Supplementary information (ESI)

For “Fusogenic Porous Silicon Nanoparticles as a Broad-Spectrum Immunotherapy against Bacterial Infections”

Supplementary Data

Supplementary Figure S1. Transmission electron microscope images of fusogenic porous silicon nanoparticles (pSiNPs) at different stages of synthesis; (a) pSiNPs post-ultrasonication, but before siRNA loading. Scale bar represents 50 nm; (b) pSiNPs with siRNA loaded via calcium silicate condensation chemistry. Scale bar represents 200 nm; (c) pSiNPs after siRNA loading and coating with fusogenic lipid. Scale bar represents 200 nm.
Supplementary Figure S2. In vitro cytotoxicity of transfectant agents in relation to incubation time. J774a.1 macrophages were incubated with equimolar doses of siRNA against Irf5 (siIRF5) with: Lipofectamine 2000 (LF-siIRF5); non-fusogenic pSiNPs loaded with siIRF5 and conjugated with CRV peptide (NF-siIRF5-CRV); fusogenic pSiNPs loaded with siIRF5 but with no homing peptide—a methoxy group was used in place of the homing peptide—(F-siIRF5-mPEG); or fusogenic pSiNPs loaded with siIRF5 and conjugated with CRV peptide (F-siIRF5-CRV). Cells were incubated with the formulations for 0.5-24 h (as indicated), and cytotoxicity was quantified using the CCK assay. Bars represent standard deviation with n = 3; * represents p < 0.05 from One-way ANOVA with Tukey’s HSD post hoc analyses.
Supplementary Figure S3. Photographs of healthy hind leg muscles and MRSA-infected hind leg muscles of mice that were intravenously injected with: PBS; free vancomycin (145 mg/kg); fusogenic pSiNPs loaded with siLuc and conjugated with CRV peptide (F-siLuc-CRV); non-fusogenic pSiNPs loaded with siIRF5 and conjugated with CRV peptide (NF-siIRF5-CRV); or fusogenic pSiNPs loaded with siIRF5 and conjugated with CRV peptide (F-siIRF5-CRV). For mice injected with the siRNA formulations, siRNA dose corresponded to 24 µg/kg siRNA. Mice were sacrificed and imaged at days 3, 7, and 14 post-injection. Dotted lines outline abscess, and white arrowheads indicate popliteal lymph nodes.
**Supplementary Table S1.** Average hydrodynamic size ($d_h$) and zeta-potential ($\zeta$-pot) of the core pSiNPs before loading (pSiNP), after siRNA loading and Ca$^{2+}$-induced condensation (pSiNP-siRNA), and the final fusogenic lipid-coated structure consisting of pSiNP-siRNA clusters and pendant CRV targeting peptides (F-siRNA-CRV), as measured by dynamic light scattering (n=7). ‘(F)’ represents formulation prepared using a combination of lipids that is fusogenic, and ‘(N)’ represents a control formulation prepared using a non-fusogenic lipid combination, as described in the text.

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<thead>
<tr>
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<th>pSiNP</th>
<th>pSiNP-siRNA</th>
<th>F-siRNA-CRV</th>
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<tbody>
<tr>
<td>$d_h$ (nm)</td>
<td>54.1 ± 12.9</td>
<td>74.7 ± 10.2</td>
<td>224.9 ± 15.6 (F)</td>
</tr>
<tr>
<td>$\zeta$-pot (mV)</td>
<td>-21.3 ± 4.5</td>
<td>-13.2 ± 3.4</td>
<td>7.1 ± 2.6 (F)</td>
</tr>
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</table>
Supplementary Experimental

**Bacterial culture**
All bacterial work was performed in an approved BSL-2 facility with a clean hood. MRSA was cultured by incubating 50 µL of the bacteria in 10 mL of cation-adjusted Mueller Hinton Broth (CAMHB), and PA01 was cultured by incubating 50 µL of bacteria in 10 mL of brain heart (BH) infusion broth (Fisher Scientific) for 16h in a shaking incubator at 37°C and shaking at 200 RPM with the cap loose. The culture was re-introduced to the lag phase from the stationary phase by sub-culturing at 1:100, 1:250, and 1:400 dilutions in fresh aliquots (5 mL) of the broth for 2h in a shaking incubator at 37°C and shaking at 200 RPM with the cap loose.

**Cell culture and confocal microscopy**
J774a.1 macrophage cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. All cells were incubated at 37°C in 5% CO₂.

All confocal microscope images are representative of at least three independent trials and of at least 1 x 10⁶ cells per slide. Lysosomal co-localization with Dil-loaded or siRNA-loaded particles was observed by seeding 0.3 x 10⁶ cells on top of 22 mm round coverslips (BD Biocoat Collagen Coverslip, 22 mm) in a 6-well plate, growing to 80% confluence. The cells were pre-stained with LysoTracker Green (Thermo Fisher Scientific) for 1 h at 37°C in 5% CO₂ according to manufacturer’s instructions. The cells were then washed with PBS three times, and treated with 10 µL of Dil-loaded nanoparticles for 10 min by incubation at 37°C in 5% CO₂. The cells were washed with PBS three times to remove any particles that were not taken up, and the wells were filled with 1 mL of PBS and immediately subjected to live-cell imaging by confocal microscopy (Zeiss LSM 710 NLO).

For imaging Cy3-tagged siRF5 as a model siRNA, 0.3 x 10⁶ cells were seeded on 35 mm petri dishes and grown to 80% confluence. The cells were then treated with 10 µL of DiO-loaded fusogenic or non-fusogenic particles loaded with Cy3-tagged siRF5 for 10 min at 37°C in 5% CO₂. The cells were washed with PBS three times to remove any particles that were not taken up, then fixed in 1% paraformaldehyde (PFA, Santa Cruz Biotechnology) for 10 min at 4°C, then washed with PBS three times. The coverslips were mounted on glass slides with ProLong® Diamond Antifade Mountant with DAPI (Life Technologies), dried and kept in the dark until examined by confocal microscopy (Zeiss LSM 710 NLO). For Cy3 imaging, λₜₐₓ = 550 nm and λₐₑₘ = 580-630 nm band-pass filters were used.

**In vitro knockdown quantification**
In vitro knockdown efficiencies of the nanoformulations were quantified using two-step quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR, Roche LightCycler 96). J774a.1 cells were seeded on a 24-well plate at 2 x 10⁴ cells per well and grown to 50% confluence overnight. The cells were incubated with the desired
nanoformulations or Lipofectamine 2000 at 20 pmol siRNA dose per well for 1 h, then the treatment media was removed, cells were washed with PBS three times, and fresh media was added. Then, 24 h or 7 days post-incubation, the cell media was removed and RNA was purified using the QIAshredder and RNeasy Mini Kit (Qiagen, Valencia, Ca). cDNA was transcribed from the purified RNA using the BIORAD iScript cDNA Synthesis Kit and heat-treated in an Eppendorf Vapo.protect Mastercycler thermal cycler. cDNA was mixed with IRF5 primers, or the control HPRT primers (IRF5 forward: aataccacccacccatta; IRF5 reverse: ttgagatccgggtttgagat; HPRT forward: GTCAACGGGGGACATAAAAG; HPRT reverse: CAACAATCAAGACATT-CTTTCCA) and iQ SYBR Green Supermix according to the manufacturer's instructions. qRT-PCR analysis was performed in BIORAD 96-well white Multiplate PCR Plates using a Roche LightCycler 96. The quantification was performed at n=6 and in a RNAse- and DNase-free laminar flow hood dedicated to RNA work. Quantified knockdown was statistically evaluated using One-way ANOVA with Tukey’s HSD post-hoc analysis.

**In vivo IRF5 knockdown efficiency**

8 week-old male Balb/C mice were infected as described in the Experimental section. Infected mice were intravenously injected with 100 µL of PBS or siLuc- or siIRF5-loaded fusogenic and non-fusogenic pSiNPs with or without CRV at 23.2 µmol/kg lipid, corresponding to 69 µg/kg siRNA, and 0.3 mg/kg pSi in 100 µL PBS. Twenty-four hours post-injection and circulation, MRSA infected mice were sacrificed for popliteal lymph node and muscle harvest, and PA01-infected mice were sacrificed for brochoalveolar lavage (BAL) and lung harvest. BAL was performed by intratracheal instillation of a mouse catheter, with a suture tied around the trachea to prevent leakage. 1 mL of PBS was injected into the lungs through the catheter, and aspirated back out. The process was repeated three times to collect up to 2.5 mL of the BAL fluid. Harvested lymph nodes, muscles and lungs were homogenized, and along with BAL fluid, the homogenates were separately treated with Dead Cell Removal MicroBeads (Miltenyi Biotec) for magnetic separation on an LS column according to the manufacturer's instructions. The collected cells were then treated with Anti-F4/80 microbeads (Miltenyi Biotec) for magnetic separation on an LS column according to the manufacturer's instructions for macrophage purification.

The *in vivo* knockdown of IRF5 was quantified using two-step quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR, Roche LightCycler 96). The cell pellets or the lung homogenates were lysed and RNA was purified using the QIAshredder and RNeasy Mini Kit (Qiagen, Valencia, Ca). cDNA was transcribed from the purified RNA using the BIORAD iScript cDNA Synthesis Kit and heat-treated in the Eppendorf Vapo.protect Mastercycler thermal cycler. cDNA was mixed with IRF5 primers, or the control HPRT primers (IRF5 forward: aataccacccacccatta; IRF5 reverse: ttgagatccgggtttgagat; HPRT forward: GTCAACGGGGGACATAAAAG; HPRT reverse: CAACAATCAAGACATT-CTTTCCA) and iQ SYBR Green Supermix according to the manufacturer's instructions. qRT-PCR analysis was performed in the BIORAD 96-well
white Multiplate PCR Plates using the Roche LightCycler 96. The quantification was performed at \( n = 6 \) and in RNase- and DNase-free laminar flow hood dedicated to RNA work. Relative knockdown was statistically evaluated using One-way ANOVA with Tukey’s HSD post-hoc analysis.

**Nanoparticle targeting and biodistribution**

6-8 week old male Balb/C mice were infected as described above. 3 days (MRSA) or 24 h (PA01) post-infection, infected and healthy mice were intravenously injected with Dil- and siIRF5-loaded fusogenic pSiNPs with or without CRV conjugation, at 23.2 \( \mu \text{mol/kg lipid} \), corresponding to 24 \( \mu \text{g/kg siRNA} \), and 0.5 mg/kg pSi 100 \( \mu \text{L} \) in PBS. For MRSA infection models, the Dil-loaded particle localization in the ipsilateral (right) and contralateral (left) muscles was visualized using the IVIS 200 (Perkin Elmer) with 4 s exposure time on the DsRed excitation and emission filters. Both healthy and infected animals were sacrificed and harvested for organs 1 h and 24 h post-injection. ImageJ was used to quantify the fluorescence of each muscle, and averaged over the three mice per group.

For the PA10 infection model, homing to infected lungs was validated using flow cytometry. 24 h post-infection, mice were intravenously injected with Dil- and siIRF5-loaded fusogenic pSiNPs with or without CRV conjugation, at 23.2 \( \mu \text{mol/kg lipid} \), corresponding to 24 \( \mu \text{g/kg siRNA} \), and 0.5 mg/kg pSi 100 \( \mu \text{L} \) in PBS. 1 h and 24 h post-injection, the mice were sacrificed, and BAL fluid and lungs were harvested. The BAL fluid and the lung homogenates were treated with Dead Cell Removal MicroBeads (Miltenyi Biotec) for magnetic separation on an LS column according to the manufacturer's instructions. The collected cells were then treated with Anti-F4/80 microbeads (Miltenyi Biotec) for magnetic separation on an LS column according to the manufacturer's instructions to collect only the macrophages. The macrophages were then processed with the LSRFortessa flow cytometer (BD Biosciences) to analyze the number of cells containing the particles’ Dil signals (ex: 561 nm/50 mW; em: 582 \( \pm 15 \) nm), and analyzed using the Flowing Software.

**In vivo therapeutic efficacy in MRSA muscle infection**

8 week-old male Balb/C mice were infected as described. 3 days post-infection, infected mice were intravenously injected with 200 \( \mu \text{L} \) of PBS, 145 mg/kg of vancomycin, non-fusogenic particles with siIRF5 and CRV conjugation, fusogenic particles with sham siRNA (siLuc, luciferase encoding siRNA) conjugated with CRV, or fusogenic particles with siIRF5 and CRV conjugation at 23.2 \( \mu \text{mol/kg lipid} \), corresponding to 24 \( \mu \text{g/kg siRNA} \), and 0.5 mg/kg pSi in 100 \( \mu \text{L} \) PBS. To confirm clearance of bacteria from the muscles, the number of colony-forming units (CFU) of MRSA was determined from titer of muscle homogenates. At days 3, 7, and 14 post-IV injection, mice were sacrificed for photographic record of muscle abscess and muscle harvest. The muscles were weighed, gently washed in PBS, and then homogenized. The homogenates were serially diluted to a dilution factors of \( 10^2, 10^6, 10^{12}, \) and \( 10^{16} \), and plated on agar-coated petri dishes and incubated at 37 \( \degree \)C overnight at \( n = 2 \) per dilution factor. The colonies were counted (\( n = 4 \)) for each dilution factor, and divided by the muscle mass. The average CFU/g was quantified using counts from 2 plates at equivalent dilution factors from 3 mice (\( n = 2 \) plates \( \times 3 \) mice = 6).
In vivo therapeutic efficacy in PA01 lung infection

8 week-old male Balb/C mice were infected as described. 24 h post-infection, infected mice were intravenously injected with 200 µL of PBS, 100 mg/kg of tobramycin, non-fusogenic particles with siIRF5 and CRV conjugation, fusogenic particles with sham siRNA (siLuc, luciferase encoding siRNA) conjugated with CRV, or fusogenic particles with siIRF5 and CRV conjugation at 23.2 µmol/kg lipid, corresponding to 24 µg/kg siRNA, and 0.5 mg/kg pSi in 100 µL PBS. At 7 days post-injection (healthy and F-siIRF5-CRV) or at ad mortem (infected and NF-siIRF5-CRV), the lungs were inflated and harvested for fixation in 4% paraformaldehyde (PFA). The fixed lungs were paraffinized and sectioned for hematoxylin and eosin (H&E) and Gram staining. The stained slides were histopathologically evaluated by Dr. Kent Osborn (Associate Director, Animal Care Program, UCSD).

To confirm clearance of bacteria from the lungs, the number of colony-forming units (CFU) of PA01 was determined from titer of lung homogenates. At 7 days post-IV injection, mice were sacrificed for lung harvest. The lungs were weighed, gently washed in PBS, and then homogenized. The homogenates were serially diluted to a dilution factors of $10^2$ and $10^{12}$, and plated on agar-coated petri dishes and incubated at 37 °C overnight at n = 3 per dilution factor. The colonies were counted for each dilution factor, and divided by the muscle mass. The average CFU/g was quantified using counts from 2 plates at equivalent dilution factors from 2 mice (n = 3 plates x 2 mice = 6).

Finally, a survival challenge was performed with infected mice, who were intravenously injected 24 h post-infection with the treatment compounds. Each group had 7 mice, which were tallied daily for survival. Moribund mice that showed signs of expiring within 5 h were sacrificed according to the IACUC guidelines. The resulting data were statistically evaluated using one-way ANOVA and post hoc comparisons using Tukey’s HSD test at p < 0.05.