affinity IgG antibodies and IFN- $\gamma$ -mediated phagocyte activation for an effective resolution of bacterial infection. However, the lack of known CD4 T cell antigens from *S. aureus* has made it difficult to design effective vaccines. The goal of this study was to identify *S. aureus* proteins recognized by immune CD4 T cells. Using a reverse genetics approach, 43 antigens were selected from the *S. aureus* Newman strain. These included lipoproteins, proteases, transcription regulators, an alkaline shock protein, conserved-domain proteins, hemolysins, fibrinogen-binding protein, staphylokinase, exotoxin, enterotoxin, sortase, and protein A. Screening of expressed proteins for recall T cell responses in outbred, immune calves identified 13 proteins that share over 80% sequence identity among MRSA or IMI strains. These may be useful for inclusion in a broadly protective multiantigen vaccine against MRSA or IMI.

*Staphylococcus aureus* is a Gram-positive, opportunistic pathogen associated with asymptomatic colonization of the skin and mucosal surfaces of healthy animals and humans. In cattle, *S. aureus* is the leading cause of mastitis, a disease affecting the mammary gland. Mastitis results in significant losses to U.S. (>\$1.8 billion annually) and global dairy industries (76). *S. aureus*-induced mastitis is often subclinical and results in chronic bovine intramammary infections (IMI), even in animals placed under antibiotic regime (2, 25). The emergence of beta-lactam and methicillin-resistant *S. aureus* (MRSA) strains resistant to vancomycin and of recalcitrant staphylococcal IMI in cattle has necessitated the development of alternative therapeutic strategies (2, 25, 26, 31–33, 37, 51, 64). Furthermore, *S. aureus* can be transmitted from farm and companion animals to humans (15, 24, 42, 46, 80, 81). Currently, there are no commercially available vaccines available for MRSA or IMI.

Effective resolution of infection by bacterial pathogens is mediated by a combination of neutralizing antibody and inflammatory responses of activated macrophages and neutrophils. Therefore, to prevent *S. aureus* infection, a vaccine should be comprised of a broad repertoire of antigens that elicit IgG, driven by a CD4 Th1 microenvironment. However, decades of conventional vaccination attempts using various *S. aureus* antigens have yielded only a few defined proteins as vaccine candidates, of which many have proven to be ineffective (35, 69, 72). One consideration for vaccine failure is the interaction of *S. aureus* protein A with the Fc $\gamma$  region of immunoglobulin molecule, which interferes with antibody function of opsonization and downstream phagocytosis and induction of apoptosis of marginal-zone and B-1 B cells (14). A second reason for vaccine failure has been the lack of consideration of antigens that elicit CD4 T cell responses.

To address this second problem, we identified 43 surface proteins and virulence factors from the *S. aureus* Newman strain by using a reverse genetics approach with a goal of identifying novel

CD4 T cell antigens. A comparative proteomic and genomic analysis was performed using the *S. aureus* Newman strain and different bovine IMI and MRSA isolates to identify predicted surface-exposed proteins that are known virulence factors and virulence-associated factors that could be targeted by antibody and that are conserved across strains (11, 28). Holstein calves expressing different major histocompatibility complex class II (MHC-II) molecules were immunized with heat-killed *S. aureus* until an *S. aureus*-specific immune response was obtained. The 43 antigens were individually expressed by *in vitro* transcription and translation (IVTT), affinity-purified, and tested for immunogenicity using lymphocyte proliferation assays. Using this rapid screening method, we identified 13 novel antigens from the *S. aureus* Newman strain which have potential for use in a vaccine against *S. aureus* infection of cattle and, perhaps, humans. Until now, the dearth of knowledge of CD4 T cell antigens from *S. aureus* has hindered the rational design of multivalent vaccines for this pathogen, and the results of this investigation begin to bridge that critical knowledge gap.

## MATERIALS AND METHODS

***S. aureus.*** *S. aureus* strain Newman was used to prepare formalin- or heat-inactivated bacterial suspensions for immunization and *in vitro* assays. The *S. aureus* Newman strain genome does not have genes encoding

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11 out of 12 known superantigens (*tst-1*, *seb*, *seg*, *sek*, *sei*, *sek*, *sem*, *sen*, *seo*, *sep*, and *seq*), and only the *sea* gene is present (48). Bacteria were cultured overnight from frozen stocks in Trypticase soy broth (TSB) at 37°C with shaking (250 rpm), diluted 1:200 in fresh TSB the following morning, and then cultured to the early stationary phase of growth (optical density at 600 nm [OD<sub>600</sub>] of 2.0). To prepare inactivated suspensions, bacteria were harvested by centrifugation (8,000 × g for 10 min) and washed three times with phosphate-buffered saline (PBS), pH 7.2, to remove secreted proteins, including SEA, and resuspended in PBS. Serial 10-fold dilutions in PBS were then plated on Trypticase soy agar plates to determine the number of CFU. Bacterial suspensions were heat inactivated at 60°C for 1 h. For formalin inactivation, bacteria were resuspended in 3% formaldehyde in PBS and incubated overnight at room temperature with shaking at 60 rpm. Bacteria were then centrifuged, washed three times, and resuspended in the same volume of PBS and stored at 4°C until use. For both inactivation methods, 0.1 ml of each suspension was plated on blood agar and incubated at 37°C for up to 1 week to check for lack of growth.

**Cattle.** Five 3-month-old Holstein steers (animals 30864, 30900, 30904, 30916, and 30919) with different MHC class II haplotypes were purchased from a local dairy, quarantined for 2 weeks, and immunized six times over a period of 18 weeks until a strong *S. aureus*-specific immune response was obtained. Immune serum collected before immunization and 2 weeks after each immunization was stored at -20°C. The animals were immunized initially by subcutaneous inoculation of 1.0 ml of 3.3 × 10<sup>9</sup> formalin-killed *S. aureus* bacteria emulsified in complete Freund's adjuvant (Sigma-Aldrich) and with the same number of bacteria in incomplete Freund's adjuvant 3 and 6 weeks later. Antibody responses were not detected following this regime, so animals were then immunized subcutaneously three times 3 weeks apart with 10<sup>10</sup> heat-killed *S. aureus* bacteria emulsified in 2 ml of 5 mg/ml Quil-A-saponin (Sigma-Aldrich) in PBS, which was divided into two 1.0-ml subcutaneous inoculations given on each side of the neck. Antibody responses were detected after immunization number four and thereafter. Quil-A-saponin is used routinely in our lab to stimulate strong type 1 immune responses and protective immunity against *Anaplasma marginale* (9, 55, 56).

The bovine leukocyte antigen (BoLA) DRB3 alleles were determined by the PCR restriction fragment length polymorphism (PCR-RFLP) method as described previously (91). The nomenclature of bovine MHC class II genes can be found at the following websites: <http://www.ebi.ac.uk/ipd/mhc/bola> and <http://www.projects.roslin.ac.uk/bola/bolahome.html>. BoLA-DRB3-RFLP haplotypes for the cattle in this study are as follows: animal 30864, 12/16 (where 12/16 indicates RFLP haplotypes designated DRB3 12 and DRB3 16, each inherited from one parent); animal 30900, 23/27; animal 30904, 11/16; animal 30916, 3/24; and animal 30919, 10/22. During the course of this study, all animals maintained recall T cell responses, which enabled the development of *S. aureus*-specific short-term T cell lines. Two calves expressing different MHC-II haplotypes, 16/22 (animal 35287) and 8/23 (animal 583), and immunized with *A. marginale* outer membranes (OM) as described previously (55, 65) were used as a source of peripheral blood mononuclear cells (PBMC) for lymphocyte proliferation assays with *S. aureus* antigens as negative controls. Animals used in this study were in compliance with the Washington State University Institutional Animal Care and Use Committee.

**Identification and cloning of ORFs encoding surface exposed antigens.** By employing a reverse genetics and comparative genomics approach using *S. aureus* Newman strain (GenBank accession number AP009351) and MRSA strains COL and USA300 as well as a bovine mastitis isolate RF122 (GenBank accession numbers CP000046, CP000255, and AJ938182.1, respectively), we identified 43 surface proteins and virulence factors from *S. aureus* Newman strain, a human isolate (4). The proteome data based on matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) in conjunction with immuno-proteomics were used to verify the surface exported proteins analyzed by *in silico* software (17, 82, 92). The protein class of open reading frames (ORFs) coding for known cytoplasmic functions was eliminated, whereas the other classes of

proteins were selected for further analysis. A second screening step aimed at identifying putative proteins with a cellular localization spanning the inner membrane to outside the bacterium was applied. BLAST, FASTA, MOTIFS, FINDPATTERNS, and PSORT (<http://psort.nibb.ac.jp>), as well as the ProDom, Pfam, and Blocks databases, were used to predict features typical of surface-associated proteins such as transmembrane domains, leader peptides, homologies to known surface proteins, lipoprotein signature, outer membrane anchoring motifs, and host cell binding domains such as RGD. We also included antigens identified as anchorless cell wall proteins, deemed to be potentially immunogenic by subtractive proteome analysis (28). The ORFs selected were >80% identical over 80% of the length at nucleotide and amino acid levels across the four strains (Table 1). All 43 ORFs were amplified from the *S. aureus* Newman strain using freshly isolated genomic DNA (Qiagen) by high-fidelity polymerase (*Pfu* Ultra II Hot Start; Agilent Technologies). Subsequently, the PCR products were gel eluted (Qiagen) and cloned into the primary entry vector pENTR/DTOPO (Invitrogen) according to manufacturer's instructions. The cloning was directional, as dictated by the CACC overhang incorporated into the forward gene-specific primer. The reverse primer was designed with a FLAG sequence, which resulted in a carboxy-terminal FLAG epitope (Table 1). Positive colonies were identified by colony PCR using M13 forward and reverse primers. All positive clones were sequenced bidirectionally using M13 forward and reverse primers, and their integrity was confirmed by BLAST analysis. For making final expression constructs containing a carboxy-terminal FLAG and two amino-terminal epitopes (six-histidine [His<sub>6</sub>] and Xpress), the plasmid DNAs containing the 43 ORFs (pENTR/DTOPO) were isolated individually and recombined into the pEXP1-DEST vector using Clonase LR (Invitrogen). TOP 10 *Escherichia coli* cells transformed with individual plasmids were selected on carbenicillin (100 µg/ml)–Luria-Bertani (LBC) plates. The positive clones were sequenced, and their integrity was confirmed again using T7 forward and reverse primers. The gene-specific primers used to amplify individual ORFs are listed in Table 1.

**Expression of antigens by IVTT and bead purification.** Approximately 600 ng of each pEXP1-DEST clone was used as a template in a 50-µl *in vitro* transcription and translation (IVTT) reaction volume using an RTS-100 *E. coli* HY kit (5-Prime) to achieve expression of cell-free recombinant protein according to the manufacturer's instructions. Three separate expression experiments were performed. The IVTT protein products were purified as previously described, with slight modifications (53, 86). Briefly, 100 µl of protein G carboxylate latex microspheres that had 526 µg of protein G/ml of beads (Polysciences, Inc.) was washed in A/G buffer (0.1 M Tris-HCl, 0.15 M NaCl, pH 7.4) three times. The washed beads were bound to 300 µg of anti-FLAG monoclonal antibody (MAB) (Sigma-Aldrich) in a 300-µl volume of A/G buffer by incubation at 4°C on a continuous rocking shaker. After 2 h the anti-FLAG MAB-coupled protein G latex microspheres were pelleted at 10,000 × g for 5 min, washed three times in A/G buffer, and resuspended in 400 µl of A/G buffer. This anti-FLAG MAB-protein G bead stock solution was diluted to a concentration of 10 µg of protein G beads/ml and bound to 10 µl of each IVTT product individually in a 200-µl volume of A/G buffer in a 1.5-ml microcentrifuge tube at 4°C for 2 h on a continuous shaker. The mixture was washed three times in A/G buffer and resuspended in 200 µl of complete RPMI medium to give stock solutions of 2 and 10 µg of protein G beads/ml for proliferation assays. Protein quantity in IVTT reaction mixtures was estimated by dot blot analysis using a MAB specific for the FLAG epitope as previously described, with slight modifications (86). A Hybrid-Dot Manifold (BRL Life Technologies, Inc.) was used to spot a prewetted nitrocellulose membrane with 1 µl of IVTT product diluted in 200 µl of PBS (pH 7.0). The membranes were washed in I-Block reagent (I-Block124; Applied Biosystems) containing 0.05% Tween 20 (Bio-Rad) (referred to here as I-Block) for 1 h, with a subsequent 1 h of incubation with either anti-FLAG MAB or anti-Xpress MAB (Invitrogen) diluted 1:10,000 in blocking reagent and extensive washing. The membranes were then incubated for 1 h with alkaline phosphatase-conjugated goat anti-

TABLE 1 Primers designed to amplify *S. aureus* Newman strain ORFs for protein expression

Gene locus no.	Function	Forward primer (5' to 3') <sup>a</sup>	Reverse primer (5' to 3') <sup>b</sup>
NWMN_0161	Putative lipoprotein	ATGGGAAAATCAAAAATTTATATCTTG	TGAATCATCTCCAAAAATTTATG
NWMN_0364	Putative lipoprotein	ATGGGAAAATTTAAATCATTAGCAG	GTGATCTTGTCTACTCTTTAATAC
NWMN_0389	Enterotoxin	ATGGGAAAATGAAAAATTTGC	TGCTTTTATAACTTTTGATTTTC
NWMN_0390	Exotoxin 8	ATGGGAAAATGAAAAATTTGC	CTTATCATTAGCATTATTTTG
NWMN_0429	Alanine amidase	ATGGGAGTGCAAAAAAGTAATTGCAGC	GTGAATATATCTATAATTATTTAC
NWMN_0458	Erythritol kinase	ATGGGAATATATGAAACGGCACCAG	TCCTAATAGTCTAACTAAGTAC
NWMN_0601	Putative lipoprotein	ATGGGAAAAAATAGTACCTTTA	TTTACGTTTATCACTTGGTACG
NWMN_0668	Fructose PO <sub>4</sub> kinase	ATGGGAATTTATACAGTGACTTTC	CTCCCCATCAAGTACGCTAAT
NWMN_0724	LysM domain protein	ATGGGAAAAAATCTCTTACAGTG	GTGGATGTAATATATTTTCCCTG
NWMN_0734	CofD-like proteins	ATGGGAAGACAAATAAAAGTTGTAC	TTTACGTTTATCACTTGGTACG
NWMN_0738	Putative protein	ATGGGATCGAATCAAAATTACG	GTTATCATTAGCATTATTTTG
NWMN_0753	Putative lipoprotein	ATGGGAAAAAAGTAATGGGGATA	TACTTCATCTAAACCACTGTGG
NWMN_0791	CBS domain protein	ATGGGAGTGATCATTGCCATAATTA	TATAGATACGTTGCGATTTTCCG
NWMN_0925	LytR domain protein	ATGGGAAATAAATTTTTAAATAC	ATTTACAACATTTTGG
NWMN_1067	FPRL1 inhibitory protein	ATGGGAAAAAATATCACAAAAAC	ATACCAAGTAATCGAGTCGATTTTC
NWMN_1069	Fibrinogen-binding protein	ATGGGAAAAAATAAATTTGATAGC	TTTAACTAATCCCTGTTTTAATAC
NWMN_1236	Thermonuclease	ATGGGAAAGTCAAAATAAATCGCTTG	TTTACTCCAAATATTTAATTTTC
NWMN_1398	Putative lipoprotein	ATGGGAAAAAATTCATTGGATCAG	TTTACTCTTTACTGTGCACCCCATATTC
NWMN_1607	Thiol peroxidase	ATGGGAACTGAAATAACATTCAAAG	AATATTTTTGTATGCAGCTAAAGC
NWMN_1623	Glycosyl transferase	ATGGGAACGAATCAAGACAATC	ACGCTTTTTTCGATTTAACAATG
NWMN_1690	Putative lipoprotein	ATGGGAAATTCAAAGCTATCGTTG	TTTAACTTTAGTTTCTTTCAG
NWMN_1701	Serine protease SplF	ATGGGAAATAAATAATAATCATCAAAAGTATTGCAG	TTTATCTAAATATCTGCAATG
NWMN_1703	Serine protease SplD	ATGGGAAATAAATAATAATCATCAAAAGTATTGCAG	TTTATCTAAATATCTGCAATAAATTC
NWMN_1704	Serine protease SplC	ATGGGAAATAAATAATAAGTCATAAAAGC	TTGTTCAATGTGCTTTTGAATAAAATC
NWMN_1705	Serine protease SplB	ATGGGAAACAAAAACGTAGTCATCAAG	TTTATCTATGTTTCTGCAATG
NWMN_1706	Serine protease SplA	ATGGGAAATAAATAATAAGTAAAG	TTTTTCAATATATTTTGAATAAATG
NWMN_1726	RNAIII-activating protein	ATGGGAAAGAAACTATATACATC	TTTATGCTTCCGCTGTACAGTTTC
NWMN_1733	Foldase protein	ATGGGAAAGATGATAACAAATTAATC	TTGGCTCATGCCGCTTGTTC
NWMN_1876	Complement inhibitor	ATGGGAAAAATTAGAAAATCTATAC	ATATTTACTTTTTAGTGCTTC
NWMN_1880	Staphylokinase	ATGGGACTCAAAAGAGGTTTATTA	TTTCTTTTCTATAATAACCTTTG
NWMN_1926	$\beta$ -Hemolysin	ATGGGATATCCAACTGGGGGC	TTTACTATAGGCTTTGATTGG
NWMN_1999	Transglycosylase <i>sceD</i>	ATGGGAAAGAAAACATTACTCGCATC	TGCAGTAACCAATGTCCAGC
NWMN_2043	Dps family protein	ATGGGAAGTAATCAACAAGATGTTG	GCTTAAGTAAGATTTAAACATC
NWMN_2086	Alkaline shock protein	ATGGGAACGTAGATAACAATAAAG	TTGTAACCTTGTCTTTCTTGG
NWMN_2270	Putative lipoprotein	ATGGGAAAAAGATTAGTTACAGGGTTAC	TTGTTGGTAGTTGGATCAGTAAC
NWMN_2318	$\gamma$ -Hemolysin A	ATGGGAATTTAAAAATAAATAATTAAC	CTTAGGTGATGCTTTTAATTTTAC
NWMN_2319	$\gamma$ -Hemolysin C	ATGGGACTTAAAAATAAATAATTAAC	ATTCTGTCTTTACCTTGATTTC
NWMN_2320	$\gamma$ -Hemolysin B	ATGGGAAAAATGAATAAATTAGTC	TTTATTGTTTTAGTTTCTTTTG
NWMN_2426	Sortase A	ATGGGAAAAAATGGACAATCG	TTTGACTTCTGTAGCTACAAAG
NWMN_2469	Antigen A	ATGGGAAAAAAGACAATTATGGCATC	GAATCCCCAACCTAAACCTTG
NWMN_2537	Antigen B	ATGGGAAATAAAACCAAGTAAAGTTTG	TTTACTGTTTTGATGGTGATG
NWMN_2544	Isochorismatase	ATGGGATCTCGAAAACGGCGCTATTAG	TAAACTACTTTTCCATGACTC
NWMN_2579	Putative lipoprotein	ATGGGACATAAAAAATATTTATTGC	TCTTCATAATCCCGATTCCC

<sup>a</sup> The forward primers were designed with a CACC overhang at the 5' end to facilitate directional cloning.

<sup>b</sup> The reverse primers were designed with a TCATTGTGTCGTCTTTATAGTC overhang to incorporate a FLAG epitope and a stop codon at the 3' end.

mouse IgG (Applied Biosystems) diluted 1:10,000 in blocking reagent and washed. Antibody binding was detected with a Western-Star reagent system (Applied Biosystems). Dot blot densitometry was analyzed using AlphaEase FC, version 4.0.0, software (Alpha Innotech) to determine relative expression of each IVTT product. As only proteins expressing the carboxy-terminal FLAG epitope were certain to be full-length, protein concentrations were based on microgram levels of the FLAG epitope on recombinant merozoite surface antigen-1 (MSA1) from the M07 strain *Babesia bovis* (85), as a protein standard.

**Expression of recombinant proteins in *E. coli*.** NWMN\_0601, NWMN\_0364, NWMN\_2086, and *A. marginale* control protein VirB9-2 were expressed in *E. coli* and purified for use in Western blot assays. The plasmids (pEXP1-DEST, expression vector backbone, encoding His<sub>6</sub> and FLAG epitopes) containing these five individual ORFs were transformed, according to the manufacturers' specifications, into BL21(DE3) LysS One Shot chemically competent *E. coli* (Invitrogen). Individual colonies were

selected and grown in LB broth supplemented with 100  $\mu$ g/ml carbenicillin (LBC broth). To produce recombinant proteins, individual bacterial colonies containing each antigen of interest were expanded in LBC broth overnight, diluted 1:100 in fresh LBC broth, grown for 3 h at 37°C, and induced with 1 mM isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) for 8 h. Bacteria were harvested after centrifugation at 4,000  $\times$  g for 20 min and resuspended in 6 M guanidine hydrochloride, 20 mM dibasic sodium phosphate, and 500 mM sodium chloride, pH 7.8, for 30 min and then sonicated four times at 350 W for 45 s each time. Proteins were extracted from the *E. coli* lysate using nickel chelating resin (ProBond purification system) following the manufacturer's protocol (Invitrogen). Recombinant proteins were purified using a ProBond system two consecutive times, followed by dialysis against PBS using Slide-A-Lyzer dialysis cassettes with a 10-kDa molecular-mass cutoff (Thermo Fisher Scientific). Protein concentrations were determined using a Quick Start Bradford assay, and proteins were frozen at -20°C.



**Western blot assays.** Western blot analysis was performed to detect *S. aureus*-specific antibody responses and to determine titers against individual recombinant antigens. Briefly,  $10^{10}$  heat-killed *S. aureus* bacteria were lysed in 200  $\mu$ l of lysis buffer (130  $\mu$ l of 20 mM Tris-HCl, pH 8.0, 2 mM EDTA, 1.2% Triton X-100, 10  $\mu$ l of 2 mM phenylmethylsulfonyl fluoride [PMSF], 60  $\mu$ l of 2 mg/ml lysostaphin, 1  $\mu$ l of 5 U/ml Benzamide) and incubated at 37°C for 1 h. The reaction was stopped by the addition of 20  $\mu$ l of stop buffer (20 mM Tris-HCl, pH 8.0, 2 mM EDTA, 20% SDS) and incubated for 5 min at 95°C. Protein concentrations were estimated with a Quick Start Bradford assay (Bio-Rad), and lysates were diluted to 1  $\mu$ g/ $\mu$ l in PBS. Forty micrograms of lysate was added to sample loading buffer (10  $\mu$ l of 1 M dithiothreitol [DTT] 50  $\mu$ l of Nupage loading blue [Invitrogen]) and incubated at 95°C for 10 min. Next, 15  $\mu$ l of the reaction mixture was loaded on a 4 to 12% Tris-HCl gradient for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Criterion gels; Bio-Rad) and electrophoresed under denaturing conditions for 1 h at 100 V. The proteins were transferred to 0.45- $\mu$ m-pore-size polyvinylidene difluoride (PVDF) membranes at 100 V for 30 min. The PVDF membranes were blocked for 1 h in 5% milk powder (Bio-Rad) and washed three times in TBST buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20). The pre- and postimmune sera from each animal were added to individual strips at 1:100 and 1:1,000 dilutions and incubated for 6 h and then washed with TBST buffer. Alkaline phosphatase-conjugated mouse anti-bovine IgG (1:5,000) was used as a secondary antibody, and blots were developed in a substrate solution containing nitroblue tetrazolium/5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP; Bio-Rad) for 1 to 5 min.

To determine the titers of *S. aureus*-specific antibodies against recombinant NWMN\_0601, NWMN\_0364, NWMN\_2086, and *A. marginale* VirB9-2 as a control, proteins were boiled in SDS sample buffer and loaded at 1  $\mu$ g/lane on 4 to 20% SDS-PAGE precast Tris-HCl polyacrylamide Ready Gels (Bio-Rad). One gel was stained with Coomassie brilliant blue, and the second was transferred to a nitrocellulose membrane for immunoblot analysis using anti-FLAG MAb (86). Immunoblotting was also used to determine IgG1 and IgG2 antibody titers specific for *S. aureus* using pre- and postimmune sera. Serum was collected before immunization and 2 weeks after each immunization and stored at -20°C until the assays were performed simultaneously on all samples. After adsorption with *E. coli* lysate, the serum was serially diluted up to 1:4,000 in I-Block containing 5% bovine serum albumin (BSA) and 0.5% Tween 20 (New England BioLabs) as described previously, with the following modifications (34). Individual recombinant proteins were boiled in sample buffer, and 1  $\mu$ g protein/lane was electrophoresed and transferred to nitrocellulose membranes. Individual membrane strips were incubated with 1:200 dilutions of mouse anti-bovine IgG1 and mouse anti-bovine IgG2 (both from Serotec). Preimmune serum was used as a negative control. Serum from an *A. marginale*-infected calf (04B92) with known VirB9-2-specific IgG1 and IgG2 titers was used as a positive control to standardize the immunoblots (34, 55).

**Proliferation assays.** Freshly harvested PBMC obtained before immunization and 2 weeks after the last immunization were assayed in triplicate using  $2.5 \times 10^5$  viable cells/well in complete RPMI 1640 medium (Gibco) as previously described, with minor modifications (1, 34). Cells were stimulated in 96-well round-bottomed plates in a volume of 100  $\mu$ l/well with  $5 \times 10^3$  to  $5 \times 10^6$  heat-killed *S. aureus* cells. T cell growth factor (TCGF) diluted 1:10 in complete RPMI 1640 medium served as a positive control, and complete RPMI medium served as a negative control. Cells were cultured for 6 days at 37°C in a 5% CO<sub>2</sub> incubator, radiolabeled for 18 h with 0.25  $\mu$ Ci of [<sup>3</sup>H]thymidine (Dupont), and harvested onto glass filters; radionucleotide incorporation was measured with a beta counter.

For T cell proliferation assays using individual IVTT products, 2-week CD4 T cell lines were established as previously described, with slight modifications (53, 86). We have previously shown that the use of T cell-enriched lines enables a more sensitive detection of responses to specific

proteins (53). Briefly, PBMC were isolated from *S. aureus*-immunized steers, plated at  $4 \times 10^6$  cells/well in 24-well flat-bottom plates for 7 days with  $5 \times 10^4$  heat-killed *S. aureus* bacteria/well, and incubated at 37°C with 5% CO<sub>2</sub> in air. On day 7, the cells were harvested and recultured for 1 week at  $0.7 \times 10^6$  cells/well with  $2 \times 10^6$  cells/well of freshly harvested, irradiated (3,000 rads) autologous PBMC as a source of antigen presenting cells (APC) without antigen (resting). The cells were harvested after 7 days, washed repeatedly in complete RPMI medium, and tested for a proliferative response to individual affinity-purified *S. aureus* antigens (bead-bound IVTT products). Cells were cultured in triplicate wells of 96-well U-bottom plates at  $3 \times 10^4$  cells/well with  $2 \times 10^5$  APC and incubated at 37°C in 5% CO<sub>2</sub> in air with medium or various concentrations of antigen. Positive controls consisted of  $5 \times 10^4$  or  $5 \times 10^6$  heat-killed *S. aureus* bacteria/well and 10% TCGF. The negative-control antigens included a no-DNA control IVTT reaction mixture incubated with beads and IVTT-expressed *A. marginale* VirB9-2 bound to beads (53). The IVTT antigen-beads were tested at a final concentration of 1 and 5  $\mu$ g of protein G/ml in 100- $\mu$ l triplicate cultures. After 3 days, the cells were radiolabeled and harvested.

For an additional negative control to rule out nonspecific proliferation to the IVTT-expressed proteins, 2-week T cell lines were derived from two *A. marginale* outer membrane (OM)-vaccinated cattle as described previously (65). PBMC were cultured for 1 week with 1  $\mu$ g/ml *A. marginale* OM and rested for 1 week and then tested as described above in proliferation assays.

In some assays, the 2-week cell lines were depleted of CD4<sup>+</sup> cells, CD8<sup>+</sup> cells, and/or  $\gamma\delta$  T cells and cultured in 4-day proliferation assays. Depletion was accomplished by incubating cells with MAb, obtained from the Washington State Monoclonal Antibody Center, specific for bovine CD4 (ILA-11, mouse IgG2a), CD8 (7C2B, mouse IgG2a), or  $\gamma\delta$  T cells (GB21A, mouse IgG2b), at 15  $\mu$ g of MAb per  $10^7$  cells, rotating at 4°C for 30 min. Cells were repeatedly washed with complete RPMI medium and magnetic cell sorting (MACS) bead buffer (Miltenyi) containing 5% bovine serum albumin (BSA). MAb-labeled cells were incubated on ice for 20 min with 80  $\mu$ l of goat anti-mouse IgG magnetic microbeads (Miltenyi) per  $10^7$  cells. MAb- and bead-labeled cells were depleted by filtration through an LD magnetic column (Miltenyi) under a strong magnetic field, and washed with 3 ml of MACS buffer containing BSA. Depletion was determined by flow cytometry after cells were stained with MAbs ILA-11 (CD4), 7C2B (CD8), and GB21A ( $\gamma\delta$ ) or with isotype-matched negative control MAbs COLIS205B (IgG2a) and COLIS169A (IgG2b) and a 1:10,000 dilution of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories). The cells were assayed for proliferation to antigen as described above for cell lines and PBMC.

Proliferation was expressed as either mean counts per minute (cpm)  $\pm$  1 standard deviation (SD) for triplicate cultures or as a stimulation index (SI), determined as the mean cpm of cells cultured with *S. aureus* antigen divided by the mean cpm of cells cultured with negative-control antigen.

**Detection of IFN- $\gamma$  in supernatants of T cell cultures.** *S. aureus*-specific 2-week cell lines were cultured for 3 days with APC ( $2 \times 10^6$  cells per ml) and either  $1 \times 10^6$  heat-killed *S. aureus* bacteria per well or 1 and 5  $\mu$ g of protein G per ml of bead-bound IVTT proteins, and supernatants were tested for gamma interferon (IFN- $\gamma$ ) using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Bovigam; CSL Ltd.,) according to the manufacturer's protocol. The IFN- $\gamma$  activity in culture supernatants, diluted 1:2, was determined by comparison with a standard curve obtained with a recombinant bovine IFN- $\gamma$  as described previously (49). The results are presented as units of IFN- $\gamma$  per ml of supernatant.

**Statistical analyses.** T cell proliferation and IFN- $\gamma$  secretion to *S. aureus* antigens were compared with negative-control antigens VirB9-2 beads and no-DNA IVTT beads using a Student's one-tailed *t* test or a one-way analysis of variance (ANOVA) with Dunnett's posttest for multiple comparisons ( $P < 0.05$ ), as indicated (see Table 2 and Fig. 3 and 4).

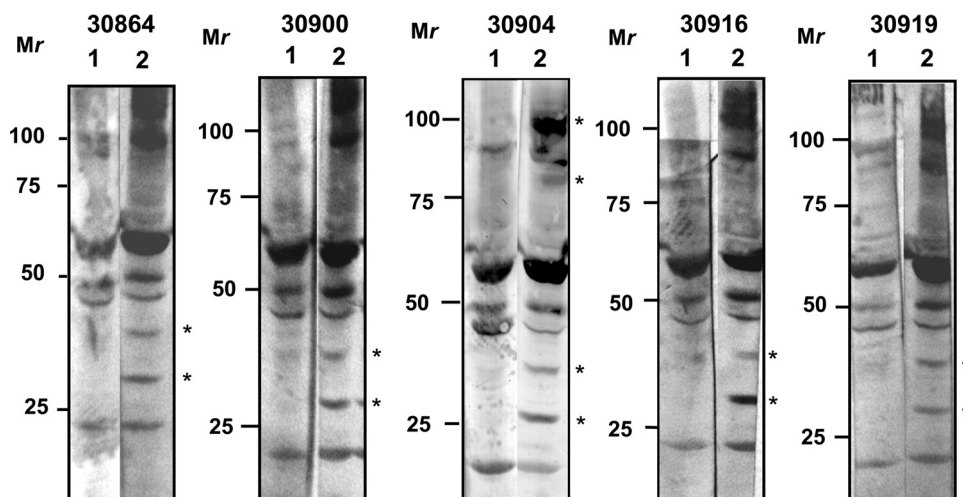


FIG 1 Immunoblotting to detect *S. aureus*-specific antibody responses. Lysate (5  $\mu$ g/lane) from heat-killed *S. aureus* was loaded and probed with preimmune (lane 1) and postimmune (lane 2) bovine serum obtained after the fourth immunization and diluted 1:100 or 1:1,000. Alkaline phosphatase-conjugated mouse anti-bovine IgG (1:5,000) was used as a secondary antibody, and blots were developed in a substrate solution containing NBT/BCIP for 1 to 5 min. Asterisks indicate unique bands which appeared after the fourth immunization.  $M_r$ , relative molecular weight in thousands.

In addition, proliferative responses to IVTT-expressed antigens were considered to be biologically relevant if the SI was  $\geq 2.0$  and if the mean cpm was  $\geq 1,000$ .

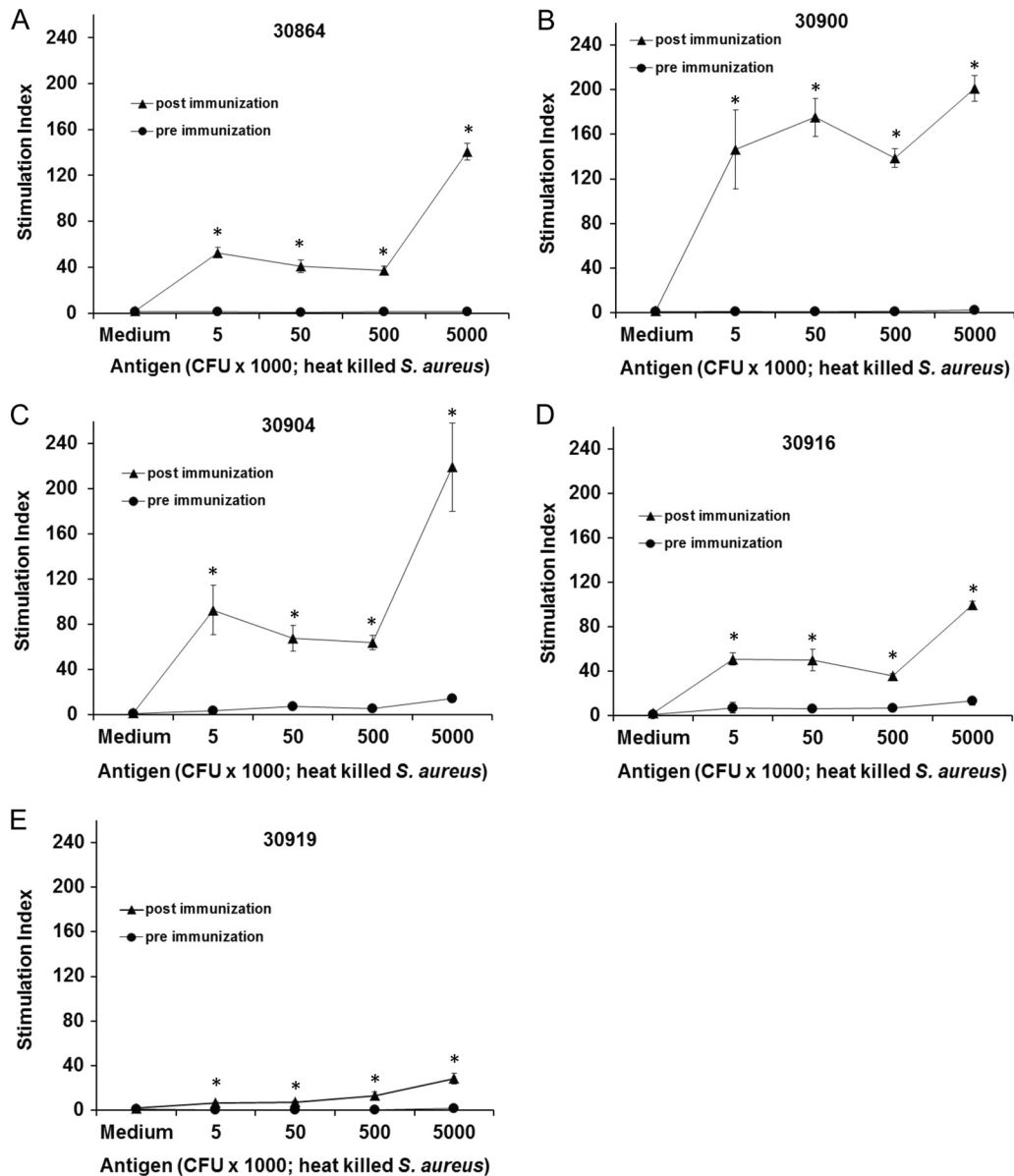
## RESULTS

**Antibody and PBMC responses from calves immunized with *S. aureus* Newman strain.** The current study was designed to screen and test the immunogenicity of selected *S. aureus* proteins that could be included in a potential vaccine in cattle that might translate to use in humans. Five age-matched Holstein steers with different MHC class II haplotypes were immunized with *S. aureus* until *S. aureus*-specific immune responses were obtained. Responses were first observed after the fourth immunization. Western blot analysis of *S. aureus* whole-cell lysates showed the presence of bands specific for *S. aureus* in all five immune sera obtained 2 weeks after the fourth immunization (Fig. 1). Bands observed in postimmunization sera that were not visible in preimmunization sera include bands at the approximate molecular masses of 80 and 100 kDa (animal 30900) and 30 and 40 kDa (all animals). In addition, the  $\sim 100$ -kDa band identified by preimmunization sera was more intense with postimmunization sera (animals 30864, 30900, 30916, and 30919). The presence of the same bands identified by both pre- and postimmunization sera indicates that the cattle may have been exposed to *S. aureus* or other bacteria that share antigenic epitopes with *S. aureus* before the animals were immunized. The identities of these proteins were not determined.

Lymphocyte responses from all five calves were determined by proliferation assays using PBMC obtained before and 2 weeks after the last immunization and heat-killed *S. aureus* as antigen. The postimmunization PBMC had a significant proliferative response compared to preimmunization PBMC (Fig. 2A to E). Furthermore, the postimmunization PBMC from all calves responded in a concentration-dependent manner to heat-killed *S. aureus*. As we typically observe for outbred cattle (53, 54), there is considerable variation in the levels of immune response between animals.

**Identification of *S. aureus* antigens that induced significant T cell and IgG responses.** The 43 antigen-encoding ORFs were expressed by IVTT, and their expression was confirmed by dot blot analysis using carboxy-terminal FLAG and amino-terminal anti-Xpress MAbs. The amount of protein expressed by IVTT varied between  $\sim 16.0$  and 36.5  $\mu$ g/ml, compared with recombinant *B. bovis* MSA1 (expressing a FLAG epitope) by dot blot densitometry. All 43 antigens from *S. aureus* Newman strain were tested with 2-week T cell lines from calves immunized with heat-killed *S. aureus*. Before testing individual antigens, we compared the proliferative response of 2-week T cell lines and freshly obtained PBMC to heat-killed *S. aureus* cells. As observed previously with *A. marginale* proteins (53), the T cell lines had a higher SI to *S. aureus* than fresh PBMC (data not shown). Therefore, 2-week T cell lines were used to assay all 43 IVTT-expressed proteins. The T cells were cultured with IVTT-expressed proteins bound to protein G beads via anti-FLAG MAb and tested at 1 and 5  $\mu$ g/ml protein G beads (based on concentration of protein G on the beads). The amount of FLAG-specific MAb bound to protein G beads ranged between 90 and 105  $\mu$ g/ml. This was determined by comparing the concentrations of FLAG-specific MAb in the supernatants before and after binding.

The 43 proteins were expressed by IVTT three separate times, but they did not express uniformly each time. During the first round of expression and screening, antigens NWMN\_2043 and NWMN\_2579 could be detected only when 3  $\mu$ l, instead of 1  $\mu$ l, of the IVTT mixture was blotted onto the membranes. However, all of the proteins were comparably expressed during the second round of screening. During the third round of expression and screening, NWMN\_1607, NWMN\_1726, NWMN\_1733, NWMN\_1926, NWMN\_1999, NWMN\_2043, and NWMN\_2086 were expressed at  $< 16$   $\mu$ g/ml, but two of these bead-bound proteins (NWMN\_1733 and NWMN\_2086) were sufficient to elicit T cell proliferation in one or more calves (Table 2). Figure 3 provides examples of assays in which *S. aureus* proteins induced significant proliferation of 2-week T cell lines from one or more calves (30864, 30900, 30904, 30916, and



**FIG 2** Proliferation assays on pre- and postimmune PBMC to confirm *S. aureus*-specific T cell responses. Heat-killed *S. aureus* cells were used as the antigen at the indicated concentrations. TCGF (interleukin-2) was used as a positive control. The assay was performed with the indicated PBMC obtained before immunization and 2 weeks after the last immunization. Responses are presented as the SI compared to the negative control (RPMI medium)  $\pm$  1 SD for triplicate cultures. Responses that were significantly greater than medium are indicated with asterisks where  $P$  is  $<0.05$  using a one-tailed Student's  $t$  test.

30919) in one or more of the assays. Negative controls included *A. marginale* VirB9-2 beads (expressed in the same way using IVTT) and no-DNA control beads. In contrast, the *S. aureus* proteins did not induce significant T cell proliferation of 2-week cell lines derived from *A. marginale* OM-immunized cattle 33583 and 35287 (Fig. 4A and B). These cell lines did respond to *A. marginale* OM and responded variably to type 4 secretion system proteins VirB9-1 (CTP) and VirB9-2 (VirBa) expressed by IVTT and bound to protein G beads, as we have shown previously (53, 65, 86).

The cumulative results of three rounds of expression and screening identified 13 T cell antigens, two of which induce serum antibodies in humans (NWMN\_2318 [ $\gamma$ -hemolysin A] and NWMN\_2469 [antigen A]) (11–13). However, the remain-

ing 11 proteins (NWMN\_0161 [putative lipoprotein], NWMN\_0364 [putative lipoprotein], NWMN\_0390 [exotoxin 8], NWMN\_0429 [alanine amidase], NWMN\_0601 [putative lipoprotein], NWMN\_1733 [foldase protein], NWMN\_1999 [transglycosylase], NWMN\_2086 [alkaline shock protein], NWMN\_2270 [putative lipoprotein], NWMN\_2319 [ $\gamma$ -hemolysin C], and NWMN\_2320 [ $\gamma$ -hemolysin B]) were not previously identified as antigenic or used as a vaccine (Table 2). The 13 T cell antigens include four putative lipoproteins, three hemolysins, various enzymes, and an alkaline shock protein. Three antigens were designated the “best” in these experiments (NWMN\_0601, NWMN\_0364, and NWMN\_2086) because they induced significant T cell proliferation in three indepen-

**TABLE 2** Summary of antigens that induced significant T cell proliferation in three experiments using 2-week cell lines from *S. aureus*-immunized calves

Expt no.	Proliferative response of T cell lines to the indicated antigen(s) by animal <sup>a</sup>									
	30864 (12/16) <sup>b</sup>		30900 (23/27)		30904 (11/16)		30916 (3/24)		30919 (10/22)	
	Antigen	SI	Antigen	SI	Antigen	SI	Antigen	SI	Antigen	SI
1	NWMN_0364	25.7 <sup>c</sup>	NWMN_2086	9.6 <sup>c</sup>	NWNM_0601	38.2 <sup>c</sup>	NWMN_0601	2.3 <sup>c</sup>	NWMN_2086	8.3 <sup>c</sup>
	NWMN_0601	24.4 <sup>c</sup>					NWMN_0364	2.4	NWMN_1999	4.3
2	NWMN_0364	46.7 <sup>c</sup>	NWMN_2086	17.2 <sup>c</sup>	NWMN_0601	6.7 <sup>c</sup>	NWMN_0601	5.3 <sup>c</sup>	NWMN_2086	9.3 <sup>c</sup>
	NWMN_0601	20.1 <sup>c</sup>	NWMN_0601	3.0 <sup>d</sup>	NWMN_0429	4.4	NWMN_2086	2.2 <sup>d</sup>	NWMN_2469	2.6 <sup>d</sup>
	NWMN_0161	25.3					NWMN_2270	2.3 <sup>d</sup>		
	NWMN_0390	10.4					NWMN_2318	7.8 <sup>d</sup>		
							NWMN_2319	7.2 <sup>d</sup>		
3	NWMN_0364	38.9 <sup>c</sup>	NWNM_2086	4.2 <sup>c</sup>	NWMN_0601	2.7 <sup>c</sup>	NWMN_0601	18.9 <sup>c</sup>	NWMN_2086	6.0 <sup>c</sup>
	NWMN_0601	19.3 <sup>c</sup>	NWMN_0601	2.4 <sup>d</sup>	NWMN_1733	2.7	NWMN_2086	12.5 <sup>d</sup>	NWMN_2469	13.7 <sup>d</sup>
	NWMN_2086	9.4	NWNM_2320	4.7			NWMN_2270	12.8 <sup>d</sup>	NWMN_2320	5.3
							NWMN_2318	29.3 <sup>d</sup>		
							NWMN_2319	24.0 <sup>d</sup>		
							NWMN_2320	16.2 <sup>d</sup>		
							NWMN_2469	34.5 <sup>d</sup>		

<sup>a</sup> Different batches of IVTT-expressed proteins were tested in each experiment. Antigens were determined to induce significant responses based on the following criteria: mean cpm of >1,000, SI of >2.0, and *P* of <0.05 compared to medium, beads, and VirB9 beads. Significant proliferation was determined using ANOVA with Dunnett's posttest for multiple comparisons (*P* < 0.05). Assays were performed using 43 antigens tested in triplicate from three expression experiments. SI was determined as the mean cpm in response to 1 or 5  $\mu$ g/ml *S. aureus* antigen/mean cpm in response to VirB9 beads, whichever was higher.

<sup>b</sup> MHC class II haplotypes were determined by RFLP analysis of exon II from the DRB3 locus. For example, 12/16 refers to the RFLP haplotypes designated DRB3 12 and DRB3 16, each inherited from one parent.

<sup>c</sup> Antigens that induced significant proliferation in three independent assays for each animal.

<sup>d</sup> Antigens that induced significant proliferation in two independent assays for each animal.

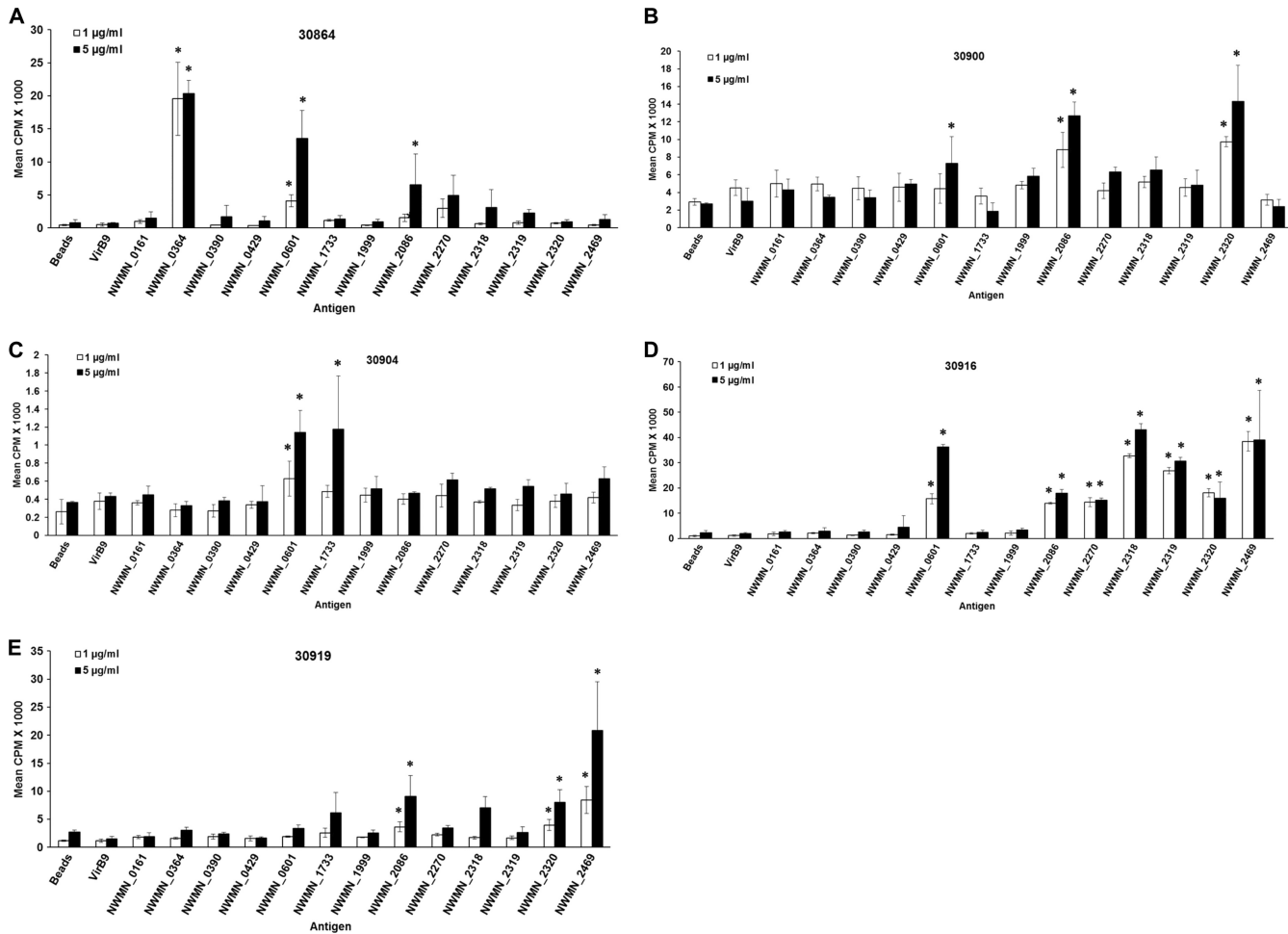
dent assays in at least one animal and stimulated responses from two or more animals that expressed different MHC class II haplotypes (Table 2). Antigens NWMN\_0601 and NWMN\_2086 each stimulated T cell responses from four cattle, and antigen NWMN\_0364 stimulated responses from two cattle. Furthermore, only two cattle shared one MHC class II haplotype; animals 30864 (12/16) and 30904 (11/16) have the DRB3 RFLP haplotype 16. Thus, the finding that these three antigens stimulate recall responses from two to four animals with completely different haplotypes and the fact that these haplotypes are common among North American Holstein cattle (65, 77) suggest that these are good candidates for inclusion in a multiantigen vaccine that would be broadly protective in Holstein cattle. Two of these antigens are putative lipoproteins, and one is the alkaline shock protein.

Antigen-specific lymphocytes in 2-week T cell lines were further characterized by depleting CD4<sup>+</sup> cells or  $\gamma\delta$  and CD8<sup>+</sup> cells and repeating proliferation assays. Flow cytometric analysis indicated that the 2-week T cell lines from all five steers contained 21 to 46% CD4<sup>+</sup> cells, 31 to 36%  $\gamma\delta$  T cells, and 7 to 11% CD8<sup>+</sup> cells. Following enrichment, CD4<sup>+</sup> T cells were increased to 94 to 96% of total T cells, with only 4 to 6%  $\gamma\delta$  and CD8 T cells remaining. Similarly, upon enrichment for  $\gamma\delta$  and CD8<sup>+</sup> cells, these increased to 95 to 96% of total T cells, while CD4<sup>+</sup> T cells fell to 4 to 5%. CD4-enriched and  $\gamma\delta$ - and CD8-enriched cell populations were then tested for proliferation to *S. aureus* and two IVTT-expressed proteins. The CD4-enriched 2-week cell lines from all three animals showed significant proliferation against *S. aureus* compared to  $\gamma\delta$ - and CD8-enriched T cells in this assay (representative data are shown in Fig. 5). Furthermore, when tested against IVTT-expressed proteins

from the third round of expression, CD4-enriched T cells from animal 30919 showed significant responses to antigens NWMN\_2086, NWMN\_1999, NWMN\_2318, and NWMN\_2469; those from animal 30904 responded significantly to NWMN\_1733, and those from animal 30864 responded significantly to NWMN\_0364 and NWMN\_0601 (data from 30864 are shown in Fig. 6). The  $\gamma\delta$  T cell- and CD8-enriched populations had insignificant proliferation to heat-killed *S. aureus* and all of the proteins tested. This indicates that the antigen-specific lymphocytes obtained following *S. aureus* immunization were CD4 T cells. Enriching for CD4<sup>+</sup> T cells increased the response of the cell lines to the individual proteins compared with untreated T cell lines, just as we have shown previously that T cell lines are more sensitive than PBMC to detect IVTT-expressed proteins (53).

Secretion of IFN- $\gamma$  is a hallmark of the Th1-type response, and IFN- $\gamma$  induces opsonizing antibodies and activates macrophages to kill intracellular bacteria and therefore likely plays an important role in immunity to *S. aureus* (5, 30, 38, 62, 70). The 13 IVTT-expressed *S. aureus* proteins shown to stimulate T cell proliferation were therefore tested, in comparison with medium and negative-control VirB9-2-beads, for inducing secretion of IFN- $\gamma$  using 2-week T cell lines. Eight of the 13 proteins induced IFN- $\gamma$  secretion in T cell lines from one or more calves (Table 3). The three best antigens, NWMN\_0601, NWMN\_0364, and NWMN\_2086, induced high levels of IFN- $\gamma$  in culture, ranging from 206 to 429 U/ml. Antigens NWMN\_1733, NWMN\_2270, NWMN\_2318, NWMN\_2319, and NWMN\_2469 induced generally lower levels of IFN- $\gamma$ , ranging from 44 to 219 U/ml. Animal 30919 had a high background response to the negative-control VirB9 beads so none of the responses to *S. aureus* proteins were significant.

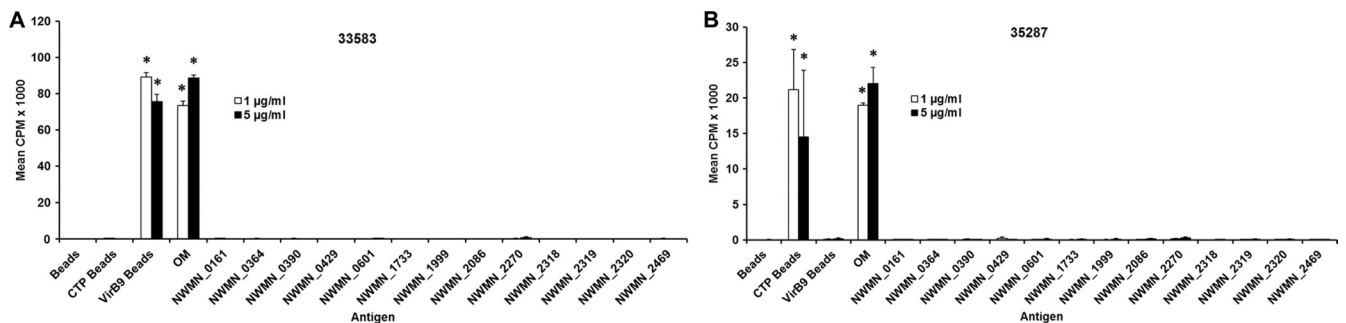




**FIG 3** Antigens that induced significant proliferation for one or more calves. Proliferation assays were performed with 2-week T cell lines from the immunized calves using 1 and 5 µg/ml protein-G bead-bound IVTT-expressed *S. aureus* proteins. Negative-control antigens include IVTT-expressed *A. marginale* VirB9-2 beads and no-DNA control beads. Data are presented from one screening assay for each animal. Responses were considered significant (indicated by asterisks) if mean the cpm was >1,000, the SI compared with negative-control antigens was >2.0, and *P* was <0.05 compared to negative-control antigens and medium (using ANOVA with Dunnett’s posttest for multiple comparisons).

IgG titers specific for the three best T cell antigens in *S. aureus*-vaccinated calves were determined using affinity-purified recombinant proteins. Animal 04B92 immunized with *A. marginale* OM had a titer (both IgG1 and IgG2) of at least 4,000 against VirB9-2,

and this serum served as a positive control (54). Recombinant *S. aureus* proteins NWMN\_0601, NWMN\_0364, and NWMN\_2086 were expressed in *E. coli* and purified, and their expression was confirmed by immunoblotting with an anti-FLAG MAb (data not



**FIG 4** *S. aureus* antigens do not induce significant proliferation of 2-week T cell lines from *A. marginale*-immunized cattle. *S. aureus* proteins were tested on 2-week T cell lines from cattle immunized with *A. marginale* OM: animal 35287 that expresses the DRB3 RFLP haplotype 16/22 (A) and animal 33583 that expresses the DRB3 RFLP haplotype 8/23 (B). *A. marginale* OM and IVTT-expressed VirB9-1 (CTP) and VirB9-2 (VirBa) bound to protein G beads were used as positive controls. Responses were considered significant (indicated by asterisks) if the mean cpm was >1,000, the SI compared with negative-control antigen was >2.0, and *P* was <0.05 compared to negative-control antigens and medium (using ANOVA with Dunnett’s posttest for multiple comparisons).



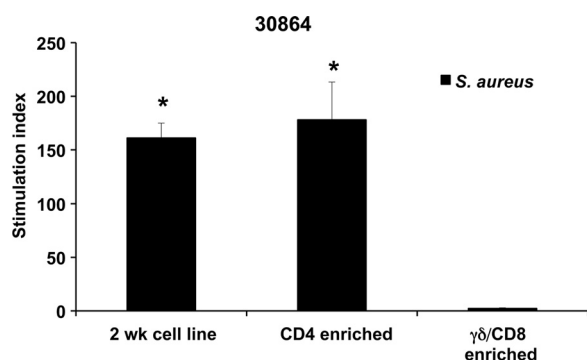


FIG 5 The cells responding to *S. aureus* immunization are predominantly CD4 T cells. Two-week cell lines were undepleted or depleted of CD4<sup>+</sup> cells or CD8<sup>+</sup> cells and  $\gamma\delta$  T cells. Heat-killed *S. aureus* ( $5 \times 10^6$  bacteria/well) was used as the antigen. Responses are presented as the SI compared to medium  $\pm$  1 SD for triplicate cultures. Significant responses of each cell population are indicated with asterisks, where  $P$  is  $<0.05$  using a one-tailed Student's  $t$  test.

shown). All five animals had positive antibody responses to all three recombinant proteins. IgG1 titers were at least 10 times higher than IgG2 titers in most of the animals for all three antigens (Table 4).

## DISCUSSION

Identifying candidate vaccine antigens for *S. aureus* has been problematic due to its complex and plastic genetic machinery, and protection using a single-component vaccine has been elusive to date (52). Identification of potential vaccine antigens using patient infection serum is a common strategy (19, 94) although the usefulness of this strategy for *S. aureus* has not been proven (16). The failure of bivalent capsular polysaccharide type 5 (CP5) and CP8 conjugate vaccine, StaphVAX, in human clinical trials can be partly explained by the fact that *S. aureus* switches to an acapsular phenotype and can still be lethal (18, 63, 66). Various vaccination trials have shown that partial protection against *S. aureus* challenge in mice can be achieved by using *S. aureus* surface carbohydrates such as poly-*N*-acetylglucosamine or poly-*N*-succinyl glucosamine or individual surface proteins such as clumping factor A or B (ClfA or ClfB, respectively), iron-regulated surface determi-

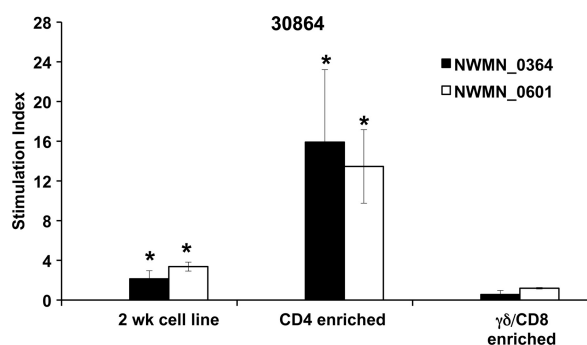


FIG 6 Response by enriched CD4 T cells to two *S. aureus* proteins. Two-week cell lines from animal 30865 were depleted of CD4<sup>+</sup> cells or CD8<sup>+</sup> cells and  $\gamma\delta$  T cells. Antigens NWMN\_0364 and NWMN\_0601 were used at 1  $\mu\text{g/ml}$  in proliferation assays with undepleted 2-week cell lines, CD4-enriched cells, and  $\gamma\delta$ - and CD8<sup>+</sup>-enriched cells. Responses are presented as the SI compared to medium  $\pm$  1 SD for triplicate cultures. Significant responses of each cell population are indicated with asterisks, where  $P$  is  $<0.05$  using a one-tailed Student's  $t$  test.

TABLE 3 IFN- $\gamma$  induced by *S. aureus* proteins

Culture condition	Amt of antigen ( $\mu\text{g/ml}$ )	IFN- $\gamma$ secretion (U/ml) by 2-week cell lines from the indicated animal <sup>a</sup>				
		30864	30900	30904	30916	30919
Medium		1.0	40.0	1.0	1.0	6.4
<i>S. aureus</i> cells		<b>429.6</b>	<b>298.0</b>	<b>596.2</b>	<b>111.5</b>	<b>294.3</b>
TCGF		<b>415.1</b>	<b>235.0</b>	<b>171.1</b>	<b>126.1</b>	<b>239.8</b>
IVTT-expressed protein						
VirB9	1	2.0	33.0	7.0	12.3	190.4
	5	8.1	60.0	24.0	21.8	251.2
NWMN_0161	1	16.1	ND	126.5	ND	ND
	5	28.5	ND	29.8	ND	ND
NWMN_0364	1	<b>325.5*</b>	ND	ND	ND	225.0
	5	<b>401.1*</b>	ND	ND	ND	219.4
NWMN_0390	1	22.5	ND	ND	ND	ND
	5	30.7	ND	ND	ND	ND
NWMN_0429	1	29.5	33.4	0.6	ND	ND
	5	16.1	33.0	1.0	ND	ND
NWMN_0601	1	<b>381.9*</b>	25.1	<b>19.8</b>	<b>39.1</b>	ND
	5	<b>429.2*</b>	43.2	11.3	<b>90.3*</b>	ND
NWMN_0724	1	2.08	ND	34.6	ND	ND
	5	17.4	ND	15.1	ND	ND
NWMN_1733	1	1	ND	<b>114.5*</b>	ND	139.8
	5	17.68	ND	<b>80.0*</b>	ND	196.5
NWMN_1880	1	ND	ND	ND	12.4	ND
	5	ND	ND	ND	24.6	ND
NWMN_1999	1	ND	ND	ND	ND	168.4
	5	ND	ND	ND	ND	211.1
NWMN_2086	1	17.1	<b>206.3*</b>	ND	13.4	179.8
	5	49.7	<b>198.0*</b>	ND	40.4	211.2
NWMN_2270	1	67.7*	55.4	ND	11.7	ND
	5	99.9	36.4	ND	25.6	ND
NWMN_2318	1	ND	66.4	ND	<b>27.5</b>	258.8
	5	ND	87.4	ND	<b>53.1*</b>	273.7
NWMN_2319	1	<b>36.8</b>	34.1	ND	<b>26.3</b>	ND
	5	<b>73.5*</b>	26.4*	ND	<b>44.4*</b>	ND
NWMN_2320	1	22.8	87.1	ND	10.9	220.1
	5	45.3	108.2	ND	20.3	213.5
NWMN_2469	1	<b>39.8</b>	ND	ND	<b>145.9*</b>	212.8
	5	<b>63.8</b>	ND	ND	<b>219.4*</b>	206.5
NWMN_2579	1	22.8	41.8	ND	11.4	ND
	5	45.3	44.6	ND	<b>60.7</b>	ND

<sup>a</sup> Data are presented as the mean U/ml of IFN- $\gamma$  secreted by 2-week T cell lines cultured in triplicate with  $1 \times 10^6$  heat-killed *S. aureus* bacteria or the indicated antigens. Boldface numbers indicate significant ( $P < 0.05$ ) IFN- $\gamma$  secretion with respect to medium and bead-bound VirB9. Asterisks (\*) indicate antigens that induced significant proliferation. ND, not determined.

nant B (IsdB), truncated fibronectin-binding protein (FnBP), live attenuated *S. aureus*, or a combination of FnBP and ClfA (3, 29, 44, 57, 60, 67, 73–75, 84, 89). Other vaccines that are currently under different stages of clinical trials by various pharmaceutical companies include CP5 and/or CP8, combined with alpha toxin and Panton-Valentine leukocidin (PVL), whole-cell bacteria, and proteins such as IsdB, siderophore receptors, porins, secreted proteins, and enterotoxin B (16). However, none of these antigens has been tested for induction of CD4 T cell responses in animals or humans (16). These approaches raise serious concerns regarding immune protection and safety since an IsdB-based vaccine (V710) was abruptly terminated during a recent phase II/III clinical trial in humans (61).

**TABLE 4** Titers of IgG1 and IgG2 to recombinant proteins in cattle immunized with *S. aureus*

Animal	<i>S. aureus</i> -specific IgG titer to the indicated antigen <sup>a</sup>					
	rNWMN_0364		rNWMN_0601		rNWMN_2086	
	IgG1	IgG2	IgG1	IgG2	IgG1	IgG2
30864	4,000	400	4,000	400	4,000	400
30900	4,000	400	4,000	400	4,000	<400 <sup>b</sup>
30904	4,000	400	4,000	400	4,000	<400
30916	4,000	400	4,000	400	4,000	<400
30919	4,000	400	4,000	400	4,000	<400

<sup>a</sup> Serum obtained 2 weeks after the last immunization were adsorbed with *E. coli* lysate, diluted 1:400 to 1:4,000, and tested for reactivity to recombinant protein (indicated by the “r” prefix) by immunoblotting. The titer is defined as the reciprocal of the highest serum dilution giving a positive signal.

<sup>b</sup> No specific bands were detected at a 1:400 dilution, and no other dilutions were tested.

Although extensive immunization trials in mice have revealed protective *S. aureus* antigens (3, 20–23, 36, 39, 41, 44, 57, 60, 67, 74, 75, 78, 79, 84, 89), these have not translated to a vaccine for cattle or humans. One promising approach is the use of a mutated SpA protein (KKAA) which has amino acid substitutions incorporated into each of the five Ig-binding domains of protein A. This protein A mutant can no longer bind to IgG and interfere with antibody function and does not induce B cell apoptosis (43). Immunization of mice with this protein elicited antibody that neutralized the native protein A molecule and provided some protection in mice against virulent MRSA (43). However, there are limitations with the mouse model. *S. aureus* is highly adapted to humans, with virulence factors more potent in humans than in mice (87, 90, 96, 98). Additionally, inbred mice are not optimal animal models for identifying antigens from *S. aureus* because of MHC differences. Cattle are evolutionarily more closely related to humans than are mice, are outbred, and have polymorphic MHC-II alleles similar to those of humans. Although the class II *DP*, *DO*, *DM*, *DQ*, and *DR* genes are generally conserved among mice, humans, and cattle, there are some important differences (45). In mice the class II genes have undergone contraction, and about half of the strains lack functional I-E (*DR* orthologue) class II molecules, whereas cattle and humans have the same number of functional *DR* and *DQ* genes, and both sets of molecules present antigen to CD4 T cells (27, 50, 88). Therefore, cattle with different haplotypes would be more likely than mice to identify a wide range of *S. aureus* antigens that would be relevant for vaccine design. Furthermore, it is very likely that antigens discovered here could be useful for vaccine development against human MRSA. Both *S. aureus*  $\gamma$ -hemolysin A and antigen A, identified by T cells in this study (Table 2), induced serum antibody responses in humans (11–13), and recognition of the same proteins by cattle and human T cells has been shown for other bacterial pathogens, such as mycobacterial ESAT-6 and Ag85 (93).

One significant omission from the mouse and human studies is the paucity of known *S. aureus* CD4 T cell antigens, making it difficult to design efficacious multivalent vaccines against human and animal infections. To address this problem, we used cattle as a large outbred animal model to screen *S. aureus* antigens, selected using reverse genetics, to identify those antigens that elicited recall CD4 T cell responses. The *S. aureus* Newman strain was chosen because it displays robust virulence properties in animal models

and has been extensively analyzed for its molecular traits of staphylococcal pathogenesis (4). Furthermore, this strain also shares high homology (>80% amino acid identity) within the 43 full-length antigen-encoding ORFs found in IMI and MRSA strains, which will increase the potential for stimulating cross-reactive immune responses for these strains. Notably, genes encoding staphylokinase (*sak*, NWMN\_1880) and staphylococcal complement inhibitor (*scin*, NWMN\_1876) were absent in *S. aureus* RF122 and COL strains. Analysis of 58 *S. aureus* genomes from 14 different clonal complex lineages revealed that 24 surface proteins involved in adhesion and 13 secreted proteins implicated in immune evasion are located on the stable core genome (28, 58). Eight of these 37 surface-exposed or secreted proteins that had ORFs of  $\leq 1.2$  kb (an optimal size) were included for expression by IVTT. These are  $\beta$ -hemolysin (NWMN\_1926), fibrinogen-binding protein (NWMN\_1069), staphylokinase (NWMN\_1880), exotoxin (NWMN\_0390), enterotoxin (NWMN\_0389), sortase (NWMN\_2426), antigen A (NWMN\_2469), and antigen B (NWMN\_2537) (28, 58). We also identified antigens through our analysis which included eight surface-exposed lipoproteins (NWMN\_0161, NWMN\_0364, NWMN\_0601, NWMN\_0753, NWMN\_1398, NWMN\_1609, NWMN\_1690, and NWMN\_2579), five proteins encoded by the serine protease operon *spIFDCBA* located in the pathogenicity island (NWMN\_1701, NWMN\_1703, NWMN\_1704, NWMN\_1705, and NWMN\_1706), global transcription regulator RNAlII activating protein (NWMN\_1726), and an alkaline shock protein (NWMN\_2086). The complete list of 43 antigens included in the initial screening to determine T cell immunogenicity is summarized in Table 1.

Over one-fourth of the candidate antigens were immunogenic for one or more cattle, and specificity was shown by lack of stimulation of T cells from control *A. marginale*-immune cattle that shared MHC class II alleles with the *S. aureus*-immune cattle. Furthermore, eight of the *S. aureus* antigens (NWMN\_0364, NWMN\_0601, NWMN\_1733, NWMN\_2086, NWMN\_2318, NWMN\_2319, NWMN\_2270, and NWMN\_2469) induced IFN- $\gamma$  secretion, the signature Th1 cytokine. Five antigens (NWMN\_0161, NWMN\_0390, NWMN\_0429, NWMN\_1999, and NWMN\_2320) that failed to induce T cell proliferation also failed to induce IFN- $\gamma$  in this assay. In cattle, IFN- $\gamma$  produced by antigen-activated T cells can correlate with T cell proliferation (8).

Five of the 13 T-cell antigens we identified are lipoproteins. The three best antigens were an alkaline shock protein (NWMN\_2086) and two lipoproteins (NWMN\_0364 and NWMN\_0601). Although these have not been previously described as immunogenic, their immunogenicity is not surprising since they are secreted and are involved in a variety of extracellular functions. The alkaline shock protein imparts pH tolerance during bacterial infection by maintaining an optimal pH (7.0 to 7.5) during the initial phase of staphylococcal colonization and probably functions as an accessory virulence factor during infection (47). Lipoproteins are an integral part of bacterial peripheral membrane and perform diverse functions, including adhesion, antibiotic resistance and prevention of phage superinfection, transportation, and secretion of enzymes and redox and sensory processes, and they serve as important virulence factors (40). Lipoproteins can also function as pathogen-associated molecular patterns, activating Toll-like receptor 2 (TLR2) signaling cascades, which can lead to increased pathology (10, 83). Putative lipoprotein, NWMN\_0601 (*mntC*) belongs to manganese transport reg-

ulator operon, *mntABCD* (71) and plays a critical role in maintaining Mn<sup>2+</sup> homeostasis during infection. Therefore, *mntC* may serve as a component of a new multivalent vaccine against *S. aureus* along with NWMN\_2086 and NWMN\_0364.

Previously identified antigens staphylokinase (NWMN\_1880) (12), immunodominant antigen A (NWMN\_2469) (11, 28), and transglycosylase *sceD* (NWMN\_1999) (28) were also immunogenic in our T cell assays, but staphylokinase failed to induce IFN- $\gamma$ . However, of the previously reported vaccine antigens that we tested, including fibrinogen-binding protein (NWMN\_1069),  $\beta$ -hemolysin (NWMN\_1926), staphylococcal complement inhibitor (NWMN\_1876), and sortase (NWMN\_2426), none was immunogenic in our T cell assays, which may explain their failure to afford protective immunity.

The predominant T cell population responding in proliferation assays is CD4 T lymphocytes. Targeting CD4 T cell antigens is important because CD4 T cells are required for T cell-dependent immunoglobulin isotype switching to produce high-affinity, opsonizing IgG antibodies and for IFN- $\gamma$ -mediated phagocyte activation (7, 56, 95), effectors important for protective immunity against intracellular bacterial pathogens (5, 30, 38, 62, 70). In mice it was shown that a Th1 response is also important for inducing optimal IgG for opsonization and complement-mediated lysis against *S. aureus* (30). Thus, in the context of appropriate adjuvants, antigens that stimulate IFN- $\gamma$  and IgG production would be good choices for vaccine development. The three best T cell antigens were also recognized by IgG1 from all five immune animals, and two of the three antigens were recognized by IgG2 although IgG1 titers were 10 times higher than IgG2. The consequence of this response regarding protection against *S. aureus* in cattle immunized with killed bacteria is unknown. However, higher titers of IgG1 and IgG2 could be induced using recombinant proteins administered with adjuvant such as CpG oligodeoxynucleotide or RIBI adjuvant (a mixture of monophosphoryl lipid A, trehalose dimycolate, and cell wall skeleton), which have been shown to stimulate these isotypes in cattle (68, 97). Nevertheless, both IgG1 and IgG2 have been shown to be opsonins and to activate macrophage-mediated phagocytosis in humans and cattle (6, 59).

In summary, we identified 13 *S. aureus* antigens that induce CD4 T cell responses in immunized cattle. Eight antigens, including the top three antigens that elicit IgG and IFN- $\gamma$  responses, are good candidates for testing in vaccination-challenge trials. These antigens may prove useful as broadly cross-protective components of multivalent vaccine against IMI in cattle and MRSA in humans.

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