RNA-Seq Analysis of the Host Response to *Staphylococcus aureus* Skin and Soft Tissue Infection in a Mouse Model

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Abstract

*Staphylococcus aureus* is a leading cause of skin and soft tissue infections (SSTI), which are primarily self-limiting. We conducted a comprehensive analysis of the host transcriptome during a *S. aureus* SSTI to provide insight on the protective mechanisms that thwart these infections. We utilized a murine SSTI model in which one ear is epicutaneously challenged while the other is not. We then harvested these infected and uninfected ears, as well as ears from naïve mice, at one, four, and seven days post-challenge, and performed RNA sequencing (RNA-seq) using the Illumina platform. RNA-seq data demonstrated a robust response at the site of infection. Comparison of gene expression profiles between infected ears and the non-infected ears of challenged mice defined the local response to infection, while comparisons of expression profiles of non-infected ears from challenged mice to ears of naïve mice revealed changes in gene expression levels away from the site indicative of a systemic response. Over 1000 genes exhibited increased expression locally at all tested time points. The local response was more robust than the systemic response. Through evaluation of the RNA-seq data using the Upstream Regulator Analytic as part of the Ingenuity Pathway Analysis software package, we found that changes in the activation and inhibition of regulatory pathways happen first locally, and lag behind systemically. The activated pathways are highly similar at all three time points during SSTI, suggesting a stable global response over time. Transcript increases and pathway activation involve pro- and anti-inflammatory mediators, chemotaxis, cell signaling, keratins, and TH1/TH17 cytokines. Transcript decreases and pathway inhibition demonstrate that metabolic genes and anti-inflammatory pathways are repressed. These data provide insight on the host responses that may aid in resolution of this self-limited *S. aureus* infection, and may shed light on potential immune correlates of protection for staphylococcal SSTI.
Introduction

Staphylococcus aureus is a normal colonizer of the human nose that is found in almost one-third of the population [1]. *S. aureus* features a broad and highly redundant repertoire of virulence factors that allow it to colonize and damage the host, as well as evade host immune responses and cause a variety of diseases in all areas of the body. As a result, this bacterium is a leading cause of both nosocomial and community-acquired infections in the United States [2,3]. In recent years, *S. aureus* infections have become more prevalent in the community, infecting patients with no predisposing risk factors [3].

Most community-acquired *S. aureus* infections occur in the skin and soft tissue and include boils, impetigo, cellulitis, folliculitis, and abscesses, often caused by isolates of the USA300 subtype [4]. While these infections are largely self-limiting in nature, severe invasive illness can result [5]. Therefore, an understanding of the protective immune response in the skin is important in order to elucidate potential new ways of combatting *S. aureus* while it remains localized in this area.

Investigators are beginning to understand some facets of the host response to *S. aureus* SSTI. The skin itself acts as an immunologic barrier to infection, with its surface maintaining an unsuitable temperature and pH for bacterial growth [6]. Antimicrobial peptides such as β-defensin, as well as skin commensals such as *Staphylococcus epidermidis* and their secreted products (e.g., phenol-soluble modulins [7]) also work to inhibit *S. aureus* growth [6]. If an infection does take hold, neutrophil recruitment to the site of infection has been demonstrated as crucial for subsequent protection [8], mediated by pro-inflammatory cytokines such as IL-1β [9,10] and by IL-17, the product of a TH17 adaptive response [11].

Much still remains unknown about the host response to *S. aureus* SSTI, and, specifically, what correlates with resolution of these infections. While microarray analyses have been performed examining the murine gene expression profile in *S. aureus*-infected skin [9], limitations to microarray studies can lead to incomplete results. Next generation sequencing, such as RNA-seq, can provide greater sensitivity and eliminates issues with hybridization and nonspecific detection [12]. Therefore, in this study we utilized RNA-seq to generate transcriptional profiles of skin from the ears of mice infected with *S. aureus* sub-type USA300 using an epicutaneous infection model [13,14]. We compared the differential gene expression of skin from infected ears to that from uninfected ears from the same challenged mice over time in order to evaluate, for the first time, the local response to infection directly at the site of SSTI. We also compared the non-infected ears from challenged mice to naïve mice in order to elucidate the systemic response to SSTI challenge. The data generated provide further insight into the host response to *S. aureus* over the course of a self-limited infection, both locally and systemically, which may be useful in determining potentially novel pathways that are important for clearance of the pathogen.

Materials and Methods

Ethics Statement

For all animal studies, protocols were reviewed and approved by the Institutional Animal Care and Use Committees (IACUC) of the Center for Biologics Evaluation and Research (Silver Spring, MD; permit number 2010–04). All surgeries were performed under ketamine/xylazine anesthesia, and all efforts were made to minimize animal suffering. Animals were sacrificed at the time points indicated below using CO2 inhalation.
Strains, mice, and reagents

*S. aureus* USA300 strain SAP149 [15] was used to infect mice. Ten week old female BALB/c mice were obtained from NCI (Frederick, MD). All animal experiments were approved by the CBER Institutional Animal Care and Use Committee. Unless indicated, all reagents were obtained from ThermoFisher Scientific (Rockville, MD).

Epicutaneous *S. aureus* infection

The epicutaneous *S. aureus* challenge was performed as described by Prabhakara *et al.* [13]. Briefly, tryptic soy broth (TSB) was inoculated 1:50 with an overnight culture of SAP149, and then grown at 37°C with shaking until an absorbance at 600 nm (A600) of 0.8 was reached. The culture was then centrifuged at 3000 × g for 15 minutes and the pellet was resuspended in sterile PBS. The bacteria were then counted using a Petroff Hauser cell counter (Hausser Scientific, Horsham, PA). The bacteria were centrifuged again and then resuspended in PBS to a density of 1×10^11 CFU/mL.

Mice were anesthetized using 2 mg Ketamine (Ketaject, Phoenix Pharmaceutical, St. Joseph, MO) and 0.1 mg Xylazine (AnaSed, Akorn, Decatur, IL) and the left ears were cleaned with 70% ethanol. The left ears were then pricked 10 times with a Morrow Brown allergy test needle (Morrow Brown Allergy Diagnostics, Oakhurst, NJ) containing a 10 μL drop of the *S. aureus* suspension. After infection, animals were monitored daily. Because the infection causes minimal distress, no analgesics were given. At each subsequent time point, subsets of mice were euthanized and ears were excised with scissors. Each ear was homogenized immediately in 5 mL of TRIzol (Life Technologies, Grand Island, NY). The homogenized ears were placed at -80°C until RNA isolation. For Western blotting, ears were excised and placed into lysis buffer on ice.

RNA isolation

Frozen ear homogenates were thawed on ice, and the 5 mL TRIzol/homogenate mixture was then added to an additional 15 mL of TRIzol in a 50 mL conical tube. The tubes were incubated at room temperature for five minutes, and then 5 mL of chloroform was added and the tubes were shaken vigorously for 15 seconds. The tubes were incubated at room temperature for an additional three minutes, and then centrifuged at 5000 × g for 30 minutes at 4°C. The upper aqueous phase was retained in a new 50 mL conical tube and 10 mL of isopropanol was added and vortexed. The tubes were incubated at room temperature for 10 minutes and then centrifuged again at 5000 × g, 4°C, for one hour. The supernatant was decanted and the pellet was resuspended in ice cold 75% ethanol and placed at -20°C overnight. The next day, the precipitated RNA was isolated by centrifugation at 5000 × g at 4°C for one hour, the supernatant was removed, and the pellets were dried in a laminar flow hood for 20 minutes with the tubes inverted. The pellets were then resuspended in diethylpyrocarbonate (DEPC) treated water (Life Technologies) and quantified spectrophotometrically (NanoVue, GE Life Sciences, Pittsburgh, PA). The RNA was then alcohol precipitated a second time, resuspended again in DEPC water, and re-quantified spectrophotometrically prior to RNA-seq analysis. RNA integrity was evaluated both by agarose gel electrophoresis and BioAnalyzer analysis.

RNA-seq and gene expression analysis

All RNA-seq libraries (non-strand-specific, paired end) were prepared with the TruSeq RNA Sample Prep kit (Illumina, San Diego, CA). The total RNA samples isolated from the SSTI infections were subject to poly(A) enrichment as part of the TruSeq protocol, and 100 nt of sequence was determined from both ends of each cDNA fragment using the HiSeq platform.
(Illumina) per the manufacturer’s protocol. Sequencing reads were annotated and aligned to the UCSC BALB/c mouse reference genome using TopHat [16]. For analysis of the infection data, the alignment files from TopHat were used to generate read counts for each gene and a statistical analysis of differential gene expression was performed using the DESeq package from Bioconductor [17]. We made comparisons between naïve uninfected mice and unchallenged ears from infected mice, as well as between challenged and unchallenged ears from the same infected mouse, at one, four, and seven days post-challenge. Three naïve and three infected mice were evaluated at each time point. A gene was considered differentially expressed if the false discovery rate (FDR) for differential expression was less than 0.01 and the fold change was at least two-fold (Log₂ fold change (LFC) of ≥1).

Selection of top differentially expressed genes

All significantly differentially expressed genes for all time points are presented in the S1 Table. For simplicity of analysis, we chose to present only the 50 genes demonstrating the greatest increase or decrease in transcript levels from each time point within the manuscript. Under most tested conditions, we note that a proportion of differentially expressed genes show an up-regulation level of “inf”. These values occur when readings from the comparator ear (the non-infected ear from challenged mice when examining the local response, or the naïve ear from unchallenged mice when examining the systemic response) are below the limit of detection (i.e. no reads mapped to this gene in these samples). However, it is unrealistic biologically that the readings are truly zero. Because any level of reads in the test ear (i.e., the infected ear when examining the local response, or the uninfected ear from challenged mice when evaluating the systemic response) will then give a LFC value of inf, the magnitude of the actual difference is lost; we are unable to differentiate whether this difference is a few reads or thousands of reads. Therefore, for genes with LFC values of inf, we looked at the raw read data to determine the actual read levels in the test ear. In order to report a gene with a LFC of inf as a top differentially expressed gene (Tables 1–4), we chose to include only those genes that had a read level in the test ear that was higher than the smallest number of reads seen for any gene within the top 50 list that had a LFC of a numerical value. Alternatively, we also included genes with lower read values than this minimum, but a LFC of inf at multiple time points. We hypothesize that low read numbers consistently detected in test ears with undetectable reads in the comparator ears in different cohorts of mice, at multiple time points, likely reflect a real result. We also included genes that had a LFC of inf at one time point, but a significant differential expression with a numerical LFC at another time point. Through these criteria, we believe we have narrowed the genes with a LFC of inf to most likely reflect meaningful expression changes.

Upstream Regulator Analysis

In order to identify which signaling pathways were potentially activated or repressed during S. aureus SSTI, we used the Upstream Regulator Analytic (URA) of IPA (Ingenuity Systems, www.ingenuity.com). This software evaluates the overlap among experimentally derived gene lists and the Ingenuity Knowledge Base, a comprehensive database of genetic data containing approximately 5 million findings manually selected from the scientific literature or third party databases [18]. URA calculates the statistical significance of the overlap between the experimental dataset and the database’s regulatory pathways and reports a p value using Fisher’s Exact Test. URA also analyzes the direction of the differential gene expression to predict the activation or repression of these pathways (for example, if Regulator X is reported in the literature to cause up-regulation of genes A, B, C, and D, and these four genes are found experimentally to have significantly increased transcript levels, URA will predict the pathway regulated by X is
Table 1. Top Genes with Significantly Increased Transcript Levels at the Site of SSTI1.

| Symbol | Gene Name | Function | LFC3 | Day 1 | Day 4 | Day 7 |
|--------|-----------|----------|------|-------|-------|-------|
| Nlrp12 | NLR family, pyrin domain containing 12 | Suppression of inflammation | Inf  | Inf   | Inf   | Inf   |
| Pdyn   | Prodynorphin | Pain/Stress perception; anti-apoptotic | Inf  | Inf   | Inf   | Inf   |
| Slc32a1| solute carrier family 32 (GABA vesicular transporter), member 1 | GABA uptake into synaptic vesicles | Inf  | Inf   | 6.27  | Inf   |
| Chst4  | carbohydrate (chondroitin 6/keratan) sulfotransferase 4 | L-selectin biosynthesis | Inf  | Inf   | 5.49  | Inf   |
| IL-17a | interleukin 17A | Cytokine | 5.13 | Inf   | 2.88  | Inf   |
| Cxcl5  | chemokine (C-X-C motif) ligand 5 | Chemotactic for neutrophils | 9.01 | 8.10  | 7.00  |       |
| Cxcl3  | chemokine (C-X-C motif) ligand 3 | Chemotactic for neutrophils | 8.97 | 6.87  | 6.78  |       |
| Cxcl2  | chemokine (C-X-C motif) ligand 2 | Chemokine produced at sites of inflammation | 8.73 | 6.49  | 6.45  |       |
| Ilg1   | immunoresponsive gene 1 | Suppression of inflammation | 8.62 | 6.67  | 5.84  | Inf   |
| Ccl4   | chemokine (C-C motif) ligand 4 | Inflammatory chemokine | 8.62 | 6.12  | 5.70  | Inf   |
| Trem1  | triggering receptor expressed on myeloid cells 1 | Inflammation | 8.56 | 6.81  | 5.98  | Inf   |
| Cs3f3  | colony stimulating factor 3 (granulocyte) | GCSF; cytokine involved in granulocyte production | 8.36 | 6.59  | 6.49  | Inf   |
| Fpr1   | formyl peptide receptor 1 | Neutrophil activation | 8.29 | 6.48  | 5.93  | Inf   |
| Il-1b  | Interleukin 1 beta | Pro-inflammatory cytokine | 8.08 | 6.36  | 5.87  | Inf   |
| Cc3    | chemokine (C-C motif) ligand 3 | Inflammatory cytokine | 8.02 | 5.85  | 5.75  | Inf   |
| Saa3   | Serum amyloid A 3 | Acute phase protein | 7.79 | 8.02  | 6.34  | Inf   |
| Il-19  | interleukin 19 | Immunosuppressive during skin infection | 7.73 | 7.21  | 6.33  | Inf   |
| Clec4e | C-type lectin domain family 4, member e | Pro-inflammatory receptor | 7.71 | 6.15  | 5.46  | Inf   |
| IL-6   | Interleukin 6 | Lymphocyte differentiation; induces acute phase response | 7.64 | 6.03  | 3.97  | Inf   |
| S100a9 | S100 calcium binding protein A9 (calgranulin B) | Component of calprotectin | 7.55 | 7.07  | 6.33  | Inf   |
| Reg3g  | regenerating islet-derived 3 gamma | Antimicrobial | 7.44 | 7.77  | 7.96  | Inf   |
| Olfm4  | olfactomedin 4 | Neutrophil granule protein; negative regulator of host immunity | 7.43 | 7.45  | 5.19  | Inf   |
| IL-24  | Interleukin 24 | Cytokine | 7.36 | 7.80  | 6.76  | Inf   |
| S100a8 | S100 calcium binding protein A8 | Component of calprotectin | 7.35 | 7.38  | 6.41  | Inf   |
| Cxcr1  | chemokine (C-X-C motif) receptor 1 | IL8 receptor | 7.05 | 5.80  | 4.33  | Inf   |
| Treml4 | triggering receptor expressed on myeloid cells-like 4 | Antigen presentation | 6.95 | 5.24  | 5.93  | Inf   |
| Defb3  | Defensin beta 3 | Antimicrobial | 6.19 | 7.57  | 6.09  | Inf   |
| Ly6g   | lymphocyte antigen 6 complex, locus G | Signaling; neutrophil marker | 5.88 | 5.07  | 3.42  | Inf   |
| Il-22  | interleukin 22 | Pro-inflammatory cytokine | 4.19 | 7.17  | 4.37  | Inf   |
| Cxcl9  | chemokine (C-X-C motif) ligand 9 | Potentially involved in T cell trafficking | 2.14 | 3.52  | 6.51  | Inf   |
| Defa-ps12 | Alpha defensin, pseudogene 12 | Pseudogene | —   | Inf   | Inf   | Inf   |
| Mcpt1  | mast cell protease 1 | Peptidase found in mast cell granules | —   | Inf   | Inf   | Inf   |
| Cmtm1  | CKLF-like MARVEL transmembrane domain containing 1 | Chemokine-like family | —   | Inf   | 6.60  | Inf   |
| Cd5l   | CD5-antigen like | Immune system regulation; inhibitor of apoptosis | —   | 4.29  | Inf   | Inf   |
| Saa2   | Serum amyloid A2 | Acute phase protein | 7.94 | 3.63  | —     | Inf   |
| Cxcl15 | Chemokine (C-X-C motif) ligand 15/IL8 | Chemokine | Inf  | —     | Inf   | Inf   |
| Csf2   | Colony stimulating factor 2 | Cytokine involved in granulocyte production | 8.37 | —     | Inf   | Inf   |
| Fcnb   | ficolin B | Pattern Recognition Receptor | 5.22 | —     | —     | Inf   |

(Continued)
Table 1. (Continued)

| Symbol  | Gene Name                                      | Function                                                                 | Day 1 | Day 4 | Day 7 |
|---------|-----------------------------------------------|--------------------------------------------------------------------------|-------|-------|-------|
| Gzmk    | granzyme K                                    | Serine protease; released from cytoplasmic granules of CTLs and NK cells |       |       | 5.58  |
| **Metabolism/Cell Proliferation/Regulation** |                                               |                                                                          |       |       |       |
| Sfa1    | Stef A1                                       | Cysteine protease inhibitor/Epidermal development                       | Inf   | 5.96  | 5.61  |
| Drd2    | dopamine receptor D2                          | Hormone regulation                                                      | 4.44  | 5.99  | Inf   |
| Klk1b27 | kallikrein 1-related peptidase b27            | Peptidase                                                               | Inf   | 3.64  | 3.30  |
| Fgt23   | fibroblast growth factor 23                   | Regulator of phosphate homeostasis                                      | 8.78  | 8.01  | 5.42  |
| Tdgf1   | teratocarcinoma-derived growth factor 1       | Growth factor                                                           | 7.87  | 7.39  | 7.09  |
| Mrgr2a2b| MAS-related GPR, member A2B                   | Receptor                                                                | 7.67  | 6.39  | 5.50  |
| Sfa2l1  | stefin A2 like 1                              | Thiol proteinase inhibitor                                              | 7.63  | 6.72  | 6.05  |
| Mrgr2a2| MAS-related GPR, member A2A                   | Receptor                                                                | 7.67  | 6.10  | 4.57  |
| Tdgf1-ps1| teratocarcinoma-derived growth factor, pseudogene 1 | Growth factor, pseudogene                                              | 7.61  | 7.21  | 6.15  |
| Sfa2    | Stef A2                                       | Thiol proteinase inhibitor                                              | 7.13  | 7.48  | 7.22  |
| Abca13  | ATP-binding cassette, sub-family A (ABC1), member 13 | Transporter                                                            | 6.08  | 4.39  | 4.33  |
| Reg1    | regenerating islet-derived 1                 | Islet cell regeneration                                                 | 5.81  | 8.58  | 7.45  |
| Uox     | Urate oxidase                                 | Converts uric acid to allantoin                                        | 4.84  | 7.60  | 7.13  |
| Lhx1    | LIM homeobox protein 1                        | Transcription factor                                                    | 4.21  | 6.66  | 5.40  |
| Cyp2j11 | cytochrome P450, family 2, subfamily j, polypeptide 11 | Arachidonic acid metabolism                                            | Inf   | Inf   | Inf   |
| Slc9c1  | solute carrier family 9, subfamily C, member 1 | Sodium-hydrogen exchanger; regulates intracellular pH of spermatozoa   | 3.76  | Inf   | Inf   |
| Dynap   | Dynactin associated protein                   | Regulation of cell proliferation                                        | Inf   | Inf   | Inf   |
| Cyp4a12a| cytochrome P450, family 4, subfamily a, polypeptide 12a  | Arachidonic acid metabolism                                            | Inf   | Inf   | Inf   |
| Vmn1r210| vomeronasal 1 receptor 210                    | Pheromone binding                                                       | Inf   | 4.81  | Inf   |
| Sali    | sal-like 1 (Drosophila)                      | Transcriptional repressor                                               | Inf   | 4.72  | Inf   |
| Prl2c3  | prolactin family 2, subfamily c, member 3     | Growth factor                                                           | Inf   | 5.03  | 7.05  |
| Chil4   | Chitinase-like 4                              | Chitin degradation                                                      | Inf   | 7.91  | Inf   |
| **Epidermal formation**               |                                               |                                                                          |       |       |       |
| Spr2a1  | small proline-rich protein 2A1                | Keratinocyte envelope protein                                            | 7.64  | 5.85  | 5.99  |
| Krt6b   | Keratin 6b                                    | Hair follicle formation                                                  | 6.94  | 7.87  | 6.50  |
| Spr2a3  | small proline-rich protein 2A3                | Keratinocyte envelope protein                                            | 6.58  | 5.14  | 6.29  |
| Spr2j-ps| small proline-rich protein 2J, pseudogene    | Epithelial cell envelope                                                | 5.51  | 7.47  | 6.20  |
| Spr3    | Small proline-rich protein 3                  | Keratinocyte envelope protein                                            | Inf   | 5.98  | Inf   |
| Spr2k   | small proline-rich protein 2K                 | Epithelial cell envelope                                                | Inf   | 8.00  | 7.97  |
| **Unknown Function**                  |                                               |                                                                          |       |       |       |
| Gm5581  | Predicted gene 5581                           | Unknown                                                                 | Inf   | Inf   | Inf   |
| Gm14039 | predicted gene 14039                          | Unknown                                                                 | Inf   | Inf   | Inf   |
| Gm24801 | predicted gene, 24801                         | Unknown                                                                 | Inf   | Inf   | Inf   |
| Gm11345 | predicted gene 11345                          | Unknown                                                                 | Inf   | Inf   | Inf   |
| Gm25465 | predicted gene, 25465                         | Unknown                                                                 | Inf   | 5.65  | Inf   |
| Gm15133 | predicted gene 15133                          | Unknown                                                                 | Inf   | Inf   | 5.40  |
| Fam71a  | family with sequence similarity 71, member A  | Unknown                                                                 | Inf   | Inf   | 4.83  |
| Gm590   | predicted gene 590                            | Unknown                                                                 | Inf   | Inf   | Inf   |

(Continued)
activated; if genes A, B, C, and D have significantly decreased transcript levels, URA will predict the pathway is inhibited) and reports an Activation Z-score. Activation Z-scores ≥2 are considered “activated” and scores ≤2 are considered “inhibited”.

**Sequencing Data Access**

All raw sequencing reads have been submitted to the NCBI Sequence Read Archive (http://www.ncbi.nlm.nih.gov/sra) under the accession number SRP040121. The processed gene
### Table 2. Top Genes with Significantly Increased Transcript Levels Systemically During SSTI1.

| Symbol | Gene Name | Function2 | LFC3 |
|--------|-----------|-----------|------|
|        |           | Day 1     | Day 4 | Day 7 |
|        |           | Day 4     | Day 7 |
|        |           | Day 7     |       |
| **Immune Function** | | | |
| Cxcl2 | chemokine (C-X-C motif) ligand 2 | Chemokine | 2.54 | 3.31 | 1.74 |
| Cxcl3 | chemokine (C-X-C motif) ligand 3 | Chemokine | 2.29 | 3.09 | 2.29 |
| Irg1 | immunoresponsive gene 1 | Suppression of inflammation | 2.17 | 3.35 | 1.95 |
| S100a9 | S100 calcium binding protein A9 (calgranulin B) | Component of calprotectin; regulation of immune response | 1.19 | 1.77 | — |
| IL-1b | interleukin 1 beta | Pro-inflammatory cytokine | — | 1.65 | 1.37 |
| Saa3 | serum amyloid A 3 | Acute phase protein | 2.38 | — | — |
| Ccrnn | cornulin | May play role in epithelial immune response | 2.29 | — | — |
| Cd8b1 | CD8 antigen, beta chain 1 | Coreceptor on cytotoxic T cells | 2.10 | — | — |
| Tmem1 | triggering receptor expressed on myeloid cells 1 | Inflammation | — | 2.86 | — |
| Ccl20 | chemokine (C-C motif) ligand 20 | Chemotactic for lymphocytes | — | — | 3.23 |
| IL-20 | interleukin 20 | Cytokine | — | — | 3.05 |
| Retnlg | resistin like gamma | Potentially inflammatory hormone | — | — | 2.10 |
| Cxcl1 | chemokine (C-X-C motif) ligand 1 | Chemokine | — | — | 2.05 |
| Clec4e | C-type lectin domain family 4, member e | Pathogen receptor | — | — | 1.96 |
| Ccl4 | chemokine (C-C motif) ligand 4 | Chemokine | — | — | 1.83 |
| Chil1 | chitinase-like 1 | Potentially inflammatory | — | — | 1.48 |
| Tnf | tumor necrosis factor | Pro-inflammatory cytokine | — | — | 1.43 |
| Slfn4 | schlafen 4 | Immune cell development | — | — | 1.38 |
| Fos | FBJ osteosarcoma oncogene | Cellular development/inflammation in skin | — | — | 1.15 |
| Ddefb6 | defensin beta 6 | Antimicrobial | — | — | 1.05 |
| **Metabolism/Cell Proliferation/Regulation** | | | |
| mt-Co2 | mitochondrially encoded cytochrome c oxidase II | Respiratory chain | — | 2.75 | 1.13 |
| mt-Atp6 | mitochondrially encoded ATP synthase 6 | ATP synthesis | — | 2.74 | 1.12 |
| mt-Co3 | mitochondrially encoded cytochrome c oxidase III | Respiratory chain | — | 2.74 | 1.12 |
| Csn3 | casein kappa | Stabilizes micelles | Inf | — | — |
| Kcne1 | potassium voltage-gated channel, Isk-related subfamily, member 1 | Potassium transport | 3.11 | — | — |
| Sct | secretin | Hormone | 2.96 | — | — |
| Slc5a5 | solute carrier family 5 (sodium iodide symporter), member 5 | Iodine uptake | 2.30 | — | — |
| Slc4a1 | solute carrier family 4 (anion exchanger), member 1 | Anion exchange in erythrocytes | 2.21 | — | — |
| Crisp1 | cysteine-rich secretory protein 1 | Sperm-egg fusion | 2.17 | — | — |
| Kcnk16 | potassium channel, subfamily K, member 16 | Outward rectifying potassium channel | 2.14 | — | — |
| Clcn14 | claudin 14 | Tight junction protein | 2.11 | — | — |
| mtl-Tc | mitochondrially encoded tRNA cysteine | RNA gene | — | 5.14 | — |
| mtl-Nd4l | mitochondrially encoded NADH dehydrogenase 4L | Electron transport | — | 4.72 | — |
| mtl-Atp8 | mitochondrially encoded ATP synthase 8 | Electron transport | — | 4.36 | — |
| Aldoart2 | aldolase 1 A, retrogene 2 | Glycolysis | — | 3.84 | — |
| Pnon1 | paraoxonase 1 | Enzyme | — | 2.45 | — |
| GainT2 | UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 2 | Protein glycosylation | — | 2.21 | — |
| mtl-Nd3 | mitochondrially encoded NADH dehydrogenase 3 | Respiratory chain | — | 2.13 | — |
| Adrb3 | adrenergic receptor, beta 3 | Regulation of lipolysis | — | 2.11 | — |

(Continued)
| Symbol | Gene Name                          | Function                           | Day 1 | Day 4 | Day 7 |
|--------|-----------------------------------|------------------------------------|-------|-------|-------|
| Thrsp  | thyroid hormone responsive        | Regulation of lipogenesis          | —     | 1.83  | —     |
| Chat   | choline acetyltransferase         | Acetylcholine production           | —     | 1.59  | —     |
| Gsdma3 | gasdermin A3                      | Pro-apoptotic                      | —     | —     | 1.35  |
| Syt4   | synaptotagmin IV                  | Cellular transport                 | —     | —     | 1.01  |
| Gpr128 | G protein-coupled receptor 128    | Receptor                           | -3.42 | 2.37  | —     |
| Rpph1  | ribonuclease P RNA component H1   | Endonuclease                        | —     | 3.83  | -3.25 |
| Rnu3a  | U3A small nuclear RNA             | Non-coding RNA                     | —     | 3.10  | -2.43 |
| Snord17| small nucleolar RNA, C/D box 17   | Non-coding RNA                     | —     | 3.07  | -2.60 |
| Rn7sk  | RNA, 7SK, nuclear                 | Pre-mRNA processing                | —     | 3.02  | -2.59 |
| Yam1   | YY1 associated myogenesis RNA 1   | Non-coding RNA                     | —     | 1.95  | -1.57 |
| Gys2   | glycogen synthase 2               | Glycogen production                | —     | 1.85  | -1.86 |
| Lars2  | leucyl-tRNA synthetase, mitochondrial | tRNA synthesis                   | —     | 1.83  | -1.41 |

### Epidermal Formation

| Symbol | Gene Name                          | Function                           | Day 1 | Day 4 | Day 7 |
|--------|-----------------------------------|------------------------------------|-------|-------|-------|
| Krtap20-2 | keratin associated protein 20–2  | Hair formation                     | 3.34  | —     | —     |
| Krt72  | keratin 72                         | Hair formation                     | 3.02  | —     | —     |
| Krtap9-5 | keratin associated protein 9–5    | Hair formation                     | 2.83  | —     | —     |
| Krtap31-2 | keratin associated protein 31–2  | Hair formation                     | 2.72  | —     | —     |
| Krt26  | keratin 26                         | Hair formation                     | 2.59  | —     | —     |
| Krtap4-2 | keratin associated protein 4–2    | Hair formation                     | 2.56  | —     | —     |
| Krt28  | keratin 28                         | Hair formation                     | 2.47  | —     | —     |
| Krtap4-13 | keratin associated protein 4–13  | Hair formation                     | 2.47  | —     | —     |
| Krtap31-1 | keratin associated protein 31–1  | Hair formation                     | 2.40  | —     | —     |
| Krt74  | keratin 74                         | Hair formation                     | 2.33  | —     | —     |
| Tchh1  | trichohyalin-like 1                | Hair formation                     | 2.23  | —     | —     |
| Krtap4-9 | keratin associated protein 4–9    | Hair formation                     | 2.18  | —     | —     |
| Krt27  | keratin 27                         | Hair formation                     | 2.17  | —     | —     |
| Tchh   | trichohyalin                      | Epithelial tissue strength         | 2.14  | —     | —     |
| Padi1  | peptidyl arginine deiminase, type I | Epidermal differentiation    | 2.02  | —     | —     |
| Fgf5   | fibroblast growth factor 5        | Regulation of hair growth          | 2.01  | —     | —     |
| Padi3  | peptidyl arginine deiminase, type III | Modulates hair structural proteins  | 1.99  | —     | —     |
| Krtap4-8 | keratin associated protein 4–8    | Hair formation                     | 1.95  | —     | —     |
| Stfa21 | stefin A2 like 1                   | Epidermal hair development        | —     | 2.35  | —     |

### Unknown Function

| Symbol | Gene Name                          | Function                           | Day 1 | Day 4 | Day 7 |
|--------|-----------------------------------|------------------------------------|-------|-------|-------|
| Gm10243 | predicted gene 10243              | Unknown                            | —     | 2.85  | 1.64  |
| Rps11-ps4 | ribosomal protein S11, pseudogene 4 | Pseudogene                       | —     | 2.27  | 1.50  |
| Rpl19-ps1 | ribosomal protein L19, pseudogene 1 | Pseudogene                       | —     | 1.85  | 1.37  |
| Gm8810  | predicted gene 8810               | Unknown                            | —     | 1.77  | 1.65  |
| Gm9789  | predicted gene 9789               | Unknown                            | 4.26  | —     | —     |
| Gm11569 | predicted gene 11569              | Unknown                            | 4.25  | —     | —     |
| Gm14180 | predicted gene 14180              | Unknown                            | 3.64  | —     | —     |
| Gm11564 | predicted gene 11564              | Unknown                            | 3.62  | —     | —     |
| Gm11554 | predicted gene 11554              | Unknown                            | 3.61  | —     | —     |
| D130052B06Rik | RIKEN cDNA D130052B06 gene      | Unknown                            | 3.37  | —     | —     |
| Gm14182 | predicted gene 14182              | Unknown                            | 3.23  | —     | —     |

(Continued)
| Symbol                | Gene Name                          | Function \(^2\) | LFC\(^3\) |
|----------------------|------------------------------------|-----------------|-----------|
|                      |                                    |                 | Day 1     |
|                      |                                    |                 | Day 4     |
|                      |                                    |                 | Day 7     |
| 1110057P08Rik        | RIKEN cDNA 1110057P08 gene         | Unknown         | 3.07      |
| Gm6358               | predicted gene 6358                | Unknown         | 2.98      |
| Gm11596              | predicted gene 11596               | Unknown         | 2.87      |
| Gm7735               | predicted gene 7735                | Unknown         | 2.69      |
| Gm5278               | predicted pseudogene 5278          | Unknown         | 2.67      |
| Gm10061              | predicted gene 10061               | Unknown         | 2.48      |
| 2300002M23Rik        | RIKEN cDNA 2300002M23 gene         | Unknown         | 2.21      |
| Gm11595              | predicted gene 11595               | Unknown         | 2.08      |
| Crym                 | crystallin, mu                     | Unknown         | 2.04      |
| Gm11563              | predicted gene 11563               | Unknown         | 1.93      |
| Gm14513              | predicted gene 14513               | Unknown         | 4.30      |
| Mup-ps22             | major urinary protein, pseudogene 22 | Pseudogene     | 3.56      |
| Rps13-ps1            | ribosomal protein S13, pseudogene 1 | Pseudogene      | 3.46      |
| Gm5483               | predicted gene 5483                | Unknown         | 2.79      |
| Gm12883              | predicted gene 12883               | Unknown         | 2.69      |
| Mup18                | major urinary protein 18           | Unknown         | 2.40      |
| Gm14323              | predicted gene 14323               | Unknown         | 2.02      |
| Rpl31-ps16           | ribosomal protein L31, pseudogene 16 | Pseudogene     | 1.84      |
| Rps15a-ps3           | ribosomal protein S15A, pseudogene 3 | Pseudogene     | 1.84      |
| Gm10132              | predicted gene 10132               | Unknown         | 1.83      |
| Slpi                 | secretory leukocyte peptidase inhibitor | Unknown        | 1.82      |
| Gm13675              | predicted gene 13675               | Unknown         | 1.70      |
| Gm5453               | predicted gene 5453                | Unknown         | 1.70      |
| Calr-ps              | calreticulin, pseudogene           | Pseudogene      | 1.34      |
| Rpl31-ps11           | ribosomal protein L31, pseudogene 11 | Pseudogene     | 1.33      |
| Gm8080               | predicted gene 8080                | Unknown         | 1.20      |
| Gm12482              | predicted gene 12482               | Unknown         | 1.14      |
| Rpl13-ps3            | ribosomal protein L13, pseudogene 3 | Pseudogene     | 1.02      |
| Gm12248              | predicted gene 12248               | Unknown         | -1.06     |
| Gm24407              | predicted gene, 24407              | Unknown         | 2.19      |
| Gm24265              | predicted gene, 24265              | Unknown         | 1.74      |
| Rprl3                | ribonuclease P RNA-like 3          | Unknown         | Inf       |
| Gm14279              | predicted gene 14279               | Unknown         | -1.65     |
| Gm24270              | predicted gene, 24270              | Unknown         | 2.77      |
| Gm24187              | predicted gene, 24187              | Unknown         | 2.49      |
| Gm23935              | predicted gene, 23935              | Unknown         | 3.55      |
| Gm15564              | predicted gene 15564               | Unknown         | 2.21      |
| BC100530             | cDNA sequence BC100530             | Unknown         | -1.74     |

\(^1\)Top 50 genes with greatest increase of LFC comparing uninfected ears from challenged mice to naïve mice for each time point represented; for Day 7, all genes with significant levels of transcript increases are listed.

\(^2\)Function determined via Entrez (www.ncbi.nlm.nih.gov) or Uniprot (www.uniprot.org)

\(^3\)LFC = Log Fold Change

Italicized values indicate transcripts are significantly decreased at the indicated time point.

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Table 3. Top Genes with Significantly Decreased Transcript Levels at the Site of SSTI1.

| Symbol | Gene Name | Function | Day 1 | Day 4 | Day 7 |
|--------|-----------|----------|-------|-------|-------|
| **Immune Function** | | | | | |
| Fcer2a | Fc receptor, IgE, low affinity II, alpha polypeptide | Receptor for IgE; B cell differentiation | -1.61 | -1.87 | -1.50 |
| Serpinb1c | serine (or cysteine) peptidase inhibitor, clade B, member 1c | Inhibits neutrophil-derived proteinases | — | -1.11 | -1.65 |
| Marco | macrophage receptor with collagenous structure | Pattern recognition receptor | — | -1.61 | — |
| Bpifb2 | BPI fold containing family B, member 2 | LPS binding | — | — | -2.10 |
| Lyg2 | lysozyme G-like 2 | cleaves peptidoglcan | — | — | -1.67 |
| **Metabolism/Cell Proliferation/Regulation** | | | | | |
| Inmt | indolethylamine N-methyltransferase | Indole N-methylation | -4.40 | -1.63 | -1.33 |
| Nell2 | NEL-like 2 | Protein kinase C binding | -1.81 | -1.81 | -2.04 |
| Cyp11b1 | cytochrome P450, family 11, subfamily b, polypeptide 1 | Conversion of progesterone to cortisol | -1.45 | -1.88 | -2.04 |
| Sost | sclerostin | Negative regulator of bone growth | -3.43 | -2.06 | — |
| Mrgrp | MAS-related GPR, member G | Pain sensation/modulation | -3.22 | -1.65 | — |
| Gkn3 | gastrokine 3 | May inhibit gastric cell proliferation | -3.05 | -1.63 | — |
| Spock3 | sparc/osteonectin, cwcv and kazal-like domains proteoglycan 3 | Inhibition of matrix metalloproteinase processing | -2.96 | -1.82 | — |
| Hrk | harakiri, BCL2 interacting protein (contains only BH3 domain) | Pro-apoptotic | -2.64 | -2.19 | — |
| Thrsp | thyroid hormone responsive | Regulation of lipogenesis | -2.35 | -1.18 | — |
| Mettl1b | methyltransferase like 11B | Methyltransferase | -2.25 | -1.95 | — |
| Cntnap2 | contactin associated protein-like 2 | Neurogenesis | -2.23 | -1.59 | — |
| Gucy2f | guanylate cyclase 2f [Source:MGI Symbol;Acc: MGI:105119] | cGMP resynthesis | -1.49 | -1.87 | — |
| Ms4a10 | membrane-spanning 4-domains, subfamily A, member 10 | Signal transduction | -1.35 | -1.61 | — |
| Sun5 | Sad1 and UNCP4 domain containing 5 | Spermatogenesis | — | -4.05 | -2.29 |
| My3 | myosin, light polypeptide 3 | Light chain of myosin | — | -2.03 | -1.45 |
| Ptgsd | prostaglandin D2 synthase (brain) | Prostaglandin synthesis | — | -1.97 | -1.22 |
| Octl2b | 3-oxoacid CoA transferase 2B | Metabolism | -3.91 | — | — |
| Gmnc | gerninin coiled-coil domain containing | Regulation of DNA replication | -3.35 | — | — |
| Odf4 | outer dense fiber of sperm tails 4 | Spermatogenesis | -3.27 | — | — |
| Cntn4 | contactin 4 | Neuronal development | -2.72 | — | — |
| Rergl | RERG/RAS-like | GTPase | -2.67 | — | — |
| Mettl21e | methyltransferase like 21E | Lysine methyltransferase | -2.65 | — | — |
| Prss51 | proteasome, serine 51 | Endopeptidase | -2.57 | — | — |
| Mylk4 | myosin light chain kinase family, member 4 | Muscle development | -2.55 | — | — |
| Unc5d | unc-5 homolog D (C. elegans) | Netrin receptor; may be required for apoptosis | -2.52 | — | — |
| Mrgrp | MAS-related GPR, member H | G protein coupled receptor | -2.47 | — | — |
| Olfr4120 | olfactory receptor 1420 | Sensory perception of smell | -2.44 | — | — |
| Abca6 | ATP-binding cassette, sub-family A (ABC1), member 6 | Probable transporter | -2.40 | — | — |
| Fhl5 | four and a half LIM domains 5 | Transcriptional activator | -2.30 | — | — |
| Nlgn1 | neureilgin 1 | Synapse formation | -2.26 | — | — |
| Ocm | onconemodulin | Calcium binding regulator | -2.24 | — | — |
| Kcnt1 | potassium channel, subfamily T, member 1 | Potassium transport | -2.21 | — | — |
| Ky | kyphoscoliosis peptidase | Muscle growth | -2.21 | — | — |
| mt-Atp8 | mitochondrially encoded ATP synthase 8 | Electron transport | — | -5.32 | — |
| mt-Co3 | mitochondrially encoded cytochrome c oxidase III | Electron transport | — | -3.73 | — |

(Continued)
| Symbol  | Gene Name                                                                 | Function                    | LFC<sup>3</sup> | Day 1 | Day 4 | Day 7 |
|---------|---------------------------------------------------------------------------|-----------------------------|------------------|-------|-------|-------|
| mt-Co2  | mitochondrially encoded cytochrome c oxidase II                           | Electron transport          | -3.49           |       |       |       |
| Aldoart2| aldolase 1 A, retrogene 2                                                  | Glycolysis                  | -3.32           |       |       |       |
| mt-Tc   | mitochondrially encoded tRNA cysteine                                      | RNA gene                    | -3.06           |       |       |       |
| mt-Atp6 | mitochondrially encoded ATP synthase 6                                    | ATP synthesis               | -3.02           |       |       |       |
| mt-Nd3  | mitochondrially encoded NADH dehydrogenase 3                              | Respiratory chain           | -2.76           |       |       |       |
| Dbx1    | developing brain homeobox 1                                               | Neurogenesis                 | -2.74           |       |       |       |
| ElcAb6  | EF-hand calcium binding domain 6                                           | Negatively regulates androgen receptor | -1.82           |       |       |       |
| Myl2    | myosin, light polypeptide 2, regulatory, cardiac, slow                     | Muscle development          | -1.64           |       |       |       |
| Kif12   | kinesin family member 12                                                  | Intracellular transport     | -2.17           |       |       |       |
| Lrp1b   | low density lipoprotein-related protein 1B (deleted in tumors)            | Cell surface protein that may bind ligands for endocytosis | -2.01           |       |       |       |
| Actb2   | actin, beta-like 2                                                         | Cell motility               | -1.94           |       |       |       |

**Epidermal Formation**

| Symbol  | Gene Name                                                                 | Function                  | LFC<sup>3</sup> | Day 1 | Day 4 | Day 7 |
|---------|---------------------------------------------------------------------------|---------------------------|------------------|-------|-------|-------|
| Ros1    | Ros1 proto-oncogene                                                       | Epithelial cell differentiation | -2.19 | -2.26 | -2.04 |
| Krt24   | keratin 24                                                                | Epithelial cytoskeleton   | -1.68           |       |       |       |
| Krtap11-1| keratin associated protein 11–1                                           | Hair formation             | -1.05           |       |       |       |
| Krtap26-1| keratin associated protein 26–1                                           | Hair formation             | -1.05           |       |       |       |
| Krtap4-8 | keratin associated protein 4–8                                            | Hair formation             | -1.03           |       |       |       |
| Krtap12-1| keratin associated protein 12–1                                           | Hair formation             | -1.95           |       |       |       |
| Krtap16-1| keratin associated protein 16–1                                           | Hair formation             | -1.87           |       |       |       |
| Krtap24-1| keratin associated protein 24–1                                           | Hair formation             | -1.75           |       |       |       |
| Krt82   | keratin 82                                                                | Hair formation             | -1.72           |       |       |       |
| Krtap10-4| keratin associated protein 10–4                                           | Hair formation             | -1.69           |       |       |       |
| Krtap4-9 | keratin associated protein 4–9                                            | Hair formation             | -1.69           |       |       |       |
| Krtap10-10| keratin associated protein 10–10                                          | Hair formation             | -1.65           |       |       |       |
| Krtap1-3  | keratin associated protein 1–3                                           | Hair formation             | -1.62           |       |       |       |
| Krtap1-4  | keratin associated protein 1–4                                           | Hair formation             | -1.62           |       |       |       |
| Krtap5-5  | keratin associated protein 5–5                                           | Hair formation             | -1.60           |       |       |       |

**Unknown Function**

| Symbol  | Gene Name                                                                 | Function                  | LFC<sup>3</sup> | Day 1 | Day 4 | Day 7 |
|---------|---------------------------------------------------------------------------|---------------------------|------------------|-------|-------|-------|
| 4921504E06Rik | RIKEN cDNA 4921504E06 gene                                           | Unknown                   | -2.27           | -2.25 | -1.98 |
| 1190003K10Rik | RIKEN cDNA 1190003K10 gene                                              | Unknown                   | -1.98           | -1.74 | -1.50 |
| Gm4810  | predicted gene 4810                                                       | Unknown                   | -1.90           | -2.53 | -2.30 |
| 5430427M07Rik | RIKEN cDNA 5430427M07 gene                                            | Unknown                   | -1.74           | -1.79 | -1.23 |
| Fam150b | family with sequence similarity 150, member B                           | Unknown                   | -2.19           | -1.60 |       |
| Serpina4-ps1 | serine (or cysteine) peptidase inhibitor, clade A, member 4, pseudogene 1 | Pseudogene               | -1.67           | -1.70 |       |
| Snim18  | small integral membrane protein 18                                       | Unknown                   | -1.57           | -2.11 |       |
| Fam196b | family with sequence similarity 196, member B                            | Unknown                   | -1.40           | -1.69 |       |
| 2410137M14Rik | RIKEN cDNA 2410137M14 gene                                              | Unknown                   | -1.29           | -1.83 |       |
| Gm11433 | predicted gene 11433                                                      | Unknown                   | -1.80           | -1.91 |       |
| Gm8810  | predicted gene 8810                                                       | Unknown                   | -1.67           | 1.00  |       |
| Gm11564 | predicted gene 11564                                                      | Unknown                   | -1.55           | -2.39 |       |
| 2310005G13Rik | RIKEN cDNA 2310005G13 gene                                             | Unknown                   | -1.53           | -1.59 |       |
| Gm11568 | predicted gene 11568                                                      | Unknown                   | -1.03           | -1.64 |       |

(Continued)
Table 3. (Continued)

| Symbol          | Gene Name                                    | Function² | LFC³  |
|-----------------|----------------------------------------------|-----------|-------|
|                 |                                              |           | Day 1 | Day 4 | Day 7 |
| 1700019B03Rik   | RIKEN cDNA 1700019B03 gene                   | Unknown   | -1.27 | -1.68 |
| Arrdc5          | arrestin domain containing 5                | Unknown   | -3.95 | —     |
| Gm13441         | predicted gene 13441                        | Unknown   | -3.56 | —     |
| Gm17025         | predicted gene 17025                        | Unknown   | -3.47 | —     |
| A330094K24Rik   | RIKEN cDNA A330094K24 gene                  | Unknown   | -3.25 | —     |
| Tsrg13          | testis specific gene A13                    | Unknown   | -3.01 | —     |
| Gm13273         | predicted gene 13273                       | Unknown   | -2.85 | —     |
| Gm1305          | predicted gene 1305                        | Unknown   | -2.83 | —     |
| Gm14411         | predicted gene 14411                       | Unknown   | -2.78 | —     |
| teddm1          | transmembrane epididymal protein 1          | Unknown   | -2.60 | —     |
| Gm27195         | predicted gene 27195                       | Unknown   | -2.59 | —     |
| A230108P19Rik   | RIKEN cDNA A230108P19 gene                  | Unknown   | -2.56 | —     |
| Gm9947          | predicted gene 9947                        | Unknown   | -2.56 | —     |
| 1700018A04Rik   | RIKEN cDNA 1700018A04 gene                  | Unknown   | -2.47 | —     |
| Prr32           | proline rich 32                             | Unknown   | -2.39 | —     |
| BB218582        | expressed sequence BB218582                | Unknown   | -2.39 | —     |
| 170011911Rik    | RIKEN cDNA 170011911 gene                   | Unknown   | -2.38 | —     |
| 1600029O15Rik   | RIKEN cDNA 1600029O15 gene                  | Unknown   | -2.34 | —     |
| Lrc30           | leucine rich repeat containing 30          | Unknown   | -2.32 | —     |
| Tcerg1l         | transcription elongation regulator 1-like   | Unknown   | -2.23 | —     |
| Gm14513         | predicted gene 14513                       | Unknown   | —     | -5.22 |
| Gm5590          | predicted gene 5590                        | Unknown   | —     | -4.83 |
| Mup-ps22        | major urinary protein, pseudogene 22       | Pseudogene| —     | -3.13 |
| Rps13-ps1       | ribosomal protein S13, pseudogene 1        | Pseudogene| —     | -2.86 |
| Rpl31-ps16      | ribosomal protein L31, pseudogene 16       | Pseudogene| —     | -2.49 |
| Rps11-ps4       | ribosomal protein S11, pseudogene 4        | Pseudogene| —     | -2.42 |
| Rpl19-ps1       | ribosomal protein L19, pseudogene 1        | Pseudogene| —     | -2.01 |
| Gm17597         | predicted gene, 17597                      | Unknown   | —     | -1.72 |
| Gm14279         | predicted gene 14279                       | Unknown   | 2.08  | -1.86 | 4.76  |
| Gm11571         | predicted gene 11571                       | Unknown   | —     | —     | -2.73 |
| G630018N14Rik   | RIKEN cDNA G630018N14 gene                  | Unknown   | —     | -2.03 |
| Gm9507          | predicted gene 9507                        | Unknown   | —     | -1.94 |
| Fam26d          | family with sequence similarity 26, member D| Unknown   | —     | -1.93 |
| Gm3250          | predicted gene 3250                        | Unknown   | —     | -1.93 |
| Gm7579          | predicted gene 7579                        | Unknown   | —     | -1.84 |
| Gm10100         | predicted gene 10100                       | Unknown   | —     | -1.61 |
| Gm11555         | predicted gene 11555                       | Unknown   | —     | -1.79 |
| Gm32133         | predicted gene 3233                        | Unknown   | —     | -1.76 |
| Gm7138          | predicted gene 7138                        | Unknown   | —     | -1.77 |
| Gm19402         | predicted gene, 19402                      | Unknown   | —     | -1.75 |
| Gm11567         | predicted gene 11567                       | Unknown   | —     | -1.74 |
| Gm2431          | predicted gene 2431                        | Unknown   | —     | -1.73 |
| Gm4559          | predicted gene 4559                        | Unknown   | —     | -1.69 |
| Gm3238          | predicted gene 3238                        | Unknown   | —     | -1.62 |
| Gm11596         | predicted gene 11596                       | Unknown   | —     | -1.61 |

(Continued)
expression data have been submitted to the NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE56227.

Western Blotting

Ears were harvested as indicated above and placed into 1 mL of lysis buffer (5 mM Tris pH 8, 150 mM NaCl, 1% NP-40 [Surfact-Amps, ThermoFisher], 1x protease inhibitor cocktail [Halt, ThermoFisher]) on ice, and then homogenized. Ear homogenates were centrifuged at 12000 × g for 10 minutes. The protein concentration of the supernatants was determined using BCA (Pierce BCA Protein Assay Kit, ThermoFisher), and lysates were kept at -80°C until use.

For Western blots, 15 μg total protein content per lane was resolved on 10–20% Tris-Glycine gels (Novex, ThermoFisher) or 4–12% Bis-Tris gels (NuPage, ThermoFisher). Proteins were transferred to nitrocellulose using the iBlot system and blots were blocked for 1 hour with 5% milk in TBS with 0.1% Tween 20 (TBS-T) or PBS with 0.1% Tween 20 (PBS-T) at RT with gentle shaking. Blots were then incubated with primary antibodies (anti-IL1β Mouse mAb #12242, Cell Signaling Technology, Danvers, MA; anti-S100A8/Calgranulin A pAb sc-8112, anti-S100A8/Calgranulin B pAb sc-8115, and anti-GAPDH pAb sc-20357, Santa Cruz Biotechnology, Dallas, TX) overnight at 4°C in TBS-T with 5% BSA (IL-1β, S100A9, and GAPDH), or for 1 hour at RT in PBS-T with 5% milk (S100A8). Blots were washed three times for 10 minutes each in TBS-T or PBS-T, and then incubated with HRP-conjugated goat anti-mouse IgG (IL-1β; KPL, Gaithersburg, MD) or donkey anti-goat IgG (S100A8, S100A9, and GAPDH; Santa Cruz Biotechnology) diluted in TBS-T or PBS-T for one hour at RT. Blots were imaged using ECL (GE Healthcare, Pittsburgh, PA).

Results

RNA-Seq of murine skin during S. aureus SSTI

We compared cDNA profiles of infected and uninfected murine ears at one, four, and seven days post-challenge. Sequencing reads were aligned to the mouse genome and were analyzed as described in the Materials and Methods. We defined differentially expressed genes as having a minimum of two-fold change in expression (log₂ fold change ≥1, P < 0.01) when comparing infected ears to uninfected ears from challenged mice to evaluate the local response at the site of infection, or when comparing uninfected ears from challenged mice to naïve mice to examine the systemic response.
Table 4. Top Genes with Significantly Decreased Transcript Levels Systemically During SSTI¹.

| Symbol | Gene Name | Function² | Day 1 | Day 4 | Day 7 |
|--------|-----------|-----------|-------|-------|-------|
| **Immune Function** | | | | | |
| Serpinb3a | serine (or cysteine) peptidase inhibitor, clade B (ovalbumin), member 3A | May modulate immune response and apoptosis | -2.80 | 1.02 | — |
| Spon2 | spondin 2, extracellular matrix protein | Adhesin; can function as opsonin for phagocytosis of bacteria | -1.04 | — | — |
| Glycam1 | glycosylation dependent cell adhesion molecule 1 | Ligand for L-selectin | — | — | -4.55 |
| Ighg2c | immunoglobulin heavy constant gamma 2C | Immunoglobulin | — | — | -3.62 |
| Reg3g | regenerating islet-derived 3 gamma | Anti-bacterial C-type lectin | — | — | -3.27 |
| Pax5 | paired box 5 | B cell differentiation | — | — | -3.16 |
| Igkc | immunoglobulin kappa constant | Immunoglobulin | — | — | -2.81 |
| Chi4 | chitinase-like 4 | Chemotactic for eosinophils; potentially inflammatory | — | — | -2.97 |
| Ms4a1 | membrane-spanning 4-domains, subfamily A, member 1 | Regulation of B cell activation/proliferation | — | — | -2.34 |
| Hamp2 | hepcidin antimicrobial peptide 2 | Antimicrobial; iron storage regulation | — | — | -2.18 |
| Cd22 | CD22 antigen | Mediates B-B cell interactions | — | — | -1.94 |
| Cd99 | CD99 antigen | T cell adhesion | — | — | -1.19 |
| **Metabolism/Cell Proliferation/Regulation** | | | | | |
| Gpr128 | G protein-coupled receptor 128 | Receptor | -3.42 | 2.37 | — |
| Serpin3k | serine (or cysteine) peptidase inhibitor, clade A, member 3K | Protease inhibitor | -3.24 | — | — |
| Jakmip2 | janus kinase and microtubule interacting protein 2 | Structural scaffold of Golgi | -2.48 | — | — |
| Otor | otoraplin | Cartilage development | -2.19 | — | — |
| Spink8 | serine peptidase inhibitor, Kazal type 8 | Serine protease inhibitor | -1.93 | — | — |
| Akr1c19 | aldo-keto reductase family 1, member C19 | Metabolism | -1.89 | — | — |
| Mmp13 | matrix metalloproteinase 13 | Extracellular matrix degradation | -1.65 | — | — |
| Epyc | epiphycin | Bone/cartilage formation | -1.20 | — | — |
| Str4p | secreted frizzled-related protein 4 | Regulation of cell growth | -1.09 | — | — |
| Cyp26a1 | cytochrome P450, family 26, subfamily a, polypeptide 1 | Retinoic acid metabolism | -1.03 | — | — |
| Akr1c18 | aldo-keto reductase family 1, member C18 | Metabolism | -1.02 | — | — |
| Cyp2a5 | cytochrome P450, family 2, subfamily a, polypeptide 5 | Metabolism | — | — | -1.58 |
| Nr1h4 | nuclear receptor subfamily 1, group H, member 4 | Transcription factor | 1.49 | -1.46 | — |
| Serpin3j | serine (or cysteine) peptidase inhibitor, clade A, member 3J | Protease inhibitor | — | -1.05 | — |
| Rpph1 | ribonuclease P RNA component H1 | tRNA formation | — | 3.83 | -3.25 |
| Gdf7 | growth differentiation factor 7 | Neuronal development | — | — | -2.94 |
| Reg1 | regenerating islet-derived 1 | Metabolism | — | — | -2.85 |
| Snord17 | small nucleolar RNA, C/D box 17 | Non-coding RNA | — | 3.07 | -2.60 |
| Rn7sk | RNA, 7SK, nuclear | Non-coding RNA | — | 3.06 | -2.59 |
| Bsnd | Bartter syndrome, infantile, with sensorineural deafness (Barttin) | Chloride channel formation | — | — | -2.45 |
| Rnu3a | U3A small nuclear RNA | Non-coding RNA | — | 3.10 | -2.43 |
| Pyy | peptide YY | Reduces pancreatic secretions; vasoconstrictory | — | — | -2.39 |
| Serpinb6e | serine (or cysteine) peptidase inhibitor, clade B, member 6e | Inner ear; protects against lysosomal leakage during stress | — | — | -1.96 |
| Gys2 | glycogen synthase 2 | Glycogen synthesis | 1.85 | — | -1.86 |
| Yam1 | YY1 associated myogenesis RNA 1 | Non-coding RNA | — | 1.95 | -1.57 |

(Continued)
| Symbol | Gene Name                                                                 | Function                                                                 | LFC^3 |
|--------|---------------------------------------------------------------------------|--------------------------------------------------------------------------|-------|
|        |                                                                           |                                                                          | Day 1 | Day 4 | Day 7 |
| Ntrk1  | neurotrophic tyrosine kinase, receptor, type 1                            | Nervous system development                                                | —     | —     | -1.48 |
| Lars2  | leucyl-tRNA synthetase, mitochondrial                                     | tRNA formation                                                            | —     | 1.83  | -1.41 |
| Hist1h2al | histone cluster 1, H2al                                                      | Nucleosome component                                                      | —     | —     | -1.38 |
| Scand1 | SCAN domain-containing 1                                                   | Transcriptional regulator                                                 | —     | —     | -1.38 |
|        |                                                                           |                                                                          |       |       |       |
|        | **Epidermal Development**                                                 |                                                                          |       |       |       |
| H60c   | histocompatibility 60c                                                    | Epithelial integrity                                                      | -1.01 | —     | —     |
| Stfa2  | stefin A2                                                                 | Cysteine protease inhibitor/Epidermal development                        | —     | —     | -1.65 |
| Krt6b  | keratin 6B                                                                | Epithelial development                                                    | —     | —     | -1.50 |
| Sprt1b | small proline-rich protein 1B                                              | Keratinocyte protein                                                      | —     | —     | -1.07 |
|        |                                                                           |                                                                          |       |       |       |
|        | **Unknown**                                                               |                                                                          |       |       |       |
| Cyp2g1 | cytochrome P450, family 2, subfamily g, polypeptide 1                      | Pseudogene                                                               | —     | -2.81 | -2.31 |
| Gm8221 | predicted gene 8221                                                       | Unknown                                                                  | —     | -2.50 | -2.20 |
| Sprr2a2| small proline-rich protein 2A2                                             | Unknown                                                                  | -2.06 | —     | -3.41 |
| Gsdmc  | gsdemrin C                                                                | Unkonwn                                                                  | -1.89 | —     | -2.03 |
| Sprr2a3| small proline-rich protein 2A3                                             | Unknown                                                                  | -1.86 | —     | -2.10 |
| BC100530 | cDNA sequence BC100530                                                  | Unknown                                                                  | -1.74 | 1.62  | -1.20 |
| Mup9   | major urinary protein 9                                                   |                                                                         | 1.05  | —     | -1.36 |
| Gm5478 | predicted pseudogene 5478                                                 | Unknown                                                                  | —     | —     | -1.34 |
| Gm6484 | predicted gene 6484                                                       | Unknown                                                                  | —     | —     | -1.34 |
| Gm15564| predicted gene 15564                                                      | Unknown                                                                  | 2.21  | —     | -1.31 |
| TMEM134| transmembrane protein 134 (Tmem134), transcript variant 1, mRNA          | Unknown                                                                  | —     | —     | -1.30 |
| 2610528A11Rik | RIKEN cDNA 2610528A11 gene                                           | **Unknown**                                                               | —     | —     | -1.27 |
| Rps2-ps10 | ribosomal protein S2, pseudogene 10                                      | Pseudogene                                                               | —     | —     | -1.21 |
| Apol11b| apolipoprotein L 11b                                                      |                                                                         | -3.04 | —     | —     |
| Gm14279| predicted gene 14279                                                      | Unknown                                                                  | -1.65 | 5.34  | —     |
| Gm12248| predicted gene 12248                                                      | Unknown                                                                  | -1.07 | —     | 1.21  |
| Rps8-ps2| ribosomal protein S8, pseudogene 2                                        | Pseudogene                                                               | —     | -3.71 | —     |
| Rpl15-ps2| ribosomal protein L15, pseudogene 2                                      | Pseudogene                                                               | —     | -2.08 | —     |
| Gm9581 | predicted gene 9581                                                       | Unknown                                                                  | —     | -1.38 | —     |
| Apol9a | apolipoprotein L 9a                                                       |                                                                         | -1.25 | —     | —     |
| Gm24407| predicted gene, 24407                                                     | Unknown                                                                  |       |       | -1.27 |
| Gsdmc2 | gsdemrin C2                                                               |                                                                         | 2.19  | 4.66  | -4.01 |
| Gm25360| predicted gene, 25360                                                     | Unknown                                                                  | —     | —     | -3.89 |
| Gm24265| predicted gene, 24265                                                     | Unknown                                                                  | 1.74  | 4.93  | -3.54 |
| Rprl3  | ribonuclease P RNA-like 3                                                 |                                                                         | —     | Inf   | -3.14 |
| Gm19980| predicted gene, 19980                                                     |                                                                          | —     | —     | -2.49 |
| Gm24187| predicted gene, 24187                                                     |                                                                          | 2.49  | -2.47 | —     |
| Akr1cl | aldo-keto reductase family 1, member C-like                               |                                                                         | —     | —     | -2.43 |
| Mup7   | major urinary protein 7                                                   |                                                                         | —     | —     | -2.36 |
| Gm23935| predicted gene, 23935                                                     |                                                                         | 3.54  | -2.31 | —     |
| Gm4841 | predicted gene 4841                                                      |                                                                         | —     | —     | -1.95 |
| BC117090 | cDNA sequence BC117090                                                  | **Unknown**                                                               | —     | —     | -1.95 |
| Gm24270| predicted gene, 24270                                                     |                                                                         | 2.77  | -1.88 | —     |

(Continued)
We found 1477, 1298, and 1351 genes with significantly increased transcript levels in infected ears compared to non-infected ears from the same challenged mice at days one, four, and seven, respectively. There were 585 genes that exhibited significantly decreased transcript levels at the site of infection (comparing infected ears compared to uninfected ears from challenged mice) at Day 1, 195 at Day 4, and 143 at Day 7. The complete list is available in S1 Table. In order to evaluate the systemic response, we also compared the RNA-seq profiles of the uninfected ears from challenged mice to those of naïve mice. In this analysis we found 123 genes with significantly increased transcript levels at Day 1, 66 at Day 4, and 38 at Day 7. We found 21 genes with significantly decreased transcript levels in uninfected ears from challenged mice compared to naïve mice at Day 1, 9 at Day 4, and 56 at Day 7 (S1 Table). These results demonstrate that the challenged mice exhibit a higher level of differential gene expression locally at the infection site than at more distal sites.

Because of the large amount of data generated in these studies, the top 50 differentially expressed genes at each tested time point are presented in Tables 1–4. These tables were compiled by sorting the gene expression data for each comparison (infected ears to non-infected ears from challenged mice for the local response, and non-infected ears from challenged mice to naïve mice for the systemic response) at each time point (Day 1, 4, and 7), and listing the 50 genes for each that had the highest LFC for genes with increased transcript levels, or the lowest LFC for genes that had decreased transcript levels. For each of the top 50 genes, we then determined the LFC at the other time points and added these values to the tables to show the gene’s change in expression over time. Many genes were highly altered in transcript levels at more than one time point; thus, none of Tables 1–4 contain 150 genes (50 for each of the time points).

Table 1 contains the top genes with the highest increases in transcript levels for each time point during the local response, where we compared infected ears at each time point to the corresponding uninfected ears from the same mice. Of these top genes, 39 encode proteins that have known functions in the immune response, including 17 cytokines, chemokines, and chemokine-like proteins, as well as Cxcr1, which encodes an IL-8 receptor. Several genes encoding cytotytic granule proteins (Olfm4, Mcpt1, and Gzmk) were also top up-regulated immune genes at least at one tested time point during SSTI. Overall, the majority of genes with a known immune function that have the greatest increases at the site of infection appear to have a role in the innate response. Table 1 also shows that 22 genes encoding proteins involved in metabolism, cell proliferation, and regulation are among the genes with the greatest transcript increases at the site of infection. We also found six genes encoding epidermal proteins, and 39 uncharacterized genes, in the top 50 list of genes with the highest increases in transcript levels.

| Symbol  | Gene Name                          | Function | LFC^3 | Day 1 | Day 4 | Day 7 |
|---------|------------------------------------|----------|-------|-------|-------|-------|
| Gm26917 | predicted gene, 26917               | Unknown  |       | —     | —     | -1.58 |
| Gm21887 | predicted gene, 21887               | Unknown  |       | —     | —     | -1.45 |

^1All genes that had a significantly decreased LFC when comparing uninfected ears from challenged mice to naïve mice are listed.

^2Function determined via Entrez (www.ncbi.nlm.nih.gov) or Uniprot (www.uniprot.org)

^3LFC = Log Fold Change

Italicized values indicate transcripts are significantly increased at the indicated time point.

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We also note that the majority of the genes in Table 1 had significantly increased transcript levels at more than one tested time point, suggesting that the response is consistent over time. This is reflected in the overall list of genes with locally significant increases in transcript levels (S1 Table), with 60% of these genes demonstrating significant increases in transcript levels at multiple times (Fig 1A).

Table 2 contains the genes that demonstrated the greatest increases in transcript levels systemically at each tested time point; these genes were differentially expressed when comparing the uninfected ears from challenged mice to those of naïve mice. Of these genes, 20 encode proteins with an immune function. The immune genes that are common between the local and systemic responses are Cxcl2, Cxcl3, Irg1, S100a9, Saa3, Il-1β, Trem1, and Clec4e. For each of these genes, the LFC in the local response is higher than it is in the systemic response (i.e. 8.97
vs 2.29 for the LFC of Cxcl3 at Day 1 locally and systemically, respectively). Also, while transcript levels for each of these common genes are consistently increased at each time point in the local response, only Cxcl2, Cxcl3, and Irg1 are significantly increased at all three time points when looking at the systemic response. The higher number of top genes that encode proteins that function in the immune response, as well as their greater and longer transcript level increases, suggest that the immune response is focused at the site of infection rather than systemically during staphylococcal SSTI.

Table 2 indicates that 49 genes with the greatest increases in transcript levels during the systemic response (comparing non-infected ears from challenged mice to naïve mice) are of unknown function. Of the characterized genes, 31 encode proteins that are involved in metabolism, cell proliferation, and regulation, and 20 in epithelial cell and/or hair formation. However, unlike in the local response, where many of the top genes had consistently increased transcript levels throughout the infection, most of the systemically up-regulated genes showed significant differential expression in the challenged mice compared to naïve, control mice at only one time point. Like with the local response, this trend was reflected in the entire list of systemically up-regulated genes (S1 Table and Fig 1C). We also saw that some genes had among the highest LFC at one time point, but were significantly down-regulated at another (indicated with italics, Table 2). These observations suggest that genetic changes happening systemically are more transient and that change occurs more quickly than at the site of infection, where genes with increased expression levels generally appear to exhibit more stable expression over time.

Table 3 contains the 50 genes with the greatest decreases in transcript levels at the site of infection, comparing infected ears to the uninfected ears from the same challenged mice. Again, many of these genes encode proteins with unknown function; those that have a known function appear important for cellular proliferation, differentiation, and metabolism. Only five of the genes in Table 3 encode proteins with an immune function. Fcer2a, which encodes an Fc receptor for IgE, has consistently decreased transcript levels at Days 1, 4, and 7, and Serpinb1c (which encodes a neutrophil proteinase inhibitor), is down-regulated starting at Day 4 post-challenge. Two genes that encode proteins important for binding to bacterial products (Bpifb2 and Lyg2, which encode proteins that bind LPS and peptidoglycan, respectively) have significantly decreased transcript levels at Day 7. Forty-six of the genes that have the greatest decreases in transcript levels at the site of infection encode proteins that are involved in metabolism/cell proliferation/regulation, with the majority of these genes demonstrating transcript decreases early during infection (Day 1 or Day 4 post-challenge). We also found that, by day 7, 16 of the genes with the greatest decreases in transcript levels in response to local infection encode proteins that are involved in epidermal formation. However, the majority of genes with the greatest decreases in transcript level at each time point encode proteins of unknown function, with 62 of the top genes falling into this category. Unlike the genes that had significantly increased transcript levels, where a high proportion of genes had transcript increases at more than one time point, genes with significant decreases in transcript levels were mostly not shared over multiple times, which was observed both with the genes with greatest decrease in transcription (shown in Table 3) and in the entire list of genes with significant transcript decreases at the site of infection (S1 Table and Fig 1B). Out of 769 genes that had significant decreases in transcript levels in infected ears compared to non-infected ears from challenged mice, only 126 were shared over multiple time points, suggesting that the decrease in transcript levels of locally-expressed genes in our SSTI model is also more transient than transcript increases.

Table 4 contains all of the genes that had significant decreases in transcript levels systemically as there were fewer than 50 for each time point. Twelve of these genes encode proteins with an immune function. Ten of these twelve are decreased only at Day 7 post-challenge, and of
these, six are involved in the adaptive response (*Ighg2c*, *Igk*, which encode the gamma and kappa chains of immunoglobulin; *Pax5*, which encodes a protein involved in B cell differentiation [19]; *Ms4a1*, which encodes CD20, a regulator of B cell activation and proliferation [20], *Cd22*, which encodes a protein that functions in B cell signaling [21], and *Cd99*, which encodes a protein that plays a role in T cell migration into inflamed skin [22]). The majority of genes whose transcripts were decreased in non-infected ears from challenged mice compared to ears from naïve mice encode proteins that have functions in cellular metabolism, proliferation, or regulation, or lack a known function. Several genes that exhibited significant transcript decreases at one time point showed significant transcript increases at another (highlighted in italics in Table 4). Because the RNA preps from challenged mice used for this analysis are the same ones used in other analyses (the non-infected ear RNA used here as the test RNA is the same RNA used as the control RNA for examining at the local response) where we see very consistent expression of genes over time, we do not believe these temporal differences in gene expression are aberrant. The LFC values for systemic gene expression would also be affected by the number of reads in ears from naïve mice in these comparisons. However, because we used matched control mice that were kept in the same conditions and sacrificed at the same time as the challenged mice, we feel that any changes in gene expression due to environmental factors will be similar among all mice within a time point cohort. Therefore, we hypothesize the genes transcribed during the systemic response may have a transient expression pattern with rapid increases and decreases in transcript levels as the infection progresses.

In order to evaluate whether protein production mirrors gene expression, we performed Western blotting on homogenized ear lysates using antibodies specific for three proteins encoded by genes that were increased locally during infection. Western blots indicated that protein production for IL-1β, S100A8, and S100A9 is significantly increased upon infection (Fig 2, lanes denoted by “I”), which confirms the RNA-seq data for the genes encoding these proteins and further validates the RNA-seq data as a whole.

**Upstream Regulator Analysis of murine gene expression during SSTI**

In order to funnel down the transcriptomic data, we next used the Upstream Regulator Analytic (URA) tool from the Ingenuity Pathway Analysis software package (www.ingenuity.com) to

| Day | 0 | 1 | 4 | 7 |
|-----|---|---|---|---|
|     | I | U | S | N |
|     |   |   |   |   |
| S100A8 | | | | |
| S100A9 | | | | |
| Precursor | | | | |
| IL-1 beta | | | | |
| Mature | | | | |
| GAPDH | | | | |

**Fig 2.** Protein expression for selected genes demonstrating locally increased transcripts. Western blots on ear lysates from infected ears (I), uninfected ears from the same challenged mice (U), sham-infected mice that were pricked with sterile PBS (S), and naïve, uninfected mice (N) using antibodies specific for S100A8, S100A9, IL-1β, and GAPDH at days 1, 4, and 7 post-challenge are shown. The precursor and mature forms of IL-1β are indicated.

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identify potential pathways that may be activated or repressed during *S. aureus* SSTI in the epi-cutaneous challenge model. This analysis software compares a curated database of genes and pathways to experimentally obtained transcriptomic data. It then uses statistical analysis to determine what percent of the genes within a pathway are differentially expressed in the data, and hypothesizes whether that pathway is activated or inhibited. If a high proportion of genes within a pathway are expressed in a manner that the database indicates signifies the pathway is functioning, URA will specify that the pathway is activated. Conversely, if a significant proportion of genes within a pathway are expressed in a manner opposite to what would be expected if the pathway is active, URA will indicate that the pathway is likely inhibited. Therefore, while URA is hypothetical, the analysis provides evidence-based suggestions of pathways that may be activated or inhibited based on transcriptomic data. It will also highlight activated or inhibited pathways that have upstream regulators that are not differentially expressed. These two factors allow for a global view of the transcriptomic data and for investigation into genes as pathway regulators that could otherwise be missed.

On Day 1 post-challenge, URA of the RNA-seq data for the local response (comparing infected ears to non-infected ears from the same challenged mice) indicated that 153 regulatory pathways were potentially activated at the site of infection. However, at this same time point, URA of the non-infected ears from challenged mice compared to naïve mice (systemic response) showed no activated pathways. At Day 4 post-challenge, there were 133 potentially activated pathways at the site of infection (local response), whereas URA of the systemic response showed only two activated pathways. At Day 7 post-challenge, URA of the RNA-seq data for the infected vs uninfected ears from the same mice (local response) showed indicated 127 possibly activated pathways, and URA of the RNA-seq data from the non-infected ears from challenged mice vs naïve mice (systemic response) showed 10 potentially activated pathways.

When evaluating inhibition of regulatory pathways, URA indicated 29 potentially inhibited pathways at the site of infection Day 1 post-challenge. On Day 4, 24 pathways were inhibited at the site of infection. When comparing uninfected ears from challenged mice and naïve mice to evaluate the systemic response, no pathways were inhibited on either Day 1 or Day 4. On Day 7 post-challenge, 23 pathways were potentially inhibited when analyzing the RNA-seq data from infected vs uninfected ears from challenged mice (local response). Systemically, three pathways were possibly inhibited when examining RNA-seq data from uninfected ears from challenged mice compared to naïve mice.

**Evaluation of upstream regulator activation during the course of SSTI**

We next examined the URA analyses to categorize the types of pathways that were indicated as potentially activated during SSTI, both at the site of infection and at distal skin sites. For simplification, we are presenting only the top activated and inhibited pathways here (Tables 5 and 6; full URA data are available in S2–S7 Tables). In order to choose these top pathways, we sorted the URA data based on overlap *p* value, with the most significant *p* values listed first (signifying pathways that have the most significant overlap with pathways listed in the IPA Knowledge Base). We then further sorted for top pathways by choosing the most significant pathways (based on *p* value) that had an Activation Z score ≥ 2 (for activated pathways) or ≤ -2 (for inhibition). When more than 10 pathways met these criteria, we chose the 10 with the most significant *p* values.

The top activated pathways are listed in Table 5. The majority of top pathways activated in the local response (comparing the infected ears to non-infected ears from challenged mice) were shared across all three tested time points and several were activated systemically by Day 7.
Table 5. Top Activated Upstream Regulators.

| Upstream Regulator (Pathway) | Category                  | Activation Z score | P value of overlap |
|------------------------------|---------------------------|--------------------|--------------------|
| **Day 1 Local Response**     |                           |                    |                    |
| TNF                          | cytokine                  | 9.473              | 2.55E-55           |
| NFkB (complex)               | complex                   | 7.035              | 3.92E-35           |
| IFNG                         | cytokine                  | 7.25               | 4.04E-34           |
| IL-1β                        | cytokine                  | 7.322              | 4.53E-34           |
| RELA                         | transcription regulator    | 4.806              | 2.55E-28           |
| IL-1α                        | cytokine                  | 6.22               | 3.05E-27           |
| STAT3                        | transcription regulator    | 2.822              | 3.75E-26           |
| TREM1                        | transmembrane receptor    | 3.535              | 7.68E-26           |
| JUN                          | transcription regulator    | 3.249              | 1.21E-21           |
| TCR                          | complex                   | 3.782              | 7.82E-21           |
| **Day 1 Systemic Response**  |                           |                    |                    |
|                              | No activated upstream regulators |                 |                    |
| **Day 4 Local Response**     |                           |                    |                    |
| TNF                          | cytokine                  | 8.697              | 1.48E-50           |
| IFNy                         | cytokine                  | 6.83               | 5.39E-43           |
| NFkB (complex)               | complex                   | 7.063              | 8.49E-37           |
| IL-1β                        | cytokine                  | 6.43               | 6.03E-35           |
| IL-1α                        | cytokine                  | 6.069              | 2.91E-30           |
| TCR                          | complex                   | 3.741              | 3.19E-24           |
| TREM1                        | transmembrane receptor    | 3.493              | 5.09E-23           |
| RELA                         | transcription regulator    | 4.386              | 7.45E-23           |
| JUN                          | transcription regulator    | 3.223              | 1.55E-22           |
| IL-27                        | cytokine                  | 2.403              | 5.84E-21           |
| **Day 4 Systemic Response**  |                           |                    |                    |
| IL-1α                        | cytokine                  | 2.184              | 8.80E-08           |
| TNF                          | cytokine                  | 2.572              | 1.66E-07           |
| **Day 7 Local Response**     |                           |                    |                    |
| IFNy                         | cytokine                  | 7.934              | 1.78E-52           |
| TNF                          | cytokine                  | 8.592              | 1.11E-44           |
| NFkB (complex)               | complex                   | 6.327              | 1.68E-31           |
| TCR                          | complex                   | 3.319              | 1.38E-29           |
| IL-1β                        | cytokine                  | 5.528              | 1.26E-27           |
| TGM2                         | enzyme                    | 7.107              | 9.06E-26           |
| IL-27                        | cytokine                  | 2.837              | 3.28E-25           |
| IL-12 (complex)              | complex                   | 3.029              | 3.98E-24           |
| STAT3                        | transcription regulator    | 2.78               | 6.94E-24           |
| IL-21                        | cytokine                  | 3.548              | 9.54E-24           |
| **Day 7 Systemic Response**  |                           |                    |                    |
| IL-17A                       | cytokine                  | 2.376              | 3.82E-11           |
| TNF                          | cytokine                  | 2.617              | 2.88E-10           |
| TLR7                         | transmembrane receptor    | 2.399              | 7.15E-10           |
| IL-1β                        | cytokine                  | 2.31               | 1.28E-09           |
| CAMP                         | other                     | 2.219              | 2.49E-09           |
| NFkB (complex)               | complex                   | 2.606              | 2.96E-09           |
| RELA                         | transcription regulator    | 2.343              | 3.98E-09           |
| IL-1α                        | cytokine                  | 2.416              | 5.87E-09           |

(Continued)
post-challenge. The TNF pathway is activated locally on Days 1, 4, and 7, and systemically (comparing non-infected ears from challenged mice to naïve mice) on Days 4 and 7. The NFκB and IL-1β pathways are activated locally at all three time-points, and systemically on Day 7. The IL-1α pathway was among the top activated pathways locally on Day 1, both locally and systemically on Day 4, and systemically on Day 7. It was also activated locally on Day 7, though it was not a top pathway under this condition (Z score 5.045, P value 1.04 x 10^-19; S6 Table). The RELA pathway was a top activated pathway locally at Days 1 and 4, and systemically on Day 7. This pathway was activated locally at Day 7 also, but again was not among the top pathways (Z score 3.822, P value 1.72 x 10^-19; S6 Table). Other top pathways were activated only locally at the site of infection, including IFNγ, TCR, STAT3 (not on the top pathway list at Day 4; Z score 2.459, P value 1.04 x 10^-19; S4 Table), TREM1 (not on the top pathway list at day 7; Z score 2.242, P value 3.6 x 10^-16; S6 Table), and IL-27 (not on the day 1 top activated pathway list; Z score 2.168, P value 3.6 x 10^-16; S2 Table). The top activated pathways at all three tested time points are all involved in the immune response. In particular, these pathways suggest the importance of the TH1 (IFNγ, TREM1, IL-27), TH17 (STAT3, IL-1β, IL-17A), and overall pro-inflammatory responses (NFκB, IL-1α, RELA, JUN, ERK1/2, SELPLG).

Pathway inhibition was similar across all three time points as well, with 24 out of 43 inhibited pathways shared at Days 1, 4, and 7 (S2–S7 Tables). As with the activated pathways, we also listed the top inhibited pathways in Table 6. Of these top pathways, two (JAG2 and Mir-155-5p) were inhibited locally at all three tested time points, and systemically at Day 7, and five were among the top locally inhibited pathways at all three time points but unaffected systemically (CD3, IL-1RN, TAB1, CD28, and MAPK1). Two pathways were on the top inhibited pathways lists at Days 4 and 7, and were also inhibited on Day 1, but did not make the top list for this time point (Mir-146a-5p, Z score -3.075, P value 3.6 x 10^-16; S2 Table). IL-37, which was a top locally inhibited pathway on Day 7, was also inhibited on Days 1 (Z score -2.408, P value 3.6 x 10^-16; S2 Table) and 4 (Z score -2.408, P value 1.22 x 10^-7; S4 Table) but was not a top inhibited gene at these times. The SOCS3 pathway was a top locally inhibited pathway at Day 7, and was also locally inhibited at Day 4 (Z score -2.619, P value 2.21 x 10^-7; S4 Table) but was not inhibited at day 1. These data further support the similarity of the response over time during S. aureus SSTI. As with the top activated pathways, the top inhibited pathways all function in the immune response. These pathways generally appear to be involved in the anti-inflammatory response (e.g., SOCS1 and SOCS3, which work to suppress cytokine production, and IL-37, which inhibits innate immunity [23]). Inhibition of these anti-inflammatory pathways may augment a pro-inflammatory response.

Table 5. (Continued)

| Upstream Regulator (Pathway) | Category | Activation Z score^2 | P value of overlap^3 |
|-----------------------------|----------|----------------------|---------------------|
| SELPLG                      | other    | 2                    | 2.46E-07            |
| ERK1/2                      | group    | 2.19                 | 1.45E-06            |

1Top upstream regulators selected based on p value of overlap and activation Z score. Data are ordered by p value of overlap. If more than 10 pathways were indicated as activated for a condition, the top 10 regulators were chosen as the 10 most significant regulators (based on p value) that had a Z score ≥ 2.

2Z score infers the activation states of predicted regulators based on expression of the downstream genes within the pathway; a score ≥ 2 indicates activation.

3P value of overlap evaluates whether there is a statistically significant overlap between differentially expressed genes and the genes that are regulated by the upstream regulator.

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Table 6. Top Inhibited Upstream Regulators\(^1\).

| Upstream Regulator (Pathway) | Category            | Activation Z score\(^2\) | P value of overlap\(^3\) |
|-----------------------------|---------------------|---------------------------|--------------------------|
| **Day 1 Local Response**    |                     |                           |                          |
| JAG2                        | growth factor       | -4.32                     | 3.98E-15                 |
| MAP3K7                      | kinase              | -2.316                    | 2.20E-14                 |
| IgG                         | complex             | -2.187                    | 1.97E-13                 |
| CD3                         | complex             | -4.555                    | 3.55E-13                 |
| TAB1                        | enzyme              | -3.85                     | 6.56E-13                 |
| miR-155-5p (miRNAs w/seed UAAUGCU) | mature microRNA    | -3.394                    | 9.54E-11                 |
| IL1-RN                      | cytokine            | -4.776                    | 1.35E-10                 |
| TRAF3                       | enzyme              | -2.345                    | 4.52E-10                 |
| CD28                        | transmembrane receptor | -2.823             | 4.99E-10                 |
| MAPK1                       | kinase              | -4.137                    | 6.48E-10                 |
| **Day 1 Systemic Response** |                     |                           |                          |
| No inhibited upstream regulators |                     |                           |                          |
| **Day 4 Local Response**    |                     |                           |                          |
| CD3                         | complex             | -4.135                    | 4.18E-17                 |
| IL1-RN                      | cytokine            | -4.981                    | 1.43E-15                 |
| JAG2                        | growth factor       | -3.961                    | 1.57E-13                 |
| CD28                        | transmembrane receptor | -3.064               | 4.94E-13                 |
| miR-155-5p (miRNAs w/seed UAAUGCU) | mature microRNA    | -3.406                    | 8.37E-13                 |
| TAB1                        | enzyme              | -3.582                    | 5.26E-12                 |
| miR-146a-5p (and other miRNAs w/seed GAGAACU) | mature microRNA | -3.494                    | 1.52E-10                 |
| IL-37                       | cytokine            | -2.408                    | 2.02E-07                 |
| MAPK1                       | kinase              | -3.297                    | 2.02E-07                 |
| SOCS1                       | other               | -2.959                    | 2.06E-07                 |
| **Day 4 Systemic Response** |                     |                           |                          |
| No inhibited upstream regulators |                     |                           |                          |
| **Day 7 Local Response**    |                     |                           |                          |
| CD3                         | complex             | -4.313                    | 2.37E-19                 |
| IL1-RN                      | cytokine            | -4.902                    | 4.48E-19                 |
| MAPK1                       | kinase              | -4.783                    | 1.18E-16                 |
| TAB1                        | enzyme              | -3.359                    | 3.72E-15                 |
| miR-155-5p (miRNAs w/seed UAAUGCU) | mature microRNA    | -3.159                    | 2.59E-14                 |
| CD28                        | transmembrane receptor | -3.421               | 3.47E-14                 |
| JAG2                        | growth factor       | -3.221                    | 2.31E-12                 |
| SOCS3                       | phosphatase         | -2.376                    | 2.38E-10                 |
| miR-146a-5p (and other miRNAs w/seed GAGAACU) | mature microRNA | -3.357                    | 1.22E-09                 |
| SOCS1                       | other               | -3.124                    | 1.43E-08                 |
| **Day 7 Systemic Response** |                     |                           |                          |
| miR-155-5p (miRNAs w/seed UAAUGCU) | mature microRNA    | -2.607                    | 5.40E-14                 |
| JAG2                        | growth factor       | -2.216                    | 2.49E-09                 |
| IL-13                       | cytokine            | -2.200                    | 2.12E-05                 |

\(^1\)Top upstream regulators selected based on p value of overlap and activation Z score. Data were ordered by p value of overlap. If more than 10 pathways were indicated as inhibited for a condition, the top 10 regulators were chosen as the 10 most significant regulators (based on p value) that had a Z score \(< -2\).

\(^2\)Z score infers the activation states of predicted regulators based on expression of the downstream genes within the pathway; A score \(< -2\) indicates inhibition.

\(^3\)P value of overlap evaluates whether there is a statistically significant overlap between differentially expressed genes and the genes that are regulated by the upstream regulator.

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We also found that the CD3 and CD28 pathways, both critical to T cell receptor signaling [24], were consistently inhibited at all three time points.

Though we did see a higher level of variability in the RNA-seq data in terms of differential expression of individual genes at the tested time points (refer to Tables 1–4, which demonstrate that a number of the genes that are highly differentially expressed at one time are not differentially expressed at other times), the URA suggests that global pathway activation and repression is similar over the course of infection.

Discussion

In order to better understand the effects of staphylococcal infection on the host, we performed a comprehensive genetic analysis of the mouse transcriptome using high-throughput sequencing of cDNA (RNA-Seq) prepared from RNA enriched from mouse ears using an epicutaneous infection model [13]. In this study, we chose to compare infected ears to uninfected ears from the same challenged mice in order to evaluate the local response at the site of infection. We also compared cDNA from the uninfected ears from challenged mice to cDNA from ears of naïve mice in order to determine differential gene expression systemically over the time course of infection. Through these analyses, we determined that a large number of genes are differentially expressed at the site of infection both early and late during SSTI, while a smaller number of genes are affected systemically. When we categorized these differentially expressed genes into pathways using the IPA Upstream Regulator Analytic (URA), we found that all of the pathways that showed the highest level of significance both locally and systemically were involved in the immune response.

URA bundles transcriptomic data together into potentially activated or inhibited pathways, which can give hints as to what is happening within the cellular environment to lead to the observed expression data results. URA can predict activation or inhibition of pathways whether or not the gene encoding the upstream regulator itself is differentially expressed, providing added benefit over examining gene expression data alone. The majority of the predicted activated pathways, in fact, did not have a differentially expressed upstream regulator (S2–S7 Tables). For example, both the IL-12 and IL-18 pathways were predicted to be activated at every time point (S2–S7 Tables), and while Il-12a and Il-12b show significantly increased transcript levels, the Il-18 gene itself was not differentially expressed (S1 Table). If one were only examining the gene expression data, the potential importance of the IL-18 pathway could be missed. These analyses broaden avenues of future study of staphylococcal SSTI using a rational, statistically-based categorization of gene expression data. However, it is important to note that the URA is theoretical, and follow-up experiments are necessary to confirm the activation or inhibition of these pathways.

The immune-associated genes that demonstrated the highest differential expression at the site of infection (Table 1) suggest a significant local inflammatory response. Our data largely agree with the microarray analysis performed by Cho and colleagues [9,11]; all 20 top up-regulated genes at four hours post-infection in the previous dataset had significantly increased transcript levels in our SSTI model at Day 1 post-infection. The activation of the IL-1β pathway at all tested time points in our data, demonstrated through URA, also supports Cho et al.’s contention that this cytokine plays an important role in SSTI. Our URA predicts that the IL-1α pathway was also activated at the site of infection at all tested time points; this pathway was activated systemically both at Day 4 and Day 7. Several pathways that are important for the TH17 response were activated at the tested time points, including the TNF, IL-6, TGF-β1, IL-21, and GM-CSF pathways. Cho and colleagues also demonstrated the importance of IL-17 in resolution of S. aureus skin infection [11]. Il-17a was also one of the top locally up-regulated genes.
genes Day 1 post-challenge in our model, and was IL-17 was activated globally as a pathway regulator (meaning that a significant number of downstream genes were differentially expressed) at all three time points.

We feel that the high degree of similarity between our Day 1 data and Cho et al.’s four hour post-infection data [9] validate the results we obtained through RNA-seq. Future studies will follow up on the biological significance of several genes that we have found to be differentially expressed in our model.

Our data expand on Cho and colleagues’ analyses through testing more time points and through use of a more sensitive method of studying differential gene expression. We also evaluated both local and systemic responses to S. aureus SSTI instead of focusing only on the response at the site of infection. In order to best elucidate protective immune responses, we believe it is important to fully understand gene expression both at the site of infection and at distal sites.

While a significant proportion of differentially expressed genes were shared between Days 1, 4, and 7 (Fig 1), we also found many genes that are differentially expressed early and not late, or vice versa. For example, Saa2, which encodes an acute phase protein, has significantly increased transcript levels at the site of infection on Days 1 and 4, but is not differentially expressed at Day 7. SAA2 is important in the initial response to infection and its expression is activated by pro-inflammatory cytokines [25]. Csf2, which encodes a cytokine involved in granulocyte production, and the gene encoding Ficolin B, a pattern recognition receptor [26], both demonstrate significant transcript increases only at Day 1 at the site of infection. Conversely, the gene encoding granzyme K, a cytolytic granule found in CTLs and NK cells [27], had significant increases in transcript levels at the site of infection only at Day 7. These genes’ expression profiles give a sense of how the host response is changing over time.

We also found a large number of uncharacterized genes that were differentially expressed early during infection but not late, or vice versa. These genes could be potentially interesting as unknown mediators of any number of pathways, host responses, cellular metabolism, etc. Because SSTI is self-limiting [13], we hypothesize that the genes that are differentially expressed later but not early are potentially most important for direct resolution of the infection, and may provide insight into the response that leads to clearance by 14 days post-challenge. However, those genes that are expressed only early in the infection might be important for helping to stimulate later responses, or for holding bacterial levels in check, allowing for future resolution.

Through examining gene expression over time, we also were able to demonstrate that activation of systemic immune responses lags behind that of the local response. Of the immune genes with the most significantly systemically increased transcript levels only on Day 7 (see Table 2), all except for two (Il-20 and Defb6) had significant increases in transcripts locally by one day after infection (Table 1 and S1 Table). The kinetics of infection, along with the patterns of gene expression, may help aid in dissection of temporal changes that lead to clearance.

Our gene expression data suggest that a TH1 response is occurring during staphylococcal SSTI. Ifnγ had significant increases in transcript levels at the site of infection on Days 4 and 7 (LFC = 4.15 and 4.43, respectively; S1 Table), and its pathway was also predicted through URA as activated locally at Days 1, 4, and 7 after challenge because a large number of genes affected by IFNγ demonstrated expression patterns indicative of pathway activation (S2, S4 and S6 Tables). This cytokine is important for a variety of immune functions, including activating innate immune cells and skewing the adaptive response toward TH1 [28]. IL-12 and IL-18 synergistically amplify IFNγ production [29], increasing the TH1 response. Both genes encoding IL-12 had significantly increased transcript levels at the site of infection in our model (IL-12a Day 1 LFC 2.84; Day 4 LFC 2.00; IL-12b Day 4 LFC 2.23, Day 7 LFC 4.29; S1 Table). We also saw
significant local transcript increases of genes encoding the IFN\(\gamma\)-inducible TH1 chemokines CXCL9 (Table 1), CXCL10 (LFC 4.21, 4.59, and 4.87, respectively), and CXCL11 (LFC 2.09, 3.75, and 5.21, respectively) at Days 1, 4, and 7 [30]. Whether this response is beneficial is debatable, as Montgomery and colleagues demonstrated that the TH1/IFN\(\gamma\) pathway prevented protective immunity in their model of SSTI [31], and Nippe et al. demonstrated in a different subcutaneous infection model that mice skewed toward a TH1 response exhibited increased swelling at the infection site and higher bacterial loads by one week post-challenge [32]. The pathways controlled by IL-4, the hallmark cytokine of a TH2 response [28], as well as TH2 cytokine IL-13, were not activated in our model. In fact, the IL-13 pathway is predicted to be inhibited systemically by Day 7 post-challenge (S7 Table). These results are suggestive that, at least in our epicutaneous model of SSTI, the TH1 response may be augmented over the TH2 response. Previous work demonstrated that anti-alpha toxin antibody levels can correlate at least partially to protection in this model [33], but it remains unclear what mechanism of protection is most important in natural infection. The TH1 response augments the production of complement-fixing and opsonizing antibodies [34], which could be important in the response to S. aureus. Further studies will be required to dissect TH1/TH2 responses in the epicutaneous SSTI model.

Besides the mediators discussed above, a substantial number of other genes encoding proteins that are important for the innate response had significantly increased transcript levels, or their pathways were predicted as activated based on the transcriptional data, in our SSTI model. These include a variety of other cytokines and chemokines, factors important for leukocyte adhesion, components of the complement cascade, proteases found in neutrophil granules, and mast cell proteases. Since the infection is self-limiting, remains localized, and clears by day 14, it is likely that the innate response plays an important role in containing S. aureus SSTI. Neutrophils have been implicated for their importance in controlling these infections [8,9,13]. Our results suggest that other components of the innate response, such as macrophages stimulated through the TH1 response, may also play significant roles, and future work will begin to dissect these mechanisms.

Keratinocytes are now recognized for their importance in regulating the immune response in the skin [35]. These cells produce cytokines and chemokines as well as express cytokine and chemokine receptors. A considerable number of keratin genes had increased transcript levels both locally and systemically during SSTI, especially early in the infection (S1 Table). Besides their importance as major cytoskeletal proteins in the epithelia [36], some keratins have been identified as important regulators of the immune response. While keratin genes have been examined for their roles in inflammatory diseases such epidermolytic ichthyosis [37], their role in the response to staphylococcal pathogenesis has to this point remained undescribed. These genes may provide novel means of targeting these infections. The gene encoding KRT17, which promotes epithelial cell proliferation as well as a TH1/TH17 response [38], had significantly increased transcript levels at the site of infection at Days 1 and 4 post-challenge (LFC 1.76 and 1.39, respectively), and Krt16 was highly expressed at the site of infection at all three time points, with LFCs greater than 5. KRT16 was identified for its importance in innate immune regulation during epithelial infection, where it may provide an important checkpoint in the pro-inflammatory feedback loop [39]. KRT1 is involved in negative regulation of the pro-inflammatory response in the epithelia [40], and Krt1 was significantly up-regulated at Days 4 and 7 at the site of infection in our model (LFC 1.36 and 1.77, respectively). Roth and colleagues established that Krt1\(^{-/-}\) mice have markedly higher levels of the pro-inflammatory cytokine IL-33 [40]. We noted increased transcript levels of Il-33 in infected ears at Day 4 (LFC 1.38, S1 Table). However, by Day 7, Il-33 transcripts are no longer increased, while Krt1 transcripts remain increased. This suggests that KRT1 may play a role in dampening inflammation.
during *S. aureus* SSTI, possibly by modulating IL-33 levels. By Day 7, 50 keratin-associated genes had significantly decreased transcript levels at the site of infection (S1 Table). We hypothesize that keratins may be part of the immune balance in the host during *S. aureus* SSTI, through rapid early increases in keratin gene expression, perhaps to increase cell number/epithelial thickness, as well as to augment and control the innate immune response; as the infection continues, tissue damage worsens and the adaptive response begins to take over. At this point, the keratin genes are down-regulated. KRT8 has been postulated as a potential binding site for *S. aureus* adhesin ClfB [41]. Interestingly, the *Krt8* gene is significantly down-regulated at the site of infection in our model at Day 4 (LFC -1.31, S1 Table). This lowered expression may help the host to decrease bacterial adherence in an effort to limit infection. Overall, the keratins are a largely uninvestigated area of the *S. aureus*/host interaction that deserves greater attention.

While the current literature studying responses to *S. aureus* SSTI have focused on inflammatory responses, our data suggest that genes that help to keep inflammation in check may also be important in the host response. A number of differentially expressed genes at the site of infection encode proteins with immune modulating functions. These include *Nlrp12* (Table 1) [42], *Nlrp6* (significantly increased at the site of infection on Days 1 and 4; S1 Table) [43], *Irg1* (Table 1) [44], *Olfm4* (Table 1) [45], *Socs3* (locally increased transcript levels on Days 1, 4, and 7; S1 Table) [46], zinc finger protein *Zc3h12a* (locally increased transcript levels on days 1 and 4; S1 Table) [47], and several *Cd300* family members (both increased and decreased transcript levels, S1 Table) [48]. We also note that a significant proportion of inhibited pathways are involved in attenuation of inflammation (Table 6 and S2–S7 Tables). We hypothesize that the proteins encoded by these genes may help prevent dysregulated inflammation that could exacerbate skin damage; however, it is likely that a number of anti-inflammatory pathways must also be inhibited in order to obtain a level of inflammation necessary to clear *S. aureus*. This immune balance could be critical for generation of a successful response to the pathogen. Further research into these pathways is required to determine their actual role in the response to SSTI.

In summary, we present a comprehensive transcriptomic analysis of the time course of murine gene expression for up to one week after *S. aureus* challenge in an epicutaneous skin infection model. We examined differential gene expression both at the site of infection as well as systemically at three time points by comparing infected ears to uninfected ears from the same challenged mice, or comparing uninfected ears from challenged mice to naïve mice. We also used computational analysis of the transcriptomic data to generate predictions on potentially activated and inhibited pathways during both the local and systemic response to infection over time. Through these evaluations we found that the systemic response lags behind the local response in terms of pathway activation and inhibition. We determined that a majority of the differentially expressed immune response genes are critical for the innate, TH17, and TH1 responses. We also found that a large number of keratin-associated genes are differentially expressed over time, which could give insight into tissue damage, remodeling, and the potential immune function of these genes. The high level of differential gene expression, both at the site of infection and systemically, provides a great deal of potential avenues for further study into the response to this infection. RNA-seq does not provide information as to which gene(s) are responsible for resolution of infection in our model; instead, the data generated from this work allow us to develop testable hypotheses that may further our knowledge in this area. These data may help us understand how the host can contain the infection in this self-limiting model. Further study using other animal models of SSTI, including humanized mice, will help to determine if the differences in gene expression we elucidated may be applicable to human diseases.
Supporting Information

S1 Table. Log fold change (Log2) for all RNA-seq data. (XLS)

S2 Table. URA of RNA-seq data from local response, Day 1. The data used for this analysis compared infected ears to non-infected ears from challenged mice. (XLS)

S3 Table. URA of RNA-seq data from systemic response, Day 1. The data used for this analysis compared non-infected ears from challenged mice compared to naïve mice. (XLS)

S4 Table. URA of RNA-seq data for local response, Day 4. The data used for this analysis compared infected ears to non-infected ears from challenged mice. (XLS)

S5 Table. URA of RNA-seq data for systemic response, Day 4. The data used for this analysis compared non-infected ears from challenged mice compared to naïve mice. (XLS)

S6 Table. URA of RNA-seq data for local response, Day 7. The data used for this analysis compared infected ears to non-infected ears from challenged mice. (XLS)

S7 Table. URA of RNA-seq data for systemic response, Day 7. The data used for this analysis compared non-infected ears from challenged mice compared to naïve mice. (XLS)

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Author Contributions

Conceived and designed the experiments: RB VB DB. Performed the experiments: RB VB. Analyzed the data: RB VB DB. Contributed reagents/materials/analysis tools: RB VB DB. Wrote the paper: RB VB DB.

References

1. Gorwitz RJ, Kruszon-Moran D, McAllister SK, McQuillan G, McDougal LK, et al. (2008) Changes in the prevalence of nasal colonization with Staphylococcus aureus in the United States, 2001–2004. J Infect Dis 197: 1226–1234. doi: 10.1086/533494 PMID: 18422434

2. Krespi YP, Küshner V (2012) Laser-assisted nasal decolonization of Staphylococcus aureus, including methicillin-resistant Staphylococcus aureus. Am J Otolaryngol 33: 572–575. doi: 10.1016/j.amjoto.2012.02.002 PMID: 22503099

3. Klevens RM, Morrison MA, Nadle J, Petit S, Gershman K, et al. (2007) Invasive Methicillin-Resistant Staphylococcus aureus Infections in the United States. JAMA 298: 1763–1771. PMID: 17940231

4. Moran GJ, Krishnadasan A, Gorwitz RJ, Fosheim GE, McDougal LK, et al. (2006) Methicillin-resistant S. aureus infections among patients in the emergency department. N Engl J Med 355: 666–674. PMID: 16914702

5. Zetola N, Francis JS, Nuermberger EL, Bishai WR (2005) Community-acquired methicillin-resistant Staphylococcus aureus: an emerging threat. Lancet Infect Dis 5: 275–286. PMID: 15854883
6. Grice EA, Kong HH, Conlan S, Deming CB, Davis J, et al. (2009) Topographical and temporal diversity of the human skin microbiome. Science 324: 1190–1192. doi: 10.1126/science.1171700 PMID: 19478181

7. Cogen AL, Yamasaki K, Sanchez KM, Dorschner RA, Lai Y, et al. (2010) Selective antimicrobial action is provided by phenol-soluble modulins derived from *Staphylococcus epidermidis*, a normal resident of the skin. J Invest Dermatol 130: 192–200. doi: 10.1038/jid.2009.243 PMID: 19710683

8. Molne L, Verdengh M, Tarkowski A (2000) Role of neutrophil leukocytes in cutaneous infection caused by *Staphylococcus aureus*. Infect Immun 68: 6162–6167. PMID: 11035720

9. Cho JS, Guo Y, Ramos RI, Hebroni F, Plaisier SB, et al. (2012) Neutrophil-derived IL-1beta is sufficient for abscess formation in immunity against *Staphylococcus aureus* in mice. PLoS Pathog 8: e1003047. doi: 10.1371/journal.ppat.1003047 PMID: 23209417

10. Cho JS, Zussman J, Donegan NP, Ramos RI, Garcia NC, et al. (2011) Noninvasive in vivo imaging to evaluate immune responses and antimicrobial therapy against *Staphylococcus aureus* and USA300 MRSA skin infections. J Invest Dermatol 131: 907–915. doi: 10.1038/jid.2010.417 PMID: 21191403

11. Cho JS, Pietras EM, Garcia NC, Ramos RI, Farzam DM, et al. (2010) IL-17 is essential for host defense against cutaneous *Staphylococcus aureus* infection in mice. J Clin Invest 120: 1762–1773. doi: 10.1172/JCI40891 PMID: 20364087

12. Zhao S, Fung-Leung WP, Bittner A, Ngo K, Liu X (2014) Comparison of RNA-Seq and microarray in transcriptome profiling of activated T cells. PLoS One 9: e78644. doi: 10.1371/journal.pone.0078644 PMID: 24456749

13. Prabhakara R, Foreman O, De Pascalis R, Lee GM, Plaut RD, et al. (2013) Epicutaneous model of community-acquired *Staphylococcus aureus* skin infections. Infect Immun 81: 1306–1315. doi: 10.1128/IAI.01304-12 PMID: 23381997

14. Brady RA, Mocca CP, Prabhakara R, Plaut RD, Shirliff ME, et al. (2013) Evaluation of genetically inactivated alpha toxin for protection in multiple mouse models of *Staphylococcus aureus* infection. PLoS One 8: e63040. doi: 10.1371/journal.pone.0063040 PMID: 23658662

15. Plaut RD, Mocca CP, Prabhakara R, Merkel TJ, Stibitz S (2013) Stably luminescent *Staphylococcus aureus* clinical strains for use in bioluminescent imaging. PLoS One 8: e59232. doi: 10.1371/journal.pone.0059232 PMID: 23550002

16. Trapnell C, Pletcher L, Salzberg SL (2009) TopHat: discovering splice junctions with RNA-Seq. Bioinformatics 25: 1105–1111. doi: 10.1093/bioinformatics/btp120 PMID: 19289445

17. Anders S, Huber W (2010) Differential expression analysis for sequence count data. Genome Biol 11: R106. doi: 10.1186/gb-2010-11-10-r106 PMID: 20979621

18. Kramer A, Green J, Pollard J Jr, Tugendreich S (2014) Causal analysis approaches in Ingenuity Pathway Analysis. Bioinformatics 30: 523–530. doi: 10.1093/bioinformatics/btt703 PMID: 24336805

19. Cobaleda C, Schebesta A, Delogu A, Busslinger M (2007) Pax5: the guardian of B cell identity and function. Nat Immunol 8: 463–470. PMID: 17440452

20. Kuijpers TW, Bende RJ, Baars PA, Grummels A, Derks IA, et al. (2010) CD20 deficiency in humans results in impaired T cell-independent antibody responses. J Clin Invest 120: 214–222. doi: 10.1172/JCI40231 PMID: 20038800

21. Walker JA, Smith KG (2008) CD22: an inhibitory enigma. Immunology 123: 314–325. PMID: 18067554

22. Bixel G, Kloep S, Butz S, Petri B, Engelhardt B, et al. (2004) Mouse CD99 participates in T-cell recruitment into inflamed skin. Blood 104: 3205–3213. PMID: 15280198

23. Nold MF, Nold-Petry CA, Zepp JA, Palmer BE, Bufler P, et al. (2010) IL-37 is a fundamental inhibitor of innate immunity. Nat Immunol 11: 1014–1022. doi: 10.1038/ni.1944 PMID: 20935647

24. Brownlie RJ, Zamoyska R (2013) T cell receptor signalling networks: branched, diversified and bound- ed. Nat Rev Immunol 13: 257–269. doi: 10.1038/nri3403 PMID: 23524462

25. Thorn CF, Whitehead AS (2002) Differential glucocorticoid enhancement of the cytokine-driven transcriptional activation of the human acute phase serum amyloid A genes, SAA1 and SAA2. J Immunol 169: 399–406. PMID: 12077270

26. Hunold K, Weber-Steffens D, Runza VL, Jensenius JC, Mannel DN (2012) Functional analysis of mouse ficolin-B and detection in neutrophils. Immunobiology 217: 982–985. doi: 10.1016/j.imbio.2012.01.013 PMID: 22459270

27. Bratke K, Kuepper M, Bade B, Virchow JC Jr, Luttmann W (2005) Differential expression of human granzymes A, B, and K in natural killer cells and during CD8+ T cell differentiation in peripheral blood. Eur J Immunol 35: 2608–2616. PMID: 16106370

28. Schroder K, Hertzog PJ, Ravasi T, Hume DA (2004) Interferon-gamma: an overview of signals, mechanisms and functions. J Leukoc Biol 75: 163–189. PMID: 14525967
29. Nakahira M, Ahn HJ, Park WR, Gao P, Tomura M, et al. (2002) Synergy of IL-12 and IL-18 for IFN-gamma gene expression: IL-12-induced STAT4 contributes to IFN-gamma promoter activation by up-regulating the binding activity of IL-18-induced activator protein 1. J Immunol 168: 1146–1153. PMID: 11801649

30. Lacotte S, Brun S, Muller S, Dumortier H (2009) CXCR3, inflammation, and autoimmune diseases. Ann N Y Acad Sci 1173: 310–317. doi: 10.1111/j.1749-6632.2009.04813.x PMID: 19758167

31. Montgomery CP, Daniels M, Zhao F, Alegre ML, Chong AS, et al. (2014) Protective immunity against recurrent Staphylococcus aureus skin infection requires antibody and interleukin-17A. Infect Immun 82: 2125–2134. doi: 10.1128/IAI.01491-14 PMID: 24614654

32. Nippe N, Varga G, Holzinger D, Loffler B, Medina E, et al. (2011) Subcutaneous infection with S. aureus in mice reveals association of resistance with influx of neutrophils and Th2 response. J Invest Dermatol 131: 125–132. doi:10.1038/jid.2010.282 PMID: 20882039

33. Abbas AK, Murphy KM, Sher A (1996) Functional diversity of helper T lymphocytes. Nature 383: 787–793. PMID:8893001

34. Pasparakis M, Haase I, Nestle FO (2014) Mechanisms regulating skin immunity and inflammation. Nat Rev Immunol 14: 289–301. doi: 10.1038/nri3646 PMID: 24722477

35. Schweizer J, Bowden PE, Coulombe PA, Langbein L, Lane EB, et al. (2006) New consensus nomenclature for mammalian keratins. J Cell Biol 174: 169–174. PMID:16831889

36. Depianto D, Kerns ML, Dlugosz AA, Coulombe PA (2010) Keratin 17 promotes epithelial proliferation and tumor growth by polarizing the immune response in skin. Nat Genet 42: 910–914. doi: 10.1038/ng.665 PMID: 20871598

37. Li Y, Zhang P, Wang C, Han C, Meng J, et al. (2013) Immune responsive gene 1 (IRG1) promotes endotoxin tolerance by increasing A20 expression in macrophages through reactive oxygen species. J Biol Chem 288: 16225–16234. doi: 10.1074/jbc.M113.454538 PMID: 23609450

38. Carow B, Rottenberg ME (2014) SOCS3, a Major Regulator of Infection and Inflammation. Front Immunol 5: 58. doi: 10.3389/fimmu.2014.00058 PMID: 24600449

39. Brady BL, Muljo SA (2013) RNA decay tolerizes: MCPIP1 (Zc3h12a) keeps inflammation in check by cleaving 3' UTRs. Immunol Cell Biol 91: 331–332. doi: 10.1038/icb.2013.19 PMID: 23628803

40. Ascher DK, Malireddi RK, Lukens JR, Vogel P, Bertin J, et al. (2012) NLRP6 negatively regulates innate immunity and host defence against bacterial pathogens. Nature 488: 389–393. doi:10.1038/ nature11250 PMID: 22763455

41. Li Y, Zhang P, Wang C, Han C, Meng J, et al. (2013) Immune responsive gene 1 (IRG1) promotes endotoxin tolerance by increasing A20 expression in macrophages through reactive oxygen species. J Biol Chem 288: 16225–16234. doi: 10.1074/jbc.M113.454538 PMID: 23609450

42. Carow B, Rottenberg ME (2014) SOCS3, a Major Regulator of Infection and Inflammation. Front Immunol 5: 58. doi: 10.3389/fimmu.2014.00058 PMID: 24600449

43. Brady BL, Muljo SA (2013) RNA decay tolerizes: MCPIP1 (Zc3h12a) keeps inflammation in check by cleaving 3' UTRs. Immunol Cell Biol 91: 331–332. doi: 10.1038/icb.2013.19 PMID: 23628803