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Host Response Signature to *Staphylococcus aureus* Alpha-Hemolysin Implicates Pulmonary Th17 Response

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***Staphylococcus aureus* pneumonia causes significant morbidity and mortality. Alpha-hemolysin (Hla), a pore-forming cytotoxin of *S. aureus*, has been identified through animal models of pneumonia as a critical virulence factor that induces lung injury. In spite of considerable molecular knowledge of how this cytotoxin injures the host, the precise host response to Hla in the context of infection remains poorly understood. We employed whole-genome expression profiling of infected lungs to define the host response to wild-type *S. aureus* compared with the response to an Hla-deficient isogenic mutant in experimental pneumonia. These data provide a complete expression profile at 4 and at 24 h postinfection, revealing a unique response to the toxin-expressing strain. Gene ontogeny analysis revealed significant differences in the extracellular matrix and cardiomyopathy pathways, both of which govern cellular interactions in the tissue microenvironment. Evaluation of individual transcript responses to Hla-secreting staphylococci was notable for upregulation of host cytokine and chemokine genes, including the p19 subunit of interleukin-23. Consistent with this observation, the cellular immune response to infection was characterized by a prominent Th17 response to the wild-type pathogen. These findings define specific host mRNA responses to Hla-producing *S. aureus*, coupling the pulmonary Th17 response to the secretion of this cytotoxin. Expression profiling to define the host response to a single virulence factor proved to be a valuable tool in identifying pathways for further investigation in *S. aureus* pneumonia. This approach may be broadly applicable to the study of bacterial toxins, defining host pathways that can be targeted to mitigate toxin-induced disease.**

Staphylococcus aureus is a Gram-positive human pathogen that causes a considerable burden of disease characterized by a spectrum of illness ranging from mild skin infections to life-threatening infections such as sepsis, pneumonia, endocarditis, and osteomyelitis (47). Pneumonia due to *S. aureus* is one of the most common invasive forms of disease, carrying an associated mortality rate up to 50% (1, 24, 34, 39). The combination of high incidence, high pathogenicity, and frequent resistance to multiple antibiotics results in *S. aureus* being one of the most significant human pathogens.

S. aureus harbors a number of virulence factors, including several cytolytic, pore-forming toxins (19, 21). Among the best studied of these toxins is alpha-hemolysin (Hla), a secreted toxin that assembles into a membrane-perforating homoheptamer upon binding to its eukaryotic cellular receptor, ADAM10 (5, 36, 67). Hla induces pulmonary hypertension and inflammation in several animal models (52, 66) and has been demonstrated to be essential in the pathogenesis of lethal pneumonia in a murine model, causing widespread alveolar injury and concomitant epithelial barrier disruption (9, 10). Several studies shed light on the mechanism by which Hla induces inflammation and lung damage. Hla induces platelet-activating factor production in endothelial cells (25, 69, 70) and release of nitric oxide and inflammatory mediators from pulmonary epithelium-derived cells (64). The toxin also promotes cytokine release from macrophages (56) and induces cell death and cytokine release from monocytes (4). Cell death is triggered through the NLRP3-dependent program of cellular necrosis, resulting in the release of endogenous proinflammatory molecules (15); indeed, NLRP3$^{-/-}$ mice display reduced interleukin-1β (IL-1β) production in response to Hla and demonstrate more mild clinicopathologic features of *S. aureus* pneumonia (37). Hla has been shown to injure alveolar epithelial cells by upregulation of the enzymatic activity of the toxin receptor ADAM10, leading to E cadherin cleavage and disruption of the intercellular adherens junction that is essential for intact epithelial barrier function (30, 75). Conversely, innate host protection from the effects of Hla is accomplished through activation of type I interferon signaling, recently shown to be mediated by phospholipid scramblase 1 (PLSCR1)-induced mitigation of cellular ATP loss (46). Together, these studies indicate that Hla targets critical host defenses provided by both innate immune cells and the tissue barrier and highlight the complexity of the host response to the toxin.

Neutralization of key virulence factors is one approach to find effective preventative and therapeutic agents to combat staphylococcal infection; indeed, immunization and small-molecule strategies targeting Hla afford a high degree of protection against lethal pneumonia (11, 61, 62). Similarly, antivirulence strategies associated with reduced expression of Hla protect against disease (59, 60, 73). In the setting of bacterial toxin-induced disease, a viable

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alternative approach may be modulation of the host response to minimize the deleterious effects of intoxication. This strategy circumvents the challenges associated with pathogen-acquired resistance, especially to small-molecule-based therapies. We hypothesized that whole-genome transcriptional analysis of lung tissue infected with wild-type (WT) or Hla-negative (Hla$^-$) *S. aureus* would allow us to pinpoint host signature responses during the course of infection that were specifically linked to the presence of the toxin. Such studies were anticipated to enhance our understanding of the host-pathogen interaction in *S. aureus* pneumonia and suggest avenues for disease modification.

MATERIALS AND METHODS

Bacterial strains. *Staphylococcus aureus* Newman WT and its isogenic toxin-deficient mutant, designated Hla$^-$, have been previously described (9, 10).

Mouse model of lung infection. Seven-week-old female C57BL/6J mice (Jackson Laboratories, Wilmington, MA) were inoculated via the intranasal route with either phosphate-buffered saline (PBS) or the *S. aureus* Newman or Hla$^-$ strain as described previously (9, 10). Following a 1:100 dilution of an overnight culture into fresh tryptic soy broth, the bacteria were grown with shaking at 37°C to an optical density of 0.5 at 660 nm. Culture aliquots (50 ml) were sedimented by centrifugation, and the bacteria were washed and resuspended in 1.0 to 1.5 ml of PBS. Animals utilized for microarray and transcription-based analyses received inocula ranging from 3×10^8 to 5×10^8 CFU, associated with clinical signs of severe lung disease. Animals utilized for T cell subset analyses received a sublethal inoculum of 1×10^8 CFU for initial infection, followed in 8 days by a second sublethal infection of 0.8×10^8 to 2×10^8 CFU and tissue harvesting at 24 h. Animals were anesthetized with ketamine and xylazine as previously described (49). After appropriate anesthesia was documented, 30 μl of the bacterial slurry was inoculated into the left nare and the animals were held upright for 1 min postinoculation. All animals were housed under standard conditions and provided food and water *ad libitum*. At time points of 4, 8, 12, or 24 h, animals underwent bronchoalveolar lavage fluid collection and were then sacrificed for removal of the lung tissue. All animal studies were performed in concordance with principles set forth by the Animal Welfare Act and the National Institutes of Health guidelines for the care and use of animals in biomedical research and were reviewed and approved by the University of Chicago Institutional Animal Care and Use Committee.

RNA isolation. Lung tissue was snap-frozen in liquid nitrogen and stored at −80°C. At the time of processing, total mouse lung RNA was isolated by directly solubilizing lung tissue in TRIzol LS reagent (Life Technologies) using a Brinkman Polytron tissue disrupter. RNA was then purified using an RNeasy purification kit according to the manufacturer's protocol (Qiagen). Approximately 10 μg of purified, total RNA was used for analyses.

Microarray data analysis. The microarray data analysis was performed using an Affymetrix GeneChip platform and *Expression Analysis Manual* protocols (Affymetrix Inc., Santa Clara, CA). Three mice were included for each condition. The signal intensity fluorescent images produced during Affymetrix GeneChip Mouse 430_2 array hybridizations were scanned using a Hewlett-Packard GeneArray G2500A scanner. We evaluated chip quality using GeneChip operating software (GCOS), dChip (43), and the affy package (6) in Bioconductor software. All RNA samples and chips used in this study passed established quality criteria (data not shown). The expression level of each probe set was summarized by the gcrma package in Bioconductor with GC robust multichip average (GCRMA) normalization (76). To identify differentially expressed genes, pairwise comparisons were conducted using the samr package (71) in Bioconductor. Only probe sets present (determined by the mas5calls function in the affy package in Bioconductor) in three replicates of at least one group were retained. The heat map, gene ontology biological process terms, and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were generated using criteria of a false discovery rate (FDR) of <1% and a minimum of a 3-fold change between examined groups. The summary of the number of genes differentially regulated and tables of specific genes differing between *S. aureus* WT and Hla$^-$ infections, including the genes identified in the extracellular matrix (ECM) and cardiomyopathy pathways, were prepared using an FDR of <5% and a minimum of a 2-fold change. For probe sets with the same Entrez Gene or UniGene identifier, only the probe set with the lowest FDR or the highest fold changes in the gene list were included. We searched for any enriched KEGG (35) physiological pathways among the differential genes using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) (18, 29). A corrected *P* value of <0.05 (Fisher's exact test) after the Benjamini-Hochberg procedure (3) was used as the cutoff.

qRT-PCR analysis. Quantative reverse transcription-PCR (qRT-PCR) was performed with the two-step reaction protocol using SsoFast EvaGreen supermix (Bio-Rad Laboratories, Hercules, CA). First-strand cDNAs were synthesized using an iScript Select cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA) at 42°C for 70 min, followed by 85°C for 5 min and a hold at 4°C. Actin was used as the endogenous control. Primer sets used for the quantitative PCR analysis included IL1r2 (interleukin 1 receptor type II) forward (For; 5′-GTTTCTGCTTTCACCACTCCA-3′) and reverse (Rev; 5′-GAGTCCAATTTACTCCAGGTCAG-3′), Ccl11 (chemokine [C-C motif] ligand 11) For (5′-GAATCACCAACAACAGA TGCAC-3′) and Rev (5′-ATCCTGGACCCACTTCTTCTT-3′), Sdc4 (syndecan 4) For (5′-GTCCCCGGAGAGTCGATTC-3′) and Rev (5′-GC ACCAAGGGCTCAATCACTT-3′), Figf (c-fos-induced growth factor) For (5′-TCACGCTCAGCATCCCATC-3′) and Rev (5′-ACTTCTACGC ATGTCTCTCTAGG-3′), Col5a2 (procollagen type V alpha 2) For (5′-A CAGGTGAAGTGGGATTCTCA-3′) and Rev (5′-CCATAGCACCCATT GGACCA-3′), Tgfb3 (transforming growth factor beta 3) For (5′-CAGG CCAGGGCAGTCAGAG-3′) and Rev (5′-ATTTCCAGCCTAGATCCTG CC-3′), IL-17A (interleukin-17A) For (5′-CTCCAGAAGGCCCTCAGA CTAC-3′) and Rev (5′-AGCTTTCCCTCCGCATTGACACAG-3′), and Actin For (5′-TGGCATTGTTACCAACTGGGACG-3′) and Rev (5′-GC TTCTCTTTGATGTCACGCACG-3′). All qRT-PCR assays were carried out using primer sets from Integrated DNA Technologies. qRT-PCR parameters used were as follows: 95°C for 30 s and then 95°C for 5 s and 55°C for 5 s for 40 cycles. A melting curve was constructed for verification of the specificity of PCR products by increasing the temperature from 60 to 95°C in 0.5°C-step increments. PCR was performed with SsoFast EvaGreen supermix, using a CFX96 optical reaction module and C1000 thermal cycler (Bio-Rad Laboratories, Hercules, CA). The relative fold change in gene expression was calculated following actin normalization using the comparative threshold cycle (C_T) method as described previously (45, 65). The mean value for the control mice was used to calculate the fold change for each experimental mouse.

Lung processing for flow cytometry. Mice were anesthetized and lungs were perfused with 6 ml PBS with 50 units/ml heparin injected through the right ventricle. Then, the lung was surgically excised and minced with sterile glass coverslips. The pieces were incubated in a shaker at 37°C for 30 min in complete Dulbecco modified Eagle medium (DMEM; Cellgro, Manassas, VA), 5% fetal bovine serum, 0.05 mM β-mercaptoethanol, 10 mM HEPES, 100 U/ml penicillin, and 100 μg/ml streptomycin with 146 U/ml collagenase I (Invitrogen, Carlsbad, CA). Samples were pipetted up and down 50 times to break up larger tissue sections, any remaining pieces of tissue were removed by straining the solution through a Nytex filter, and the cells in suspension were centrifuged and resuspended in complete DMEM. Cells were then resuspended in 44% Percoll (Sigma, St. Louis, MO) and spun in 44% and 66% Percoll gradients. Cells were collected at the interface of the two solutions. Live cell count was determined via trypan blue exclusion, and then the cells were divided for antibody staining and *in vitro* stimulation studies. For stimulation, cells were incubated overnight at 37°C in complete DMEM with IL-2 (20 U/ml) and then stimulated with phorbol myristate acetate

TABLE 1 Comparison of gene expression for infection with WT *S. aureus*, Hla$^-$ isogenic mutant strain, or PBS control

Time (h)	Treatment		Gene expression[a]		
	Group 1	Group 2	Total	Upregulated	Downregulated
4	WT	PBS	2,459	1,430	1,029
4	Hla$^-$	PBS	2,738	1,465	1,274
4	WT	Hla$^-$	0	0	0
24	WT	PBS	7,306	2,878	4,428
24	Hla$^-$	PBS	2,704	1,241	1,463
24	WT	Hla$^-$	1,281	540	741

[a] FDR $<$ 5%; fold change $\geq$ 2; total, upregulated or downregulated for group 1.

(PMA; 20 ng/ml) plus ionomycin (400 ng/ml) for 6 h at 37°C, with brefeldin A (25 μg/ml) added for the final 2 h.

Flow cytometry. Prior to staining with antibodies, cells were washed and nonspecific antibody binding was blocked with anti-FcγR (2.4G2) antibodies. Surface staining antibodies included anti-CD3 allophycocyanin (APC) (clone 145-2c11; eBioscience, San Diego, CA) and anti-GR1 fluorescein isothiocyanate (clone 1A8; BD Biosciences Pharmingen, San Diego, CA). For intracellular staining, cells were first surface stained for 30 to 60 min and then fixed with 4% paraformaldehyde for 15 min and washed in permeabilization buffer (0.25% saponin in fluorescence-activated cell sorter buffer [0.2% bovine serum albumin and 0.012% sodium azide in PBS]), followed by intracellular staining for 60 min at 4°C. Intracellular antibodies were anti-IL-17A phycoerythrin (PE) antibody (clone eBio17B7) or isotype control antibody rat IgG2a PE (eBioscience, San Diego, CA). The samples were then washed and flow cytometric analysis was performed with an LSRII apparatus (BD Biosciences, Mountain View, CA). Data were analyzed with FlowJo software (Tree Star, Ashland, OR).

Data analysis and statistics. Data analysis and compilation were performed using Prism (version 5.0) software (GraphPad Software, San Diego, CA). Statistical analysis was performed using Student's *t* test, where a *P* value of $<$0.05 indicates a significant result.

Microarry data accession number. The microarray data obtained in this study have been deposited in the NCBI Gene Expression Omnibus (GEO) site (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE38053.

RESULTS

***S. aureus* Hla induces unique features of the host response to lung infection.** To investigate the host response to Hla, we utilized an established mouse model of pneumonia in which 7-week-old C57BL/6J mice were infected intranasally with *S. aureus* Newman (9). In this model, lung injury is induced within the first 24 h postinfection, manifest as alveolar destruction, infiltration of large numbers of immune cells, especially neutrophils, and proliferation of *S. aureus* within the lung tissue. Mice infected with an Hla$^-$ variant of WT *S. aureus* demonstrate less severe lung inflammation, recovering from infection (9, 10). To first define the host transcriptional response to *S. aureus* pneumonia, we examined the gene expression profile in infected lung tissue using RNA prepared following infection with WT *S. aureus* and compared it to that in lung tissue following control treatment with PBS. Using the Affymetrix platform (Mouse Genome 430 [version 2.0] array) and a false discovery rate of $<$5% and requiring a minimum of a 2-fold change in gene expression, we identified 2,459 genes differentially expressed between WT-infected animals and the PBS controls (Table 1, top). A more pronounced effect was noted at the 24-h time point postinfection, wherein 7,306 genes were differentially expressed between WT-infected animals and control mice (Table 1, bottom). A total of 2,878 transcripts were upregulated, while 4,428 were downregulated at 24 h. Additional controls for these studies are shown in Table S1 in the supplemental material. Both WT *S. aureus* and the toxin-deficient strain induced a brisk host response to infection; at 4 h postinfection, the number of host genes with altered regulation was quantitatively similar (Table 1, top).

To understand the host cellular pathways impacted by *S. aureus* infection, gene ontology analysis was performed by comparison of the genes differentially expressed between WT- and Hla$^-$-infected mice and PBS control-treated mice (FDR $<$ 1% and fold change $>$ 3). Prominent biological processes altered by infection with either strain included the immune response, defense response, inflammatory response, response to wounding, and chemotaxis (see Fig. S1 in the supplemental material), ontologies very consistent with those described in published studies of infectious lung damage in rodent models (42, 53, 58). Consistent with this finding, no specific host pathways were identified within 4 h of infection to be uniquely affected by the expression of Hla on the basis of gene ontogeny analysis (see Fig. S2 in the supplemental material).

Microarray analysis demonstrated 2,704 differentially regulated genes at 24 h postinfection by comparison of mice infected with the *S. aureus* Hla$^-$ strain and PBS control-treated mice (1,241 upregulated and 1,463 downregulated genes; Table 1, bottom), a number smaller than the 7,306 differentially regulated genes seen for WT. In contrast to the analysis at 4 h, a comparison of the host response to infection with WT *S. aureus* and the Hla$^-$ strain at 24 h revealed a large number of differentially regulated genes (Table 1, bottom), with the WT strain inducing upregulation of 540 genes and downregulation of 741 genes compared to the gene induction by the Hla$^-$ strain. Both quantitative and qualitative differences in gene regulation induced by these infecting strains are appreciated by heat map analysis (Fig. 1). Gene ontogeny analysis revealed three pathways that differ significantly between WT infection and Hla$^-$ infection at 24 h: dilated cardiomyopathy, ECM receptor interaction, and hypertrophic cardiomyopathy (Fig. 2). The ECM is made of macromolecules such as collagens and elastin, as well as proteoglycans such as hyaluronan, providing a scaffold that supports cells and tissue structure. The pathway is also very important for the response to injury, modulating activities such as cell proliferation, differentiation, migration, and adhesion (8, 33). As seen in Table S2 in the supplemental material, collagens, integrins, and syndecans are among the genes differentially regulated in the presence of the toxin.

A more detailed and comprehensive analysis of specific genes from many pathways, not only the three mentioned above, that exhibit differential regulation in the presence of Hla was performed, revealing genes that were upregulated (Table 2) or downregulated (Table 3) by greater than 6-fold in WT infection compared to Hla$^-$ infection. qRT-PCR analysis for a subset of genes was performed on lung RNA prepared from mice infected with either WT or Hla$^-$ *S. aureus*, validating the findings of the microarray (see Fig. S3 in the supplemental material).

Patterning of T cell response to *S. aureus* pneumonia by Hla. To appreciate the biological significance of microarray-based findings of differential host responses to Hla-expressing *S. aureus*, we chose to further examine the upregulated expression of IL-23 p19 observed in WT infection compared to Hla$^-$ infection (6.7-fold increase; Table 2). Production of IL-23 in the context of in-

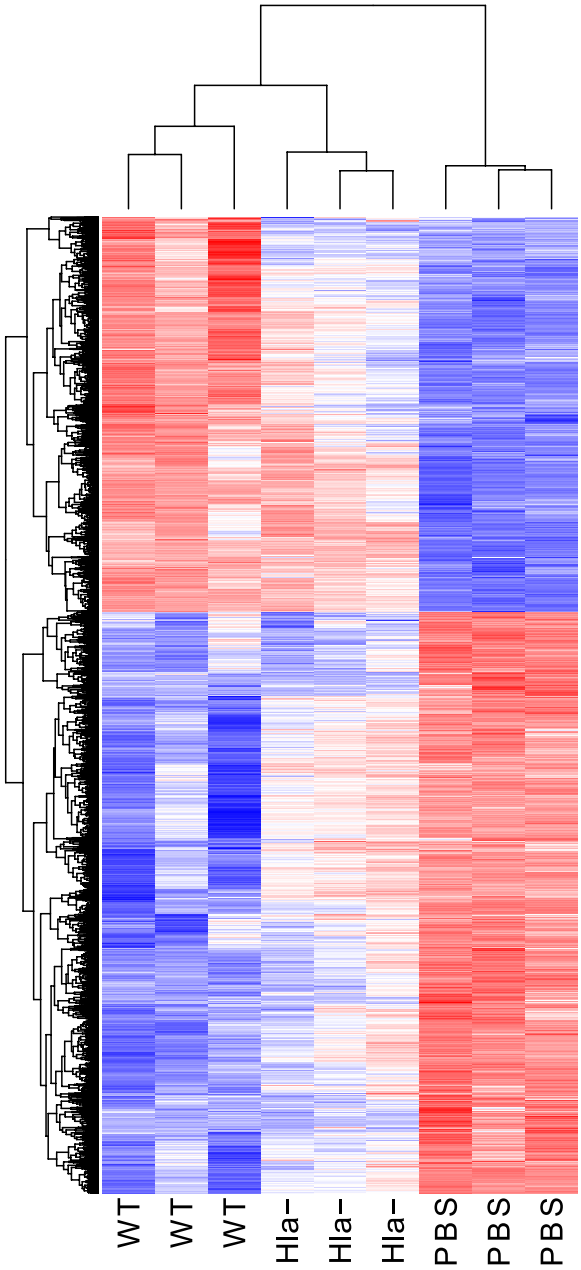

FIG 1 Heat map of gene expression levels for differentially regulated genes in the mouse lung obtained by pairwise comparison between PBS control and WT infection at 24 h postinoculation. Criteria for analysis: FDR of <1% and a minimum of a 3-fold change. Red represents increased gene expression; blue represents downregulation.

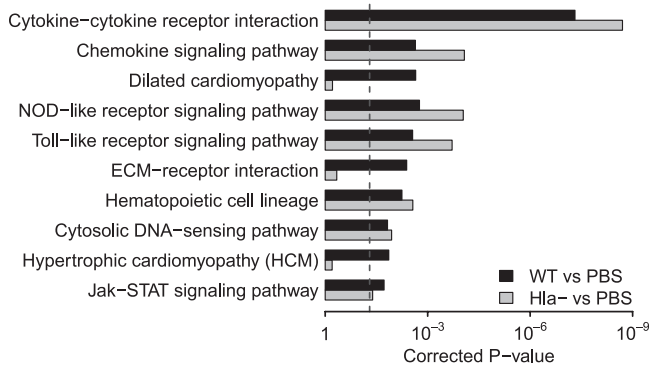

FIG 2 Top 10 significant KEGG pathways enriched by the differentially regulated genes in the mouse lung in response to WT *S. aureus* infection at 24 h postinoculation compared to PBS control. The corresponding results for the comparison between Hla$^-$ infection and PBS control are also listed. The differentially expressed genes with an FDR of <1% and a minimum of a 3-fold change were included in this analysis. The vertical dashed line indicates the cutoff of significance ($P < 0.05$). *P* values were corrected by the Benjamini-Hochberg procedure (3).

fection and autoimmunity has been linked to the differentiation of IL-17-producing CD4$^+$ T helper cells, or a Th17 response (7). The secretion of IL-17 in the context of infection promotes the mobilization of neutrophils, augments cytokine production by epithelial cells, and maintains the integrity of the tight junction. Together, these responses enhance protective immunity to extracellular pathogens (16, 38, 51, 74). IL-17 is produced both by Th17 cells and by innate immune cells as part of the immediate host defense response to bacteria. These responses are of particular relevance at epithelial surfaces, key sites of human infection with *S. aureus*. Quantitative changes in the IL-23 p19 transcript were of considerable interest to us, given our prior studies of vaccine-mediated immunity to Hla and our ongoing efforts to define novel strategies by which to protect the host against toxin-mediated injury (11, 30). Th17 responses have been demonstrated to be important in the context of *S. aureus* skin infection (14, 55) and influenza virus coinfection (40). Th17-dominant responses have been suggested to play an important role in adaptive immunity to *S. aureus* (44) and have recently been highlighted to be a potential means by which to elicit vaccine-mediated immunity (68).

qRT-PCR for IL-23 p19 was performed on RNA harvested from lungs of mice infected with WT or Hla$^-$ *S. aureus* (or treated with PBS control) at four different time points. IL-23 p19 was upregulated more than 60-fold following infection with both WT and Hla$^-$ strains as early as 4 h after inoculation (Fig. 3). At 8, 12, and 24 h, however, WT infection was characterized by amplification and persistence of the IL-23 p19 transcriptional response greater than those obtained with Hla$^-$ infection (Fig. 3). Commensurate with these findings, the IL-17A transcriptional response was also greater in WT-infected mice than Hla$^-$-infected mice at 24 h (see Fig. S4 in the supplemental material). These data indicate that the expression of IL-23 p19 is associated in part with the presence of Hla and suggest that the toxin may shape the nature of the host T cell response.

To examine the adaptive T cell immune response to *S. aureus* pneumonia, mice were infected with a sublethal dose of WT or Hla$^-$ *S. aureus* and allowed to recover. Eight days later, mice were reinfected with the same staphylococcal strain. The mice were then sacrificed at 24 h postinfection and lungs were harvested for cellular analysis. A fraction of the recovered cells was subjected to flow cytometric analysis of CD3 and GR1 expression to quantify T cell and neutrophil infiltration into the lung, respectively. Mice infected with WT *S. aureus* demonstrated an increased overall cell count in the lung compared to that for mice infected with the Hla$^-$ strain, consistent with our prior observations (Fig. 4A) (30). The absolute number of T cells and neutrophils was significantly higher in the WT infection than the Hla$^-$ infection (Fig. 4B and C). Given the initial finding that the IL-23 p19 transcript was

TABLE 2 Genes upregulated during WT *S. aureus* infection compared to expression during Hla$^-$ infection at 24 h[a]

Symbol	Gene name	Fold change
Ccl20	Chemokine (C-C motif) ligand 20	56
Cyp27b1	Cytochrome P450, family 27, subfamily b, polypeptide 1	25
Ccl11	Chemokine (C-C motif) ligand 11	23
Tmem45b	Transmembrane protein 45b	23
Aldh1a3	Aldehyde dehydrogenase family 1, subfamily A3	22
Cldn4	Claudin 4	16
Slc13a3	Solute carrier family 13 (sodium-dependent dicarboxylate transporter), member 3	14
Has1	Hyaluronan synthase 1	11
Ngp	Neutrophilic granule protein	11
Pitpnm2	Phosphatidylinositol transfer protein, membrane associated 2	11
Ceacam10	Carcinoembryonic antigen-related cell adhesion molecule 10	10
Csf3	Colony-stimulating factor 3 (granulocyte)	10
Il18rap	Interleukin-18 receptor accessory protein	9.8
Kcnk5	Potassium channel, subfamily K, member 5	9.5
Fosl1	Fos-like antigen 1	9.3
Mctp2	Multiple C_2 domains, transmembrane 2	9.0
Nipal1	NIPA-like domain containing 1	8.9
Sprr1a	Small proline-rich protein 1A	8.8
Ereg	Epiregulin	8.5
Il6	Interleukin-6	8.4
Dbn1	Drebrin 1	7.9
Lif	Leukemia inhibitory factor	7.8
Hamp	Hepcidin antimicrobial peptide	7.7
Cxcl1	Chemokine (C-X-C motif) ligand 1	7.5
Pcp4	Purkinje cell protein 4	7.2
Selp	Selectin, platelet	7.1
Zfp783	Zinc finger protein 783	6.9
Ptx3	Pentraxin-related gene	6.9
Plekha7	Pleckstrin homology domain containing, family A, member 7	6.8
Ffar2	Free fatty acid receptor 2	6.8
Cd80	CD80 antigen	6.7
Mbp	Myelin basic protein	6.7
Il23a	Interleukin-23, alpha subunit p19	6.7
Artn	Artemin	6.5
Cldn1	Claudin 1	6.3

[a] FDR < 5%.

TABLE 3 Genes downregulated during WT *S. aureus* infection compared to expression during Hla$^-$ infection at 24 h[a]

Symbol	Gene name	Fold change
Adh1	Alcohol dehydrogenase 1 (class I)	0.040
Aspn	Asporin	0.055
Ptprd	Protein tyrosine phosphatase, receptor type D	0.058
Lrrn4	Leucine rich repeat neuronal 4	0.066
Figf	c-Fos-induced growth factor	0.068
Lphn3	Latrophilin 3	0.074
Col8a1	Collagen, type viii, alpha 1	0.076
Hhip	Hedgehog-interacting protein	0.092
Prkg2	Protein kinase, cyclic GMP dependent, type II	0.10
Aard	Alanine- and arginine-rich domain-containing protein	0.10
Timp4	Tissue inhibitor of metalloproteinase 4	0.11
Kcnj13	Potassium inwardly rectifying channel, subfamily J, member 13	0.11
Mfap5	Microfibrillum-associated protein 5	0.11
Fzd2	Frizzled homolog 2 (*Drosophila*)	0.11
Prrx1	Paired related homeobox 1	0.11
Gria3	Glutamate receptor, ionotropic, AMPA3 (alpha 3)	0.11
Stmn2	Stathmin-like 2	0.11
Angpt1	Angiopoietin 1	0.11
Sema3a	Sema domain, immunoglobulin domain (Ig), short basic domain, secreted (semaphorin) 3A	0.12
Nox4	NADPH oxidase 4	0.12
Speg	SPEG complex locus	0.12
Sfrp1	Secreted frizzled-related protein 1	0.12
Agr2	Anterior gradient 2 (*Xenopus laevis*)	0.12
Cckar	Cholecystokinin A receptor	0.12
Wisp2	WNT1-inducible signaling pathway protein 2	0.12
Synm	Synemin, intermediate filament protein	0.13
Fam38b	Family with sequence similarity 38, member B	0.13
Osbpl6	Oxysterol binding protein-like 6	0.13
Gas1	Growth arrest specific 1	0.14
Pcdh12	Protocadherin 12	0.14
Vgll3	Vestigial like 3 (*Drosophila*)	0.14
Gulp1	GULP, engulfment adaptor PTB domain containing 1	0.14
Tppp	Tubulin polymerization-promoting protein	0.14
Rspo1	R-spondin homolog (*Xenopus laevis*)	0.14
Decr1	1,2-Dienoyl coenzyme A reductase 1, mitochondrial	0.15

[a] FDR < 5%.

markedly increased in mice infected with WT *S. aureus* compared to those infected with the Hla$^-$ strain, we examined the proportion of IL-17-positive (IL-17$^+$) T cells as a subset of the CD3$^+$ population. To facilitate these studies, harvested cells were plated overnight in the presence of IL-2, stimulated with PMA and ionomycin, and then analyzed for IL-17 expression. The absolute number of IL-17$^+$ cells was higher in WT-infected mice (Fig. 5A to D), consistent with an overall increase in cell recovery. More importantly, the percentage of CD3$^+$/IL-17$^+$ cells was significantly higher in mice infected with WT *S. aureus* (Fig. 5E to H), suggesting that Hla expression in the context of staphylococcal pneumonia induces a qualitatively distinct T cell response, enhancing the generation of the Th17 population. Specificity of staining was confirmed by staining of lung cells from WT-infected mice with the isotype control antibodies (data not shown). Collectively, these data demonstrate that Hla is a strong inducer of Th17 cells during staphylococcal pneumonia.

DISCUSSION

We have used a whole-transcriptome approach as a tool to discern the impact of a single bacterial virulence factor on the host response to infection. *S. aureus* is unique among bacterial pathogens, given the multitude of organ systems and tissues that can be targeted during infection and the pleiotropic manifestations of disease apparent in different individuals. The wide array of virulence factors elaborated by *S. aureus* clearly patterns these diverse disease states. The pathological features of *S. aureus* pneumonia observed in animal model systems are intimately linked with the expression of Hla, providing a window of opportunity to dissect the host response to toxin exposure.

We observed that the general host response to both wild-type and toxin-deficient *S. aureus* is characterized by alterations in genes that regulate the biological processes of inflammation, defense, the immune response, wounding, and chemotaxis, extending earlier findings (2, 58). Specific alterations observed following expression with the Hla-expressing strain, however, serve to highlight the unique role of the toxin in host injury. Gene ontogeny

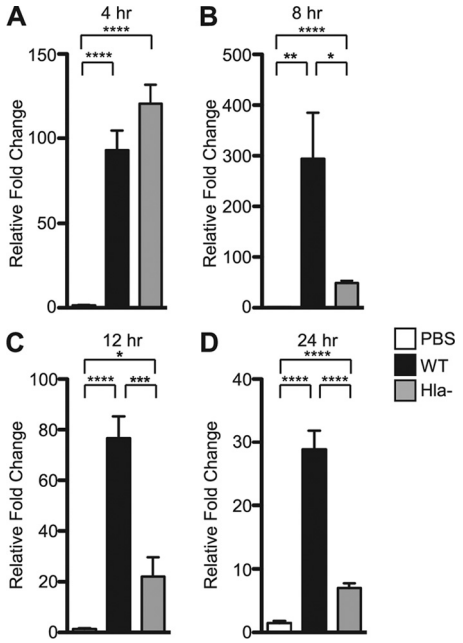

FIG 3 Sustained IL-23 p19 gene expression in *S. aureus* pneumonia depends on bacterial expression of Hla. qRT-PCR analysis of IL-23 p19 gene expression in the mouse lung in response to infection with WT or Hla$^-$ *S. aureus* or PBS control at 4 h (A), 8 h (B), 12 h (C), and 24 h (D) postinoculation. Bars represent average ± SEM. *P* values are indicated: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

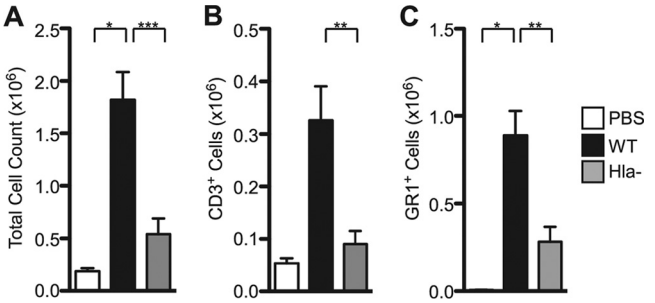

FIG 4 Infection with WT *S. aureus* is associated with increased immune cell recovery from lung tissue. Histogram plots of total cell count (A), CD3$^+$ T cells (B), and GR1-positive (GR1$^+$) neutrophils (C) recovered from the lungs of mice infected with WT *S. aureus*, Hla$^-$ *S. aureus*, or PBS control. Bars represent average ± SEM. Results are derived from 4 PBS-treated mice, 21 WT-infected mice, and 14 Hla$^-$-infected mice. *P* values are indicated: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

analysis revealed that the extracellular matrix and cardiomyopathy pathways have significantly different regulation in the presence of Hla. These findings are consistent with several prior observations, namely, that staphylococcal isolates involved in necrotizing pneumonia display increased adhesion to extracellular matrix molecules (17) and that the Hla$^-$–ADAM10 complex is associated with disruption of focal adhesions and intercellular adherens junction formation (30, 75). Future studies of the roles of differentially regulated genes in the ECM and cardiomyopathy pathways will likely lead to a more comprehensive understanding of the mechanisms of toxin-induced lung damage, perhaps also affording insight into the nascent mechanisms that underlie the host reparative response to injury.

The relevance of IL-17 to pulmonary infection has previously been established for Gram-negative bacteria (27, 28, 41, 77), and we have now examined IL-17 in the context of pneumonia due to a Gram-positive organism. Our focus on the Th17 response as a specific biological response pathway to intoxication was predicated on knowledge of the increased susceptibility of humans and mice lacking in this response to infection with *S. aureus* (48, 63), the association of Th17 cells with the host response to *S. aureus* (14, 23, 26, 31, 40), and the more recent findings from Niebuhr and colleagues that *S. aureus* Hla induced IL-17A in peripheral blood mononuclear cells and Th17 cells in the setting of atopic dermatitis (55).

Host recognition of staphylococcal cell wall components and bacterial lipopeptides leads to activation of Toll-like receptor (TLR) and Nod-like receptor (NLR) signaling on antigen-presenting cells such as monocytes and dendritic cells (12, 22, 23, 54, 72). These innate cellular responses pattern cytokine and chemokine production, immune cell recruitment, and the induction of the adaptive immune response. Collectively, these host responses clearly establish the balance between favorable immunologic clearance of the pathogen and deleterious immunopathology that contributes to host death in the setting of lung infection (2, 11, 15, 37, 50, 57, 72). Relevant to the current study, these early signaling events result in the production of IL-23, templating induction of the Th17 response (13, 20, 32, 51, 72).

The link between expression of Hla and the Th17 response observed in our studies may be accounted for by one of several molecular mechanisms. First, we have demonstrated previously that *S. aureus* recovery from the lungs of infected mice is reduced when the cellular action of Hla is antagonized during infection (11, 61, 62). In this context, the tissue bacterial load may emerge as a critical regulator of the host response. Infection with an Hla$^-$ *S. aureus* strain would be predicted to provide a lesser degree of innate immune stimulation by peptidoglycan and lipopeptides, dampening the magnitude or duration of the IL-23/IL-17 response. Indeed, we observed that the induction of IL-23 by WT and Hla$^-$ *S. aureus* was indiscernible at 4 h postinfection and then rapidly waned in response to the Hla$^-$ strain. Interestingly, a considerable number of staphylococci ($\geq 5 \times 10^6$/lung) are still recovered at 24 h postinfection in the setting of Hla antagonism (11, 61, 62), suggesting that the nature of the host response may be determined within a relatively narrow window predicated on both the absolute burden of *S. aureus* in the tissue and the length of time over which the host is exposed.

As a second plausible mechanism, the direct actions of Hla on antigen-presenting cells may shape the host adaptive response. This may be accomplished by (i) stimulation of the NLRP3 inflammasome by the toxin (15, 37), (ii) enhanced inflammatory cytokine and/or chemokine production in response to lung epithelial injury caused by Hla (2, 11), or (iii) toxin-mediated cytolytic injury to innate antigen-presenting cells (4, 56). Deciphering between these possibilities will likely shed additional light on both the nature of the antistaphylococcal response and the role of Hla in patterning this response.

We have now extended previous studies by connecting the presence of a secreted cytotoxin to the pulmonary Th17 response in staphylococcal pneumonia. This emphasizes the importance of Hla in disease pathogenesis, most specifically, in contemplating the nature of the host immunologic response. Through these stud-

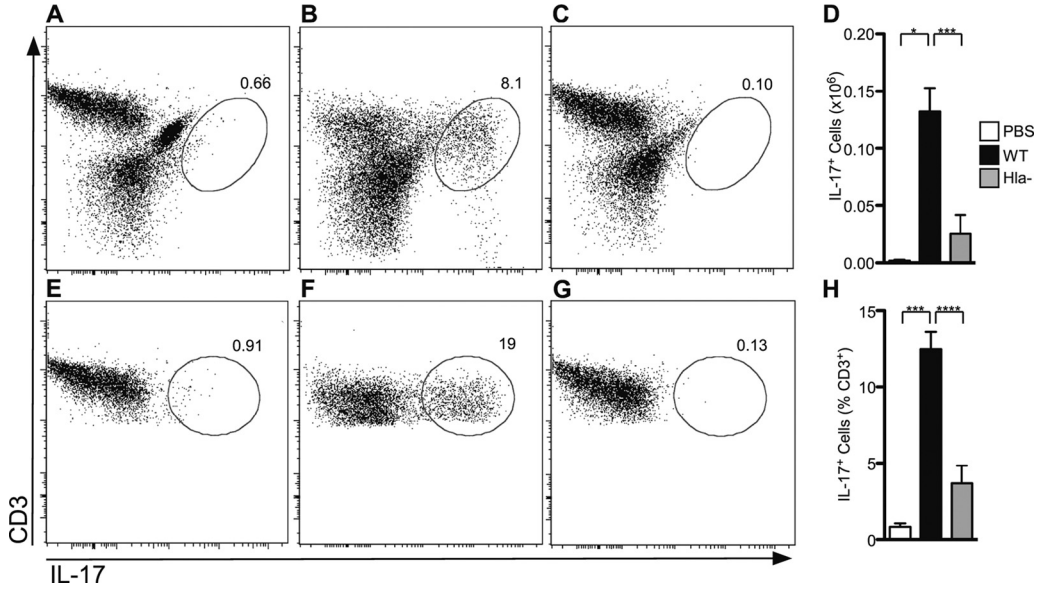

FIG 5 Hla expression is required for induction of a pulmonary Th17 response to *S. aureus* infection. Flow cytometric analysis of cells stimulated with PMA and ionomycin and then stained with anti-CD3 allophycocyanin (APC) and anti-IL-17 PE. (A to C) Live cell gate from mouse lung following infection with PBS (A), WT *S. aureus* (B), or Hla$^-$ *S. aureus* (C). (D) Histogram quantifying IL-17$^+$ cells recovered from the lungs of mice. (E to G) CD3$^+$ lymphocyte gate from mouse lung following infection with PBS (E), WT *S. aureus* (F), or Hla$^-$ *S. aureus* (G). Representative plots are shown. The percentage of IL-17$^+$ cells is indicated. (H) Histogram of the percentage CD3$^+$ cells that are IL-17$^+$. Bars represent the average ± SEM. Results are derived from 4 PBS-treated mice, 21 WT-infected mice, and 14 Hla$^-$-infected mice. *P* values are indicated: *, $P < 0.05$; ***, $P < 0.001$; ****, $P < 0.0001$.

ies, we have demonstrated microarray technology and gene ontogeny analysis to be a powerful tool in the selective identification of toxin-responsive pathways, highlighting a number of toxin-induced cellular signatures for ongoing study. As Hla has emerged as a strategic target for vaccine and passive immunotherapy approaches to *S. aureus* disease, understanding how this toxin primes the host immune system is anticipated to inform the rational design of strategies capable of specifically enhancing the generation of adaptive immunity.

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