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Engagement of the Pathogen Survival Response Used by Group A Streptococcus to Avert Destruction by Innate Host Defense

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Neutrophils are a critical component of human innate host defense and efficiently kill the vast majority of invading microorganisms. However, bacterial pathogens such as group A Streptococcus (GAS) successfully avert destruction by neutrophils to cause human infections. Relatively little is known about how pathogens detect components of the innate immune system to respond and survive within the host. In this study, we show that inactivation of a two-component gene regulatory system designated Ihk-Irr significantly attenuates streptococcal virulence in mouse models of soft tissue infection and bacteremia. Microarray analysis of wild-type and irradiation-negative mutant (irr mutant) GAS strains revealed that Ihk-Irr influenced expression of 20% of all transcripts in the pathogen genome. Notably, at least 11 genes involved in cell wall synthesis, turnover, and/or modification were down-regulated in the irr mutant strain. Compared with the wild-type strain, significantly more of the irr mutant strain was killed by human neutrophil components that destroy bacteria by targeting the cell envelope (cell wall and/or membrane). Unexpectedly, expression of ihk and irr was dramatically increased in the wild-type strain exposed to these same neutrophil products under conditions that favored cell envelope damage. We report a GAS mechanism for detection of innate host defense that initiates the pathogen survival response, in which cell wall synthesis is critical. Importantly, our studies identify specific genes in the pathogen survival response as potential targets to control human infections. The Journal of Immunology, 2004, 173: 1194–1201.

Materials and Methods

Materials

Todd-Hewitt broth containing 0.2% yeast extract (THY) was obtained from BD Biosciences ( Sparks, MD). Sterile water and 0.9% sodium chloride (both Irrigation USP; Baxter Healthcare, Deerfield, IL) were purchased from Baxter Healthcare. Dextran T-500 and Ficoll-Paque PLUS were obtained from Amersham Biosciences (Piscataway, NJ). RPMI 1640 medium was purchased from Invitrogen Life Technologies (Carlsbad, CA). Unless specified, all other reagents were from Sigma-Aldrich (St. Louis, MO).

Bacterial strains and culture

For in vitro microarray analyses, serotype M6 GAS strain JRS4 (wild-type) and isogenic irr-negative mutant strain JRS500 (irr mutant) (11) were grown in THY to early exponential phase (OD600 = 0.35), or late exponential phase (OD600 = 0.75), and harvested immediately for RNA isolation. For all other assays, GAS strains were grown to early exponential phase, washed in Dulbecco’s PBS (DPBS), and resuspended in RPMI 1640 medium containing 10 mM HEPES (RPMI/H) at 107 per milliliter. GAS strains representative of those causing invasive (serotype M1, SF370) (12) or noninvasive (serotype M18, MGAS8232) (13) disease in humans were cultured as described above.

Isolation of human neutrophils

Neutrophils were isolated from heparinized venous blood of healthy individuals with dextran sedimentation followed by Ficoll-Paque PLUS (Amersham Biosciences) gradient separation as described previously (7, 14). All studies with human blood were performed in accordance with a protocol approved by the Institutional Review Board for Human Subjects, National Institute of Allergy and Infectious Diseases.
FIGURE 1. The Ihk-Irr two-component gene regulatory system is important for GAS pathogenesis. A. Cutaneous abscess size in individual mice infected by s.c. inoculation with wild-type (blue circles) or isogenic irr mutant (red triangles) strains of GAS. Black lines represent the average abscess size on each day. *, p < 0.017 vs irr mutant strain. **, p = 0.04 vs irr mutant strain. B, Representative abscesses 3 days after infection. C. In vitro growth of the wild-type (blue circles) and irr mutant (red triangles) GAS strains. D. Bacteremia in mice after i.p. inoculation of wild-type (blue circles) and irr mutant (red triangles) GAS strains. *, p = 0.03 vs wild-type strain.

Mouse infection models

Wild-type and irr mutant GAS strains were grown to early exponential phase, washed twice with sterile DPBS, and resuspended to 10^8 GAS/200 µl. Mice were anesthetized with isoflurane and inoculated by i.p. injection with 10^8 wild-type or irr mutant GAS strains, or with sterile DPBS. There were eight mice in each treatment group for the soft tissue infection model (s.c. inoculation in the left shoulder). Size of each skin abscess was measured after initial inoculation with a sliding caliper and monitored daily for 14 days. The bacteremia experiments were performed after initial inoculation with 10^8 wild-type strain, 15 mice inoculated with the irr mutant strain, and 15 mice inoculated with sterile DPBS. There were eight mice in each treatment group for the soft tissue infection model (s.c. inoculation in the left shoulder). Size of each skin abscess was measured after initial inoculation with a sliding caliper and monitored daily for 14 days. The bacteremia experiments were performed after initial inoculation with 10^8 wild-type strain, 15 mice inoculated with the irr mutant strain, and 15 mice inoculated with sterile DPBS. Animals were bled via the saphenous vein before i.p. inoculation and at 5- and 24-h postinoculation. Blood samples were cultured overnight on blood agar plates, and animals from which GAS was cultured were considered positive for bacteremia.

GAS microarray experiments

Microarray experiments were performed as previously described with few modifications (7). Wild-type and irr mutant GAS strains were cultured to early and late exponential phases of growth as described above. We performed microarray experiments with early and late exponential-phase GAS cultures (cultures were not combined, and each culture was assayed independently), because it is unclear which phase of in vitro growth best represents that of in vivo bacterial infection. A total of 2.5 × 10^6 bacteria was lysed with 700 µl of RLT buffer (Qiagen, Valencia, CA) and the lysate homogenized with an FP120 FastPrep system (Qiobgene, Carlsbad, CA). Total RNA was isolated with RNeasy kits (Qiagen). First-strand cDNA synthesis (incorporation of biotin and fluorescein probes) and tyramide signal amplification (TSA) was performed for RNA samples using Microarray Labeling and Detection kit (PerkinElmer Life Sciences, Shleton, CT) with some modification of the manufacturer’s protocol. Random primers at 0.5 µg/ml (Promega, Madison, WI) were added to the cDNA reaction mixture. The RNA/cDNA hybridization mixtures were denatured by NaOH hydrolysis (0.3 M final concentration) at 65°C for 15 min. The cDNA was purified by using QiAquick PCR Purification kit (Qiagen) with 75% ethanol substituted for the wash buffer and nuclease-free water substituted for the elution buffer. Biotin- and fluorescein-labeled cDNA samples were combined (50 µl total), diluted 50% with SlideHyb 3 (Ambion, Austin, TX), denatured at 95°C for 2 min, and incubated with a DNA microarray containing 1705 (of 1752) M1 GAS open reading frames (ORFs) based on strain SF370 (12), and unique M18 and M3 ORFs (13, 15). PCR products derived from each ORF were printed onto CMT-GAPS Corning (Acton, MA) glass slides with a Chipwriter robotic arrayer (Bio-Rad, Hercules, CA). Hybridization of cDNA samples to microarray slides was conducted overnight in a 45°C water bath. Slides were washed sequentially with 0.5 × SSC containing 0.1% SDS (twice), 0.06× SSC containing 0.01% SDS, and 0.06× SSC. Slide signal was amplified with TSA, and slides were scanned with a ScanArray 5000 instrument (PE Biosystems, San Diego, CA). Slides were normalized to equal fluorescence intensity against serial dilutions of MGAS8232 genomic DNA standards by adjusting laser power and/or photomultiplier gain. Spot location and array alignment were adjusted with QuantArray (PE Biosystems). All genes identified as differentially expressed passed a general filter based on signal-minus-background, spot area and signal-to-noise ratio, and were at least 1 SD above controls. To compare gene expression between the wild-type and irr mutant GAS strains, fold-changes for each gene were determined by the ratio of median fluorescence intensity of microarray spots derived from RNA samples of wild-type and irr mutant strains. Analysis for microarrays was done with GENESPRING software, version 4.2 (Silicon Genetics, Redwood City, CA). Microarray experiments that directly compared wild-type and mutant strains were performed in duplicate. Each microarray slide contained 4–12 spots per gene for a total of 8 spots for each gene. A link to the Gene Expression Omnibus (GEO) containing the complete set of microarray results compliant with Minimum Information about a Microarray Experiment guidelines can be found at www.ncbi.nlm.nih.gov/geo.

H2O2 and neutrophil granule killing assays

Serotype M6 wild-type and irr mutant GAS strains (10^9) were incubated in 96-well plates with varied concentrations of H2O2 for 60 min at 37°C as indicated. GAS were plated on THY agar, and colonies were enumerated the following day. The percentage of GAS killed was calculated using the following equation: (1 – (CFU hydroperoxide control/CFU control)) × 100.

Subcellular fractionation of neutrophils was performed as previously described (16). Briefly, neutrophils were resuspended in relaxation buffer (100 mM KCl, 3 mM NaCl, 3.5 mM MgCl2, and 10 mM PIPES containing 1 mM ATP) and disrupted by nitrogen cavitation (400 psi for 20–30 min at 4°C). Neutrophil lysate was collected dropwise into 100 mM EGTA such that the final concentration was 1.25 mM. Lysates were centrifuged at 200×g for 6 min to remove unbroken cells and nuclei. Neutrophil lysates were overlaid atop Percoll step gradients (16) and centrifuged at 48,300×g for 15 min at 4°C. Fractions containing neutrophil primary (α) and secondary (β) granules were collected as described (16), and Percoll was
removed by ultracentrifugation at 100,000 × g for 90 min. To standardize bactericidal activity, aliquots of granule fractions were thawed once for use and then discarded. GAS strains were incubated with 10⁶–10⁷ neutrophil cell equivalents (as indicated) of each granule fraction for 60 min with 0.1% saponin and then plated on THY agar. Colonies were enumerated the following day and percentage of GAS killed was calculated using the following equation: (1 – (CFUgranule/CFUcontrol)) × 100.

Assays with antimicrobial peptides and proteins

GAS were cultured to early exponential growth phase and resuspended in RPMI/H to 10⁷/ml. Peptides and proteins were reconstituted in buffer as recommended by the manufacturer: human α-defensin (Chemicon International, Temecula, CA), sterile water; neutrophil elastase (EMD Biosciences, San Diego, CA), 50 mM sodium acetate (pH 5.5) containing 200 mM NaCl; neutrophil cathepsin G (EMD Biosciences), 50 mM NaOAc, and 150 mM NaCl (pH 5.5); and LL-37 (Phoenix Pharmaceuticals, Belmont, CA), 10% acetonitrile with 0.1% trifluoroacetic acid. Peptide or enzyme dilution buffer (control) was added to each GAS strain, and samples were incubated at 37°C for 60 min. Samples were plated on THY and enumerated the following day with a ProtoCol SR colony counter system (Symbiosis, Frederick, MD). The percentage of GAS killed was calculated using the following equation: (1 – (CFUpeptide/CFUcontrol)) × 100.

TaqMan real-time RT-PCR analysis

GAS strains (2.5 × 10⁷) were cultured to early exponential phase of growth and incubated with 1 mM H₂O₂ in THY medium for 30 or 60 min at 37°C. Alternatively, GAS were grown as described above, resuspended at 2.5 × 10⁷/ml in RPMI/H containing 0.1% saponin, and incubated with 10⁷ neutrophil equivalents of each granule fraction (as described above for granule killing assays) for 15, 30, and 60 min at 37°C with 5% CO₂. At the indicated times, ihk and irr transcript levels were determined with TaqMan real-time RT-PCR (ABI 7700 thermocycler; Applied Biosystems, Foster City, CA) as described (7). Samples were assayed in triplicate from two to three biological replicates as indicated.

Statistics and curve fitting

Statistics were performed with a Student’s t test, logrank test, or ANOVA with a Tukey posttest for multiple comparisons using GraphPad Prism, version 4.0 for Windows (GraphPad Software, San Diego, CA), unless indicated otherwise. Curve fitting for the antibacterial peptide assays was performed with GraphPad Prism, version 4.0 for Windows, using Boltzmann sigmoidal nonlinear regression.

Results

Inactivation of irr significantly attenuates streptococcal virulence in mouse models of soft tissue infection and bacteremia

To determine whether Ihk-Irr is important for GAS infections in tissues, mice were inoculated with wild-type or irr mutant GAS strains by s.c. injection and monitored for several days (Fig. 1). There were notable differences in tissue infection caused by the two strains. First, abscesses formed more rapidly in mice infected with the wild-type strain (Fig. 1A, day 1). Second, the average abscess size was significantly larger in mice infected with the wild-type strain (p = 0.017) (Fig. 1, A and B). Differences in development and size of abscesses between the two strains were not likely due to altered growth kinetics, because growth of the wild-type and irr mutant strains was essentially identical in vitro (Fig. 1C).

We next compared the ability of the wild-type and irr mutant GAS strains to cause bacteremia (Fig. 1D). Mice were infected with each strain by i.p. inoculation, and bacteremia was assessed at 5- and 24-h postinfection (Fig. 1D). Five hours after inoculation, 12 of 16 mice infected with the wild-type strain and 11 of 15 mice infected with the irr mutant strain were bacteremic (Fig. 1D, left panel). However, 24 h after inoculation, only 1 animal infected with the irr mutant strain remained bacteremic, compared with 6 of those infected with the wild-type strain (p = 0.03 vs wild type) (Fig. 1D). Significantly accelerated clearance of GAS from the blood of animals inoculated with the irr mutant strain could reflect increased susceptibility of that strain to effectors of innate host defense such as neutrophils (7), or an attenuated ability to dissem-
I, and supplemental Table II, which contains the complete set of microarray data for these experiments). Compared with the wild-type strain, there were significant changes in transcript levels of 351 genes in the irr mutant strain (20% of the GAS genome) (Fig. 2A, Table I, and supplemental Table II). Notably, 218 of these genes were down-regulated in the irr mutant strain (Fig. 2A). For example, genes encoding transcription regulators such as vicR (SPy0528) were up-regulated concomitantly with reduced abscess size in infected mice (Fig. 1). Because previous studies indicate sagA expression is regulated by CsrR/S (30), modulation of sagA by Ihk-Irr may be indirect or, alternatively, controlled by multiple gene regulatory systems. Regardless, these data indicate that, in contrast to CsrR/S (31, 32) or Mga (31), Ihk-Irr has limited influence on expression of known GAS virulence factors.

### Ihk-Irr controls expression of genes involved in cell wall formation

GAS genes encoding proteins involved in cell wall formation are up-regulated concomitantly with *ikh* and *irr* during neutrophil phagocytosis (7). Consistent with these findings, genes important in cell wall and/or peptidoglycan synthesis, including *gidB* (glucosylated division protein), Spy0510, putative UDP-N-acetylmuramyl tripeptide synthetase (Spy1310), D-alanyl-D-alanyl carboxypeptidase, putative UDP-N-acetylmuramoyl pentapeptide-lysine N(6)-alanyltransferase, UDP-N-acetylmuramoyl pentapeptide-lysine N(6)-alanyltransferase, and *dgk* (glucose-inhibited division protein) (Table I). These data suggest that Ihk-Irr regulates cell wall synthesis, a finding most compatible with the observation that the irr mutant strain is not readily explained by decreased *fbp*, *mf*, and *mf3* transcript levels. In contrast, *sagA* is associated with increased tissue necrosis in mouse models of GAS infection (19, 28, 29). The observation that *sagA* transcript levels are decreased in the irr mutant strain correlates with reduced abscess size in infected mice (Fig. 1). Because previous studies indicate *sagA* expression is regulated by CsrR/S (30), Ihk-Irr is essential for GAS pathogenesis (7).

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### Table I. Genes regulated by *Irk-Ihk*<sup>a</sup>

<table>
<thead>
<tr>
<th>SPy no.</th>
<th>Gene</th>
<th>Encoded Protein (or Homolog)</th>
<th>Fold Change</th>
<th>Growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>0738</td>
<td><em>sagA</em></td>
<td>Streptolysin S</td>
<td>−2.0</td>
<td>EE</td>
</tr>
<tr>
<td>1013</td>
<td><em>fhp</em></td>
<td>Fibronectin-binding protein-like protein A</td>
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<tr>
<td>1436</td>
<td><em>mf3</em></td>
<td>DNase</td>
<td>−1.8</td>
<td>LE</td>
</tr>
<tr>
<td>2043</td>
<td><em>mf</em></td>
<td>DNase</td>
<td>−1.9</td>
<td>EE</td>
</tr>
<tr>
<td>0470</td>
<td>67-kDa myosin-cross-reactive streptococcal Ag</td>
<td>+1.8</td>
<td>EE</td>
<td></td>
</tr>
</tbody>
</table>

**Oxidative stress (5)**

<table>
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<th>Gene</th>
<th>Encoded Protein (or Homolog)</th>
<th>Fold Change</th>
<th>Growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>1374</td>
<td><em>mdH</em></td>
<td>Glutaredoxin</td>
<td>−2.1 &amp; −2.9</td>
<td>EE &amp; LE</td>
</tr>
<tr>
<td>0850</td>
<td>Thiorodoxin reductase</td>
<td>−2.4</td>
<td>EE</td>
<td></td>
</tr>
<tr>
<td>1681</td>
<td>Putative NADH oxidoreductase</td>
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<td>EE</td>
<td></td>
</tr>
<tr>
<td>1835</td>
<td>Thiorodoxin</td>
<td>−1.5</td>
<td>EE</td>
<td></td>
</tr>
<tr>
<td>0605</td>
<td>Glutathione peroxidase</td>
<td>+1.7</td>
<td>EE</td>
<td></td>
</tr>
</tbody>
</table>

**Cell wall synthesis (15)**

<table>
<thead>
<tr>
<th>SPy no.</th>
<th>Gene</th>
<th>Encoded Protein (or Homolog)</th>
<th>Fold Change</th>
<th>Growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>0529</td>
<td><em>gidB</em></td>
<td>Glucose-inhibited division protein B</td>
<td>−1.5</td>
<td>LE</td>
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<tr>
<td>0530</td>
<td>Import protein for polysaccharides &amp; teichoic acids</td>
<td>−1.8</td>
<td>LE</td>
<td></td>
</tr>
<tr>
<td>0540</td>
<td>Putative glycosyltransferase</td>
<td>−1.6</td>
<td>LE</td>
<td></td>
</tr>
<tr>
<td>0540</td>
<td>Putative glycosyltransferase</td>
<td>−2.0</td>
<td>EE</td>
<td></td>
</tr>
<tr>
<td>0797</td>
<td>Oligosaccharide translocase (flippase)</td>
<td>−1.6</td>
<td>EE</td>
<td></td>
</tr>
<tr>
<td>1035</td>
<td>Putative UDP-N-acetylmuramyl tripeptide synthetase</td>
<td>−1.5</td>
<td>EE</td>
<td></td>
</tr>
<tr>
<td>1093</td>
<td>D-Alanyl-D-alanyl carboxypeptidase</td>
<td>−1.7</td>
<td>LE</td>
<td></td>
</tr>
<tr>
<td>1094</td>
<td>Putative polysaccharide deacetylase</td>
<td>−1.9</td>
<td>LE</td>
<td></td>
</tr>
<tr>
<td>1205</td>
<td>UDP-N-acetylmuramoyl pentapeptide-lysine N(6)-alanyltransferase</td>
<td>−1.9</td>
<td>LE</td>
<td></td>
</tr>
<tr>
<td>1311</td>
<td><em>dtlB</em></td>
<td>Integral membrane protein</td>
<td>−1.8</td>
<td>EE</td>
</tr>
<tr>
<td>1662</td>
<td><em>mraY</em></td>
<td>Undecaprenyl-phosphate-UDP-MurNAc-pentapeptide-phospho-MurNAc-pentapeptide transferase</td>
<td>−1.8</td>
<td>EE</td>
</tr>
<tr>
<td>0516</td>
<td>Glucosyltransferase</td>
<td>+2.1</td>
<td>EE</td>
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<tr>
<td>0786</td>
<td>RgpAc</td>
<td>Rhamnosyltransferase</td>
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<tr>
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<td>RgpAc</td>
<td>Rhamnosyltransferase</td>
<td>+1.9</td>
<td>LE</td>
</tr>
<tr>
<td>2185</td>
<td><em>gidA</em></td>
<td>Glucose-inhibited division protein</td>
<td>+1.7</td>
<td>EE</td>
</tr>
</tbody>
</table>

**Virulence associated (5)**

<table>
<thead>
<tr>
<th>SPy no.</th>
<th>Gene</th>
<th>Encoded Protein (or Homolog)</th>
<th>Fold Change</th>
<th>Growth</th>
</tr>
</thead>
</table>

<sup>a</sup> Proteins encoded by differentially expressed genes were identified by annotation or homology to known proteins (www.ncbi.nlm.nih.gov/BLAST) and assigned to categories based on description in clusters of orthologous groups of proteins (COGs). EE, Early exponential phase of growth (OD<sub>600</sub> = 0.35); LE, late exponential phase of growth (OD<sub>600</sub> = 0.75). SPy, Streptococcus pyogenes.
levels of genes involved in cell wall synthesis likely increase susceptibility of the *irr* mutant GAS strain to these peptides. This hypothesis is tested below.

**Ihk-Irr regulates expression of oxidative stress genes and protects against killing by H$_2$O$_2$**

Inasmuch as GAS elicits production of neutrophil ROS during phagocytosis (1, 7), the ability to endure neutrophil-derived ROS likely contributes to pathogen survival after phagocytosis. Therefore, one of our prominent findings was that Ihk-Irr regulates expression of genes encoding proteins that control cell redox status and protect against oxidative stress (38, 39). GAS genes encoding *irx* (thioredoxin, SPy1835), thioredoxin reductase (SPy0850), NADH peroxidase (SPy1681), *nrdH* (glutaredoxin, SPy1374), and ribonucleotide reductase (SPy1375) were down-regulated in the *irr* mutant strain (Table I). In contrast, only one gene involved in oxidative stress response, *bsaA* (SPy0605), which encodes glutathione peroxidase, was up-regulated (Table I). These data indicate that at least four possible biochemical pathways that detoxify ROS are down-regulated (Fig. 2B).

To determine whether down-regulation of genes involved in responses to oxidative stress reflects increased killing by ROS, we measured susceptibility of the wild-type and *irr* mutant GAS strains to H$_2$O$_2$ (Fig. 3A). There was concentration-dependent killing of both strains by H$_2$O$_2$ (Fig. 3A). However, the *irr* mutant strain was significantly more susceptible to H$_2$O$_2$-mediated killing than the wild-type strain (*p* < 0.016) (Fig. 3A). Although these findings suggest that Ihk-Irr protects GAS from H$_2$O$_2$-mediated killing by influencing expression of genes involved in the detoxification of ROS, it is also possible that increased killing of the *irr* mutant strain was due in part to altered cell wall synthesis.

**Ihk-Irr facilitates resistance to killing by neutrophil granule components**

In addition to eliminating pathogens with ROS, human neutrophils kill Gram-positive bacteria with antimicrobial peptides that disrupt cell envelope integrity (33–37, 40, 41). Therefore, we exposed wild-type and *irr* mutant GAS strains to human neutrophil granule fractions to determine whether there were differences in survival, thus reflecting altered cell wall synthesis. Neither strain was killed by neutrophil secondary granule components (data not shown). This finding is consistent with the idea that peptides and enzymes of secondary granules, such as hCAP-18 (42), are inactivezymogens that require proteolytic processing to obtain activity (43). In contrast, both strains were killed to various degrees by solubilized neutrophil primary granules, which contain active α-defensins, elastase, and cathepsin G (42) (Fig. 3B). Notably, the *irr* mutant strain was significantly more susceptible to killing by primary granule components compared with the wild-type strain (*p* < 0.009) (Fig. 3B). We next tested the strestiocidal activity of LL-37 (44) (the active peptide form of hCAP-18), α-defensin-1, neutrophil elastase, and neutrophil cathepsin G to determine whether differences in survival noted between the *irr* mutant and wild-type strains could be attributed to a single granule component (Fig. 3C). Although each of the strains was killed by relatively low concentrations of these antibacterial peptides (Fig. 3C), significantly more of the *irr* mutant strain was killed by LL-37 and cathepsin G, each of which targets the cell envelope (37, 41). These results suggest that Ihk-Irr protects GAS from antibacterial neutrophil peptides by regulating cell wall synthesis and/or turnover.

**ihk and *irr* are induced by H$_2$O$_2$ and neutrophil primary granules**

To further understand the role of Ihk-Irr in pathogenesis, we exposed wild-type GAS to amounts of H$_2$O$_2$ representative of that within neutrophil phagosomes and analyzed *ihk* and *irr* transcript levels with TaqMan real-time PCR (Fig. 4). Expression of *ihk* and *irr* increased following exposure to H$_2$O$_2$, although the magnitude of change compared with untreated GAS was relatively moderate (2.0-fold at 30 min) (Fig. 4A). These results are consistent with the finding that *ihk-irr* is up-regulated during neutrophil phagocytosis and accompanying production of ROS (7).

We next exposed GAS to purified neutrophil primary and secondary granule fractions and measured transcript levels of *irr* to determine whether expression of the gene is altered by these antibacterial components (Fig. 4B). Unexpectedly, exposure of the pathogen to neutrophil primary granules caused a robust increase in *irr* transcript compared with untreated bacteria (~22-fold at 30 min) (Fig. 4B). Neither secondary granules nor heat-inactivated granules altered *irr* transcript levels to the same extent (Fig. 4B). These findings indicate that increases in *irr* transcript were not simply due to the binding of primary granule components to the bacteria cell surface. Rather, the data suggest that increased *irr* expression is a pathogen response to cell wall and/or membrane damage caused by antibacterial peptides contained within neutrophil granules. Importantly, *ihk* and *irr* were each induced by neutrophil primary granule components in a time-dependent manner and in strains of GAS representative of those that cause invasive (serotype M1) and noninvasive (serotype M18) disease in humans (Fig. 4, C–F). Inasmuch as Ihk-Irr regulates expression of genes important for GAS survival in the host (Fig. 1 and Table I), these
SEM fold-change of three separate TaqMan experiments. M18 GAS strains after a 30-min incubation with 1 mM H$_2$O$_2$ or 10$^7$ cell equivalents of human neutrophil/H11006 is the mean/H11006. Results are the mean/neutrophil secondary granule fractions (H9252). The data for induction of irr by H$_2$O$_2$ was included on the same scale for comparison (see dotted line). Results are the mean ± SD fold-change of triplicate wells from a representative TaqMan experiment performed twice. C. Time-resolved change in ihk and irr transcript following exposure of the wild-type GAS strain to neutrophil primary granules. Results are the mean ± SD fold-change of triplicate wells from a representative TaqMan experiment performed at least twice for each time point. D and E. Killing of serotype M1 and M18 GAS strains by 1 mM H$_2$O$_2$ (D) or solubilized human neutrophil α-granule components (E). Percent GAS survival is the mean ± SEM of six to seven separate experiments relative to untreated bacteria (■). F. Change in irr and ihk transcript levels in serotype M1 and M18 GAS strains after a 30-min incubation with 1 mM H$_2$O$_2$ or 10$^7$ cell equivalents of human neutrophil α-granules as indicated. Results are the mean ± SEM fold-change of three separate TaqMan experiments.

FIGURE 4. ihk and irr are induced by H$_2$O$_2$ and human neutrophil primary granules. A. Induction of ihk and irr in the wild-type strain by 1 mM H$_2$O$_2$. Results are the mean ± SD fold-change of triplicate wells from a representative TaqMan experiment performed at least twice for each time point. B. Change in irr transcript levels after incubation with human neutrophil primary granule fractions (α), heat-inactivated neutrophil primary granule fractions (Δα), neutrophil secondary granule fractions (β), and heat-inactivated neutrophil secondary granule fractions (Δβ). The data for induction of irr by H$_2$O$_2$ was included on the same scale for comparison (see dotted line). Results are the mean ± SD fold-change of triplicate wells from a representative TaqMan experiment performed twice. C. Time-resolved change in ihk and irr transcript following exposure of the wild-type GAS strain to neutrophil primary granules. Results are the mean ± SD fold-change of triplicate wells from a representative TaqMan experiment performed at least twice for each time point. D and E. Killing of serotype M1 and M18 GAS strains by 1 mM H$_2$O$_2$ (D) or solubilized human neutrophil α-granule components (E). Percent GAS survival is the mean ± SEM of six to seven separate experiments relative to untreated bacteria (■). F. Change in irr and ihk transcript levels in serotype M1 and M18 GAS strains after a 30-min incubation with 1 mM H$_2$O$_2$ or 10$^7$ cell equivalents of human neutrophil α-granules as indicated. Results are the mean ± SEM fold-change of three separate TaqMan experiments.

findings provide strong support to the idea that Ihk-Irr triggers pathogen-protective responses to innate host defense.

Discussion
Our understanding of how bacterial pathogens avert neutrophil killing following phagocytosis is limited. GAS alters patterns of global gene transcription during phagocytic interaction with neutrophils and resists the effects of neutrophil microbicidal components to survive and cause neutrophil necrosis (1, 6–10). The finding that GAS lacking functional Ihk-Irr are rapidly destroyed after phagocytosis suggests that this gene regulatory system may play a role in these processes (7). Moreover, irr is highly expressed in several GAS M-serotypes isolated from patients with pharyngitis, implying that Ihk-Irr is important for GAS pathogenesis in humans (7). However, none of those studies address a mechanism for triggering pathogen-protective responses nor is the molecular basis for prolonged survival within neutrophils known.

To that end, we investigated the role of Ihk-Irr in streptococcal pathogenesis and found that inactivation of irr attenuated virulence significantly, resulting in reduced soft tissue infection and more rapid resolution of bacteremia. Microarray analyses indicated that Ihk-Irr influenced expression of genes that regulate cell redox status and cell wall synthesis, and GAS lacking irr were rapidly killed by antibacterial peptides that target the cell envelope. These observations provide strong support to the idea that regulation of cell wall synthesis is critical for GAS pathogenesis. Recent studies have hinted that bacteria cell surface modification may be involved in averting destruction by neutrophils (10, 33). Resistance of microbes to the effects of neutrophil microbicidal components through bacteria cell wall synthesis or surface modification is thus an emerging theme in bacterial pathogenesis.

Based on our findings, we propose a model in which neutrophil-derived ROS and damage resulting from neutrophil antibacterial peptides activates/induces Ihk-Irr, which in turn alters GAS gene expression (Fig. 5). Induction of Ihk-Irr-regulated genes, especially those involved in cell wall synthesis, extends GAS survival such that neutrophil lysis occurs before the pathogen is eliminated (1), thus promoting dissemination and disease. Consistent with our model for the pathogen survival response, the PhoP-PhoQ and PmrA-PmrB two-component gene regulatory systems of Salmonella typhimurium and Pseudomonas aeruginosa are activated by cationic antimicrobial peptides, and in turn, regulate resistance to antimicrobial peptides (45–48). Activation of PhoP-PhoQ in Salmonella by antimicrobial peptides alters global gene and protein expression patterns (45), a process likely similar for Ihk-Irr of GAS.

Inasmuch as neutrophils are the most prominent innate immune effector cells in humans, the host factors that induce the pathogen
survival response described in this study are prevalent in nearly all bacterial infections. The process for triggering microbe survival responses is thus a general mechanism used by pathogens to detect and evade human innate immunity. A better understanding of the host-pathogen interface at both the cell and molecular levels is critical to our understanding, treatment, and control of human diseases caused by bacteria. Importantly, our studies identify specific genes in the bacterial pathogen survival response as potential targets to control human infections.

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