Immunogene therapy with fusogenic nanoparticles modulates macrophage response to Staphylococcus aureus

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The incidence of adverse effects and pathogen resistance encountered with small molecule antibiotics is increasing. As such, there is mounting focus on immunogene therapy to augment the immune system’s response to infection and accelerate healing. A major obstacle to in vivo gene delivery is that the primary uptake pathway, cellular endocytosis, results in extracellular excretion and lysosomal degradation of genetic material. Here we show a nanosystem that bypasses endocytosis and achieves potent gene knockdown efficacy. Porous silicon nanoparticles containing an outer sheath of homing peptides and fusogenic liposome selectively target macrophages and directly introduce an oligonucleotide payload into the cytosol. Highly effective knockdown of the proinflammatory macrophage marker IRF5 enhances the clearance capability of macrophages and improves survival in a mouse model of Staphylococcus aureus pneumonia.
Deep-tissue *Staphylococcus aureus* infection is a major therapeutic challenge. *S. aureus* is a Gram-positive bacterium that predominantly infects the skin and the respiratory system causing pneumonia; local infections can become systemic in the most serious form of Staphylococcal disease, sepsis. At high levels of bacterial burden in the lungs, *Staphylococcus pneumoniae* becomes fatal due to two major factors: (1) pathogenic activity by *S. aureus* and (2) prolonged inflammation caused by the body’s immune system. The acute inflammatory response at the site of an infection involves the secretion of cytokines by alveolar macrophages, recruiting polymorphonuclear neutrophils (PMN) and monocytes from circulation that differentiate into macrophages. Alveolar inflammation causes extensive bleeding and exudation that slow down vascular flow and impede breathing, and prolonged excretion of inflammatory cytokines reduces the chances of recovery. Although the immediate inflammatory response to *Staphylococcus pneumoniae* is necessary for rapid elimination of the threat, it must be balanced with inflammation suppression and tissue repair to maintain lung homeostasis.

Owing to toxic adverse effects of small molecule antibiotics such as vancomycin and the emergence of strains resistant to these therapeutics therapies are needed to activate the immune system to treat bacterial infections. Macrophages are a potential target for such therapies owing to their polar functions as inflammatory, immune stimulatory phagocytes M1 macrophages, or as anti-inflammatory phagocytic M2 macrophages associated with bacterial phagocytosis and tissue repair functions. M1 macrophages are marked by the Irf5 gene, which upregulates tumor necrosis factor (TNF), interleukin (IL)-1, IL-6, IL-15, IL-18, and IL-23, and downregulates anti-inflammatory cytokines such as IL-10. Knockdown of Irf5 in the early stages of *Staphylococcus pneumoniae* can curtail prolonged inflammation by preventing the excretion of inflammatory cytokines, allowing the immune system to clear bacteria and repair tissue.

Despite much effort, in vivo knockdown of genes has still not been of great success. Naked RNA has a short half-life in vivo; thus, various types of nanoparticle (NP) delivery vehicles have been used to protect the oligonucleotide and deliver it intracellularly. The most common means of delivery is with lipid NPs, which are readily endocytosed by the cell, leading to extracellular excretion of 70% of the small interfering RNA (siRNA) payload, with the remaining siRNA undergoing lysosomal degradation. Typically, only 1–2% of administered siRNA escapes early endosomal uptake to potentially undergo RNAi. In order to increase the quantity of RNA delivered, polymeric and related hybrid NPs have been engineered with cationic polyethylenimine (PEI) components. Although it increases the carrying capacity of the NPs, PEI is also cytotoxic. Some lipid constituents, such as dioleoylphosphatidylethanolamine or 1,2-dioleoyl-3-trimethylammonium propane (DOTAP), impart a fusogenic nature to liposomes that enables them to fuse with the cellular membrane, mitigating toxicity, and enhancing cellular delivery of genes. Fusion is known to bypass endocytosis altogether, much like the endogenous soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE)-mediated vesicular uptake mechanism.

Although cellular penetration is important, gene therapeutics must also reach the appropriate cell to be effective. Here we present a solution to these problems that uses NPs containing a targeting peptide specific for activated macrophages and a fusogenic liposomal coating (F-pSi). Membrane fusion enables direct release of hydrophilic payloads from the core of NP directly into the cell cytoplasm, the transfer of hydrophobic molecules from the liposomal bilayer to the cell membrane bilayer, and the transfer of moieties conjugated on the outer surface of the lipid coat (including antibodies, small molecules, and peptides) to the cell membrane. By avoiding endocytosis entirely, the fusogenic coating increases the probability that siRNAs will reach the perinuclear region to undergo RNAi. In addition, in place of conventional peptide-based and polymeric NPs (such as protamine, poly-1-lysine, and PEI), we use porous silicon NPs (pSiNPs), which have been shown to be an effective gene delivery vehicle. The pSiNPs are prepared with a calcium silicate trapping chemistry that can load and protect high quantities of siRNA without the use of cytotoxic polymer stabilizers. We use siRNA against *Irf5* to inhibit the inflammatory phenotype of macrophages and favor phagocytic function. These macrophage-targeting F-pSi hybrid NPs have high siRNA knockdown efficiency in vitro and provide strong therapeutic efficacy against a *S. aureus* infection in mice, affording full recovery from a lethal dose. This study is the first successful in vivo demonstration of gene silencing for immunotherapy of deep-tissue infection, with implications for the treatment of antibiotic-resistant bacterial infections.

**Results**

**Synthesis of fusogenic lipid-coated pSiNPs.** The siRNA carrier pSiNPs, prepared by electrochemical etch of single-crystal silicon wafers, and ultrasonic fracture of the resulting porous layers into NPs. siRNA and hydrophilic fluorescent dye payload were loaded into the porous NPs by subjecting the payload and the pSiNPs to ultrasound in an aqueous solution of calcium chloride. Fusogenic liposomes were synthesized using the established film hydration method and were coated around the payload-loaded pSiNPs by co-extrusion through 200 nm polycarbonate membranes. The fusogenic (F) feature of the liposomes is derived from a controlled ratio of structural, cationic, and PEGylated lipid components (Supplementary Table 1). Control non-fusogenic (NF) NPs were prepared using the structural and PEGylated lipids, but without the cationic component—yielding a more conventional liposome. Dynamic light scattering (DLS) and microscopic data on the F-pSiNPs confirmed an average hydrodynamic diameter of 190 nm with a distribution range of 100–400 nm, with a cationic surface charge of ~10 mV (Supplementary Table 2). The F-pSi formulations were physically stable in deionized water for up to 28 days at 4°C (Supplementary Fig. 1). The loading efficiency of the siRNA payload was ~25 wt%, substantially larger than the 1–14 wt% achieved with other reported oligonucleotide–loaded nanoplatforms, such as lipid-based NPs and mesoporous Si-polymer hybrid systems (Supplementary Table 3). Notably, particles with sizes comparable to those used in the present study (200 nm) have displayed oligonucleotide loadings of <5 wt%.

**Intracellular delivery of fusogenic pSiNPs in vitro.** NPs were loaded with the hydrophilic dye calcein in the pSi core or with the lipophilic dye 1,1’dioctadecyl-3,3,3’,3’-tetramethylindocarbocyanine perchlorate (DiI) in the lipid leaflets to evaluate differences in intracellular localization and to infer uptake pathway. Calcein was chosen as the model-siRNA payload (Fig. 2a), because it shares two key characteristics with oligonucleotides: (i) it is anionic and (ii) it is membrane impermeable. The lipophilic DiI was chosen to track fusogenic uptake, because the hydrophobic molecule localizes in lipid bilayers (Fig. 2a). In the event of fusogenic uptake, DiI would be expected to diffuse from the liposomal bilayer of the NP into the plasma membrane,
whereas NF uptake would result in endocytosis and localization in the cytoplasm.

Confocal microscopy revealed that the F-pSi formulation fused with the plasma membrane of cultured J774A.1 murine macrophages and delivered the payload into the cytoplasm. The F-pSi formulation that contained DiI in the liposomal coating transferred this lipophilic dye to the cell membrane (Fig. 2b), confirming fusion, whereas the F-pSi formulation that contained calcine in the NP interior dispersed this hydrophilic dye throughout the cytoplasm (Fig. 2c). Control experiments performed using NF NPs resulted in an overall lower uptake and the DiI signal from the NF formulation were found to be concentrated within the cytoplasm (Fig. 2e). The fact that these NPs were associated with intracellular endosomes/lysosomes was confirmed using LysoTracker Red stain (Figs. 2c, 2f). By contrast, fusogenic NPs showed dispersed calcine signals that did not colocalize with lysosomal compartments. The F-pSiNPs attained an average Pearson’s correlation coefficient (PCC) = 0.04 ± 0.03, whereas the NF-pSiNPs attained an average PCC = 0.59 ± 0.18. The two values are significantly different by T-test (p = 2 × 10^{-6}), validating the lysosomal compartmentalization of the NF-pSi formulation.

The ability of the nanoparticles to be selectively targeted to macrophages was tested using a peptide selected in a phage library screen38,39 for cultured J774A.1 murine macrophages (Supplementary Fig 2a). This peptide, denoted as CRV, is a nine-amino acid peptide (sequence CRVLRSGSC) made cyclic by a disulfide bond between the side chains of the two cysteine residues. CRV labeled with a 5-FAM dye (FAM-CRV) showed higher binding to J774A.1 and Raw 264.7 macrophages relative to control peptides (Supplementary Fig. 2b). Biotin-labeled CRV was able to significantly reduce the binding of FAM-CRV to the macrophages (Supplementary Fig. 2c). All these results suggest that CRV peptide specifically binds to macrophages.

To permit coupling to the NPs, the peptide was modified by adding a third cysteine through a 6-aminohexanoic acid linker and labeled with a 5-FAM dye in order to allow tracking by fluorescence. The 3-cysteine peptide was attached to the polyethylene glycol (PEG) head of a minor fraction of the lipids via maleimide coupling chemistry. A fusogenic formulation containing CRV, the DiI membrane dye, and pSiNPs (F-DiI-CRV) showed strong colocalization of the DiI and FAM signals at specific points on the cell membrane (Fig. 2d), suggesting that CRV anchors the particles to its specific macrophage membrane receptors to allow localized fusion. The data are more consistent with a membrane fusion mechanism rather than endocytosis; substantial endocytosis would be expected to lead to increased DiI signals from the cell cytoplasm. We note that CRVs appeared to expedite the fusion process, as only half the incubation time was needed to achieve a comparable level of fusion. In contrast, the NF-DiI-CRV construct (Fig. 2g) displayed a cellular distribution similar to NF-DiI-mPEG, a NF formulation that contained mPEG (methoxy PEG) in place of the targeting peptide-conjugated PEG (Fig. 2e). The CRV-FAM and DiI signals colocalized in clusters within the cytoplasm, indicative of endosomal and lysosomal compartmentalization. These results indicate the targeting peptide is permissive for either cell entry pathway.

Transmission electron microscope (TEM) results (Fig. 2h–j) verify the different uptake pathways between fusogenic and NF formulations. Although the targeted NF particles demonstrated macropinocytosis, and were found to be localized in intracellular vesicles (Fig. 2i), the pSi cores of the targeted fusogenic particles were found degrading in the cell cytoplasm (Fig. 2j).

**Biosafety and in vitro knockdown using fusogenic pSiNPs**. To test the hypothesis that the fusogenic pathway for uptake and direct cytoplasmic release of siRNA attains higher knockdown efficiency than the endosomal uptake route, we delivered siRNA encoding the Irf5 gene to cultured Raw 264.7 macrophage cells and gene expression was analyzed by quantitative real-time PCR (qRT-PCR) (Fig. 3a). Fusogenic NP formulations (F-siIRF5) delivering 200 nM siRNA demonstrated high knockdown efficiencies with or without an added CRV macrophage-targeting peptide (96.4% and 96.6%, respectively). Knockdown was comparable to that of the standard transfection agent, Lipofectamine (95.5%). The NF formulation (NF-siIRF5-CRV) demonstrated
only 42.9% knockdown with high SD. The relatively high variance in this control is attributed to occasional incidences of endosomal escape. Administration of free siRNA achieved only 6.3% knockdown under similar conditions, statistically similar to the phosphate-buffered saline (PBS) control. The presence of CRV peptides on the particle surface had no adverse effect on knockdown ability. These experiments established that the fusogenic coatings enable higher knockdown efficiency than a conventional liposomal coating, and their siRNA payload. The primary concern with the fusogenic system is the insertion of the cationic lipid constituents and their siRNA payload. Constructs (Fig. 3b). To test in vivo biosafety, healthy Balb/C mice were intravenously injected with the fusogenic, CRV-targeted NP construct (F-siIRF5-CRV) at doses corresponding to 23.2 µmol/kg lipid, corresponding to 24 µg/kg siRNA. After 24 h circulation, the major organs were harvested and sectioned for hematoxylin and eosin (H&E) histopathological evaluation (Fig. 3c–h). All major organs were found to be normal, although the liver showed minor incidental findings also seen in control mice.

Biodistribution and targeting of fusogenic pSiNPs. We established a S. aureus pneumonia animal model involving intratracheal injection of bacteria at a dose that was lethal to untreated mice within 2 days. First, we tested the homing ability of the CRV macrophage-targeting peptide in this model, using the “free” peptide not bound to any NP, but conjugated to a fluorescein (5-FAM) dye to allow tracking. Infected and healthy control mice (n = 3 each) were intravenously injected with FAM-CR5 24 h post infection. Confocal immunofluorescence microscopy of organs collected from the healthy animals showed signs of renal clearance but no selective homing to any of the macrophage-heavy organs (Fig. 4a). However, the infected animals demonstrated clear evidence of selective homing, with CRV-FAM signals
colocalizing with the F4/80-AF555 macrophage signals in the infected lungs. The peptides cleared primarily through the kidneys, with minor localizations seen in the spleen and liver. The results demonstrate the ability of the CRV peptide to home to macrophages in *S. aureus*-infected lungs.

Next, we tested the in vivo homing ability of the CRV when attached to the fusogenic NPs. Mice were intravenously injected, 24 h post infection, with Dil-loaded F-siIRF5 NPs coated with conjugated CRV peptide, and the in vivo biodistribution was tracked by immunofluorescence microscopy of organs harvested at 1 h and 24 h post treatment. The quantified Dil fluorescence signal showed strong accumulation in the lungs after 1 h and it was still detected 24 h post treatment (Fig. 4b and Supplementary Fig. 3 shows the representative images of the organs). Control experiments using the same NP construct but replacing the CRV-conjugated fusogenic particles (F-DiI-CRV) showed minimal recruitment of peripheral macrophages. However, the CRV-conjugated NPs showed substantially enhanced uptake in the spleen of infected animals relative to controls, which is attributed to the splenic clearance and filtration of targeted macrophages that have phagocytosed senesced neutrophils and damaged cells from the infected lung.

We next studied the efficacy of CRV targeting and fusogenic uptake at the cellular level in the lungs using Dil-tagged NPs. The lungs of healthy mice intravenously injected with targeted fusogenic NPs (F-DiI-CRV) showed minimal recruitment of macrophages marked by F4/80-fluorescein isothiocyanate (FITC) and no visible F-DiI-CRV accumulation (Fig. 4c). Healthy lungs possess a baseline number of alveolar macrophages, with no significant recruitment of peripheral macrophages. However, the lungs of infected control mice (PBS injection at 24 h post infection) recruited a high number of macrophages, as expected of an inflammatory process (Fig. 4d). The group of infected mice injected (24 h post infection) with NF NPs (NF-DiI-CRV) showed minimal colocalization of macrophages with DiI from liver. These results were similar for both the targeted and the untargeted fusogenic NP constructs, in both healthy and infected animals. The clearance data are consistent with the size limitations displayed by the organs of mononuclear phagocytic system; NPs < 5.5 nm tend to be cleared by the kidneys and NPs show preferential clearance through the hepatobiliary system40,41.

We note the CRV-conjugated NPs showed substantially enhanced uptake in the spleen of infected animals relative to controls, which is attributed to the splenic clearance and filtration of targeted macrophages that have phagocytosed senesced neutrophils and damaged cells from the infected lung.

![Image](https://example.com/image1.png)

**Fig. 3** Gene knockdown in vitro and in vivo cytotoxicity of fusogenic porous Si nanoparticle constructs. a siRNA knockdown results (via qRT-PCR) from Raw 264.7 macrophage cells incubated with nanoparticles for 24 h. Error bars indicate SD (n = 6). The fusogenic formulations show substantial knockdown, comparable to standard lipofectamine transfection agent. No significant difference in knockdown efficiency is observed between the two fusogenic formulations (F-siIRF5-mPEG, F-siIRF5-CRV) and lipofectamine. Significant difference (one-way ANOVA with Tukey’s HSD post hoc test, p-level < 0.05, F (5, 30) = 28, \( p = 6.9 \times 10^{-10} \)). b Viability of J774a.1 and Raw 264.7 macrophage cells after 1 h incubation with NF-siIRF5-CRV and F-siIRF5-CRV nanoparticle constructs, containing 0.5 and 1 mg total mass of lipid as indicated. Error bar indicates SD (n = 6). ANOVA test found no statistical significance at \( p < 0.01 \); c-h H&E staining of major organs after 24 h circulation of F-siIRF5-CRV via tail vein injection (23.2 \( \mu \)mol/kg lipid, 24 \( \mu \)g/kg siRNA, 0.5 mg/kg psi) in healthy Balb/C mice; c brain; d heart; e lung; f liver; g kidney; and h spleen
these NPs (Fig. 4e). In marked contrast, infected lungs of mice injected with the targeted fusogenic NPs (F-DiI-CRV) showed high accumulation of DiI signals, which were strongly colocalized with macrophage fluorescence signals (Fig. 4f). Taken together, the results show that the combination of CRV targeting and fusogenic uptake achieves strong homing to circulating macrophages recruited to infected lungs.

**Fig. 4** Targeting peptide and fusogenic uptake enhances homing to infected lungs and macrophages. 

**a** Immunofluorescent sections of major organs of healthy and infected Balb/C mice injected with FAM-CRV peptide (green). Blue indicates cell nuclei stained with DAPI, red indicates macrophages marked by F4/80 antibody stain. **b** Quantified fluorescence signals (IVIS 200) from organs of healthy and infected Balb/C mice injected with fusogenic nanoparticles containing Dil membrane stain and sirf5 payload, with either a non-targeting (F-sirf5-mPEG) or the CRV targeting group (F-sirf5-CRV) at doses of 23.2 µmol/kg lipid, 24 µg/kg siRNA, 0.5 mg/kg pSi. “H-24” indicates healthy Balb/C organs “collected 24 h post treatment; “I-1” indicates Balb/C organs of infected harvested 1 h post treatment; and “I-24” indicates infected Balb/C organs 24 h post “ treatment. Error bars indicate SD. Data are representative of n = 3, quantified by ImageJ analyses. **c–f** Confocal microscope images of DiI-loaded fusogenic and non-fusogenic nanoparticles homed to infected lung with pendant CRV targeting peptide. These nanoparticles contained no sirf5 payload; green indicates macrophages (FITC-tagged rat anti-mouse F4/80 stain), red indicates lipophilic Dil from nanoparticles; **c** lung of healthy mouse injected with F-DiI-CRV; **d** lung of infected mouse injected with PBS control; **e** lung of infected mouse injected with NF-DiI-CRV; **f** lung of infected mouse injected with F-DiI-CRV. The data show Dil from the fusogenic, CRV-targeted nanoparticles is strongly co-localized with macrophages discussed above, we chose to deliver siRNA against the Irf5 gene of macrophages, in order to suppress inflammatory cytokine excretion of macrophages and enhance bacterial phagocytosis and tissue repair. We initially studied bacterial colonization and titers in the lungs of mice in three treatment groups as follows: (i) *S. aureus*-infected mice with no treatment (lungs collected at fatality); (ii) infected mice treated with PBS (lungs collected at fatality within 24 h post treatment); and (iii) infected mice treated with F-sirf5-CRV via tail vein injection (lungs collected 3 days post treatment). A section of a healthy lung is shown for comparison in Fig. 5a. The infected mice, when untreated (Fig. 5b) or treated with PBS only (Fig. 5c), displayed overt signs of neutrophilic pneumonia associated with bacteria. Moreover, *S. aureus* leakage into the perilymphgeal muscles was

**Therapeutic efficacy of targeted fusogenic pSiNPs.** With the cellular fusion and in vivo homing capabilities established, we next evaluated the therapeutic efficacy of the fusogenic, siRNA-loaded, CRV-targeted nanosystem (F-sirf5-CRV) against a lethal dose of *S. aureus* in the mouse pneumonia model. As
**Fig. 5** Macrophage-targeting fusogenic pSi nanoparticles loaded with siRf5 effectively treats infected mice. 

- **a-d** H&E-stained sections of Balb/C mouse lungs subjected to histopathological analyses. 
  - **a** Lung of healthy mouse with no treatment (inset shows lower magnification of the same).
  - **b** Lung of infected mouse with no treatment (inset shows large population of *S. aureus* in the perilaryngeal muscle).
  - **c** Lung of infected mouse ad mortem 24 h post-PBS treatment (inset shows gram stain of *S. aureus* populations in the lung).
  - **d** Lung of infected mouse treated with F-siIRF5-CRV nano-therapeutic (administered at 23.2 μmol/kg lipid, 24 μg/kg siRNA, 0.5 mg/kg pSi) at 3 days post treatment (inset shows lower magnification of the same).

- **e** Bacterial titer from lungs of healthy and infected mice injected with PBS, NF-siIRF5-CRV (non-fusogenic, targeted nanoparticle containing siRf5 therapeutic), F-siLuc-CRV (fusogenic, targeted nanoparticle containing siRNA against luciferase, as a negative control for siRf5), and F-siIRF5-CRV (fusogenic, targeted nanoparticle containing siRf5 therapeutic). Animals were infected on day 0 and therapeutic or control injections were given on day 1. The dashed red line indicates the average count of colony-forming units (CFU) in healthy mouse lungs. Error bars indicate SD. Each bar represents $n = 8$ animals. *Ad mortem; no measurements due to death of all mice in the cohort.

- **f** Mouse Therapeutics Injection injection

- **g** Mouse survival post-infection (at day 0) and post-therapeutic injection (at day 1) of PBS, NF-siIRF5-CRV, F-siLuc-CRV, or F-siIRF5-CRV. Each group has $n = 8$ mice. Average days of survival of mice from **f** post-infection and post-therapeutic injection. One-way ANOVA with Tukey's HSD post hoc test ($p$-level $<0.05$, $F (3, 28) = 17$, $p = 1.77 \times 10^{-6}$) revealed significant difference between F-siIRF5-CRV therapeutic and remaining three groups (PBS, NF-siIRF5-CRV, and F-siLuc-CRV). All animal experiments were performed independent of each other with different cohorts of mice.
observed in these untreated controls (Fig. 5b, inset), and Gram-positive cocci were identified in the lungs of the PBS-injected group by Gram staining (Fig. 5c inset). Three days after injection of the F-siIRF5-CRV therapeutic, the lungs of the infected mice displayed an appearance similar to healthy lungs and no bacteria were detected in the Gram stains (Fig. 5d).

We also quantified the bacterial population in the various treatment groups by preparing lung homogenates and counting the number of bacterial colonies obtained (Fig. 5e). Healthy mice showed an average baseline count of ~10^7 colony-forming unit (CFU)/g. Control groups of infected mice treated with PBS, with the NF targeted NP formulation containing siIRF5 (NF-siIRF5-CRV), or with the fusogenic targeted NP containing siRNA against luciferase as a negative control for siIRF5 (F-siLuc-CRV), were all observed to carry bacterial burdens of >10^10 CFU/g and all cohorts perished within 3 days of infection. By contrast, infected mice treated with the fusogenic targeted NP delivering the siIRF5 therapeutic (F-siIRF5-CRV) demonstrated a notable decrease in titer starting 2 days post infection and titers reached the baseline count at 8 days post infection.

To confirm that the therapeutic effect resulted from IRF5 knockdown, qRT-PCR was used to determine the relative in vivo efficiency in infected mice. Bronchoalveolar lavage (BAL) fluid collected from the lungs of infected mice was observed to have significantly lower expression of IRF5 (17%) in mice injected with the CRV-conjugated fusogenic pSiNPs relative to the PBS, free siIRF5, CRV-conjugated NF-pSiNPs, and non-targeted fusogenic pSiNP controls (Supplementary Fig. 5a).

However, the lung homogenates processed after removal of BAL fluid showed no significant difference in IRF5 expression between all groups (Supplementary Fig. 5b). BAL fluid in healthy mice consists of 98% macrophages, whereas infected mouse BAL fluid accumulates activated macrophages and neutrophils that are recruited during the initial stages of inflammation; by seven days post infection, the BAL fluid comprises 38% macrophages, 56% neutrophils, and 6% lymphocytes. As the BAL fluid was collected 48 h post infection and 24 h post-therapeutic injection, the population is expected to be primarily macrophages. On the other hand, the lung homogenate is composed of epithelial, endothelial, and interstitial cells, with a small population of macrophages. Thus, we conclude that the CRV-conjugated fusogenic particles successfully homed to the activated macrophages of the infected lungs in a selective manner and silenced IRF5 gene expression.

In a separate experiment, mouse survival was dramatically improved with the F-siIRF5-CRV nano-therapeutic; 100% of the mice administered the formulation survived the lethal challenge and showed no apparent sequelae from the infection within a 4-day post-infection observation period (Fig. 5f). Mice administered the NF-siIRF5-CRV, and mice administered the F-siLuc-CRV, with siLuc as a sham siRNA, showed limited survival, with a significantly lower average number of survival days compared with the F-siIRF5-CRV (one-way analysis of variance (ANOVA), post hoc comparisons using Tukey’s honest significant difference (HSD) test, Fig. 5g). By comparison, the first-line antibiotic vancomycin gave only 30% survival rate in the same pneumonia model when intravenously administered at a 3 mg/kg (the published ED_{50} for vancomycin in mice is in the range 0.65–4 mg/kg) single dose given one day post infection.

Discussion
A primary role of the inflammatory response during bacterial infection is to recruit additional macrophages whose function ultimately shifts to bacterial phagocytosis and tissue repair. However, an excessive inflammatory reaction can become septic, generating multi-organ failure and fatality. This study harnessed siRNA targeting the IRF5 gene, a transcriptional regulator of the inflammatory M1 macrophage phenotype that is a key inductor of proinflammatory cytokines. We hypothesized that effective silencing of this gene would suppress the inflammatory response and mitigate a lethal bacterial infection.

Silencing of IRF5 has not previously been tested as a means to slow or eliminate bacterial infections. A serious bacterial infection, such as the lethal S. aureus model studied here, requires an overwhelming and effective immune response. Although IRF5 is an attractive target, it was not clear that a strong response could be achieved using gene therapy alone, which can be notoriously inefficient in vivo. Achieving high in vivo knockdown efficiency has been a challenge due to passive clearance in circulation and endocytic uptake that causes extracellular excretion or lysosomal degradation of the oligonucleotides. In this work, we addressed these problems by incorporating three key features into the NP design: (i) a host pSiNP with high oligonucleotide loading efficiency and low systemic toxicity; (ii) a fusogenic lipid coating that effectively avoids endosomal uptake; and (iii) a targeting peptide that selectively homes the NPs to macrophages.

The first new aspect of the present approach was the NP host for the RNAi therapeutic. The carrier was based on porous silicon, a drug delivery vehicle that has shown good biocompatibility and an ability to load and protect sensitive therapeutics, such as proteins and oligonucleotides. We used a self-sealing chemistry that trapped the oligonucleotide payload within the NP in a calcium silicate matrix, loading a quantity at least 2 × greater than has been achieved with typical liposomal or related hybrid carriers (Supplementary Table 3). The calcium silicate pSiNPs follow the preparation protocol as introduced in Kang et al. In brief, large quantities of siRNA are loaded into pSi by precipitating a calcium silicate shell that simultaneously traps the payload. As the pSi matrix dissolves, the silicate product reacts with calcium (II) ion present in the CaCl_2 solvent, and forms Ca_2SiO_4 at the NP surface. To load siRNA, the oligonucleotide is added to the solvent to trap the molecules during the Ca_2SiO_4 shell formation. Between the presented work and the referenced formulation, the pSi chemistry and properties were identical, with the exception of particle size (which was ~180 nm). Thus, the pore volume in the F-pSi system is also expected to decrease by ~80% (1.36 ± 0.03 to 0.29 ± 0.04 cm^3 g^{-1}) upon shell formation. In contrast to the referenced work, the small pSiNP sizes (68.1 ± 5.8 nm; Supplementary Table 2) in the F-pSi system allows it to dissolve at an accelerated rate under physiological conditions (pH 7.4, 37°C). Moreover, the higher mass loading is important in minimizing the injected dose and maximizing gene silencing in the cells. Also, unlike the cationic polymer or oligomer stabilizers usually employed to increase loading of negatively charged oligonucleotide payloads, Ca_2^+ is an endogenous species that is essential for cellular function.

Second, we introduced a liposomal coating that protected the NP from premature degradation until cellular fusion. The coating contained a specific composition of lipids that favored fusion with the cellular membrane over endocytosis (Fig. 2b–g). The fusogenic lipid coating was composed of pro-fusogenic lipids and moieties. 1,2-Dimyristoyl-sn-glycero-3-phosphocholine (DMPC) is the major constituent that acts as the structural backbone of the liposome, with a relatively low phase transition temperature (T_m = 24 °C). The low transition temperature generates the L_a liquid crystal phase at room temperature and at body temperature. The L_a phase is the more fluidic, dynamic, and permeable structure that allows for a wide size range (100–400 nm) of extruded liposomal coatings and an easier fusion potential. DOTAP is the cationic lipid essential for the electrostatic attraction toward the anionic plasma membrane. Lastly,
PEGylated lipid was also found to be imperative in fusion\textsuperscript{31}; although the exact mechanistic role of PEG is not yet known, it is hypothesized that PEG binds water molecules to dehydrate the lipid head groups, which leads to structural asymmetry in the lipid alignment and drives double-leaflet to single-leaflet fusion as the energetically favorable route\textsuperscript{60}, similar to how SNARE proteins anchor and pull vesicles into merging with plasma membranes endogenously, in fact, neuronal SNAREs have been observed to promote PEG-mediated fusion\textsuperscript{61}. The fusion pathway peeled off the protective liposomal coating, which enhanced the rate of dissolution of the pSi carrier and release of the RNA payload after the bare NP was inserted into the cytosol, giving substantially greater knockdown of \textit{Irf5} in vitro (Fig. 3a).

Third, we used an activated macrophage-specific targeting peptide, which provided strong and selective homing to macrophages in infected lungs. The effectiveness of the targeting peptide in enhancing gene silencing was not apparent in vitro due to the high efficacy of the fusogenic coatings (Fig. 3a). However, the targeting peptide was critical for effective homing to macrophages in infected lungs in vivo. The CRV-targeted NP constructs localized in infected and not healthy lungs (Fig. 4b, c, f), whereas NPs containing the sham targeting group showed little accumulation in either infected or healthy lungs (Fig. 4b). Furthermore, the CRV-conjugated NPs strongly colocalized with macrophages in infected lungs (Fig. 4f).

The siRNA therapeutic chosen for this study focused on enhancing the macrophage response to an infection by selectively inhibiting a gene associated with inflammatory M1 macrophages. Macrophages are essential components of the innate immune system that are responsible for defense against a wide range of pathogens. On a cellular level, the alveolar macrophages respond to a challenge of infectious particles by secreting cytokines, resulting in an overall reduction in extended inflammation and accelerate tissue regeneration in mouse models of myocardial infarct and skin wounds\textsuperscript{10}. The improved healing response was attributed to attenuation of M1 macrophage polarization, which is typically the dominant macrophage phenotype in wounds shortly after injury. In these prior studies, the siRNA was delivered in a lipidoid NP vehicle\textsuperscript{95}.

Finally, it should be pointed out that the substantially improved survival afforded by the targeted gene nano-therapeutic developed in this work relative to a standardized dose of vancomycin represents a significant finding. Vancomycin is a first-line antibiotic, which is prescribed at high dosage and prolonged administration when used to treat \textit{S. aureus} infections\textsuperscript{2}. \textit{S. aureus} strains have a history of evolving antibiotic-resistance genes, such that we are currently facing vancomycin-intermediate and -resistant strains that build strong peptidoglycan walls to bind and trap vancomycin, and inhibit its therapeutic action\textsuperscript{2}. The \textit{Irf5} knockdown approach used here is unlikely to be susceptible to development of resistance.

Taken together, the combination of high payload capacity, fusogenic uptake, and macrophage-specific targeting yielded an \textit{Irf5}-silencing construct that staved all inflammation from a lethal dose of \textit{S. aureus}, and that gave the immune system time to clear the bacteria and return the lungs of the infected animals to their normal, healthy state within 7 days. The work reported here represents the first example of successful immunogene therapy against fatal deep-tissue infection. As the therapy focuses on changing the host macrophage response to suppress excessive inflammatory stimuli and enhance the antibacterial macrophage activity through \textit{Irf5} silencing, rather than attacking a phenotypic characteristic of the pathogen, the approach should be applicable to a wide range of infections.

**Methods**

**Materials.** Highly boron-doped p-type silicon wafers (~1 mΩ-cm resistivity, polished on the (100) face) were obtained from Virginia Semiconductor, Inc or Siltronix, Inc. Hydrofluoric acid (HF, 48% aqueous, ACS grade) was obtained from Fisher Scientific. Anhydrous calcium chloride was obtained from Spectrum Chemicals (Cardena, CA). Deionized (18 mΩ) water was used for all aqueous dilutions. For lipids, DMPC, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (PEG)-2000], 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleamide (PEG)-2000], and DOTAP were purchased from Avanti Polar Lipids (Alabaster, AL) and stored at ~4 °C. Fluorescent dyes Calcein (Sigma-Aldrich) and hydrophobic DiI (Life Technologies) were used and Lipofectamine\textsuperscript{2000} Transfection Reagent was obtained from Thermo Fisher Scientific. Custom siRNAs were purchased from Dharmacon (Lafayette, CO) and primers were purchased from IDT DNA (San Diego, CA). Macrophage-targeting peptide (CRV) was identified by Dr Erkki Ruoslahti’s group at Sanford Burnham Prebys Medical Discovery Center (SBPMDI, CA) and custom synthesized by CTC Scientific (Sunnyvale, CA). For in vitro studies, Raw 264.7 and J774a.1 cells were purchased from ATCC (Manassas, VA) within 6 months before all experiments. Dulbecco’s modified Eagle’s medium (DMEM) was purchased from GE Healthcare Life Sciences (HyClone, Pittsburg, PA), with supplemental fetal bovine serum (HyClone) and penicillin/streptomycin (HyClone). \textit{S. aureus} subsp. aureus Rosenbach (ATCC\textsuperscript{®} 25923\textsuperscript{®}) was purchased from ATCC within 6 months before all experiments, and 6-week-old male Balb/c were purchased from Envigo (Placentia, CA).

**Animals.** Six- to 8-week old mice (BALB/c, male) were purchased from Envigo (Indianapolis, IN). All animal experiments were performed independent of each other with different cohorts of mice. All animals for the in vivo studies were handled, anesthetized, and killed according to the Institutional Animal Care and Use Committee (IACUC) guidelines, and all experiments followed the approved protocol by the IACUC at SBPMDI.

**Preparation of pSiNPs.** pSi samples were prepared by electrochemical etching of silicon wafers in an electrolyte consisting of 3:1 (v/v) of 48% aqueous HF ethanol (caution: HF is highly toxic and proper care should be exerted to avoid contact with skin or lungs). A silicon wafer electrode with an exposed area of 8.6 cm\textsuperscript{2} was contacted on the back side with aluminum foil and mounted in a Teflon cell. The silicon wafer was then anodized in a two-electrode configuration with a platinum counter electrode, by applying an alternating current of square waveform, with lower current density of 50 mA/cm\textsuperscript{2} for 0.6 s and high current density of 400 mA/cm\textsuperscript{2} for 0.36 s repeated for 500 cycles. Then the porous layer was lifted off by...
etching at a constant current density of 3.7 mA/cm² for 250 s in a 1:20 (v:v) of 48% aqueous HF/ethanol solution, to be sonicated in deionized water for 12 h in NPs. Fluorescent dye and siRNA payloads were loaded into the pSiNPs by pore sealing with calcium silicate formation; the calcium silicate sealing chemistry has demonstrated high efficiency in loading anionic payloads previously53. Calcein was dis-solved in PBS at 100 mM. One hundred and fifty microliters of calcein was pipetted gently with 150 µl of pSiNPs, and 300 µl of 3 M calcium chloride was added. The mixture was sonicated using a sonicator for 15 min. For siRNA loading, we used siRfix5 (Irf5, sense 5′-dTdT-CUG CAG AUA AAC CUG A-A-dTdT-3′ and antisense 5′-dTdT-UCA GGG UUA UUC UCU GCA G-A-dTdT-3′), and siLc (Iuciferase, 5′-CUU ACG CUG AGU ACU UGU A-A-dTdT-3′ and antisense 5′-UGU AAG UAC UCA GGG UAA G-dTdT-3′). siRNA was dissolved in RNase-free water at 150 µg/mL and loaded into pSi with the same volume ratio and process as calcein loading with only RNase-free water used as solvent.

**Liposomal coating.** Fusogenic coating (F) and NF coating were prepared from DMPC, DSPE-PG, and DOTAP at the molar ratio of 76.2:3.8:20 and 96.2:3.8:0, respectively. The lipid films were prepared by evaporating the organic solvent, with 725.5 µg of DMPC, 151.6 µg of DSPE-PG (methoxy or maleimide terminated), and 196.3 µg of DOTAP (F) or 916.0 µg of DMPC and 151.6 µg of DSPE-PG (methoxy or maleimide terminated) (NF). The Dil-encapsulated films were added with 26.3 µg of Dil (1.25 mg/mL in 100% ethanol). The films were then hydrented with a payload-pSi solution and prepared for film formation/extrusion; the pSihydrented lipid was heated to 40°C with constant magnetic stirring for 10 min. Then the lipid mixture was loaded through 250 mesh 0.2 µm filters 30 times. CRV was conjugated to maleimide-terminated PEG by mixing 100 µl of 1 mg/mL CRV (in deionized water) in 1 mg/mL of the liposomal pSi (by lipid mass) overnight at 4°C. Particles were washed three times at each step by centrifugation in Microcon-30 kDa Centrifugal Filter Unit (EMD Millipore) by spinning at 5000 g at 25°C and then washed with PBS three times. The coverslips were mounted on glass slides with ProLong® Diamond Antifade Mountant with DAPI (4,6-diamidino-2-phenylindole) (Life Technologies), dried and kept in the dark until examined by confocal microscopy (Zeiss LSM 710 NLO). PCC for colocalization was calculated using the Coloc2 plugin from ImageJ. At least ten representative images were analyzed to obtain the average colocalization efficiency.

**For TEM of cells, particles were introduced to the Raw 264.7 and J774a.1 cells under the same conditions as above, and cells were fixed with glutaraldehyde overnight before being stained with osmium and uranyl acetate during embedding, and with lead on the TEM grids. The samples were viewed using a JEOL 1200 EX II TEM instrument.**

**In vitro knockdown.** In vitro knockdown efficiencies of the nanoformulations were quelled using a two-step qRT-PCR (Roche LightCycler 96). Raw 264.7 cells were seeded on a six-well plate at 3 × 10⁵ cells per well and grown to 80% confluency overnight. The cells were incubated with the desired nanoformulations at 0.2 nmol of siRNA in 2 ml of medium (100 nM siRNA). Forty-eight hours post incubation, the cell media was removed, and RNA was purified using the QiAshedder 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® Promaker Mastercycler thermal cycler. cDNA was mixed with Irf5 primers, or the control hypoxanthine phosphoribosyltransferase (HPRT) primers (Irf5 forward: 5′-AATACCCCAACCTTCTCTGTG-3′; Irf5 reverse: 5′-TGTAGATCTGG TGCCATTTGAG-3′; HPRT forward: 5′-CAAGAATGACATT-CCTTCCA-3′; and Iq SYBR Green mix according to the manufacturer’s instructions. RT-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.
Biofidelity of fusogenic NPs. For in vivo biofidelity validation, healthy Balb/C mice were intravenously injected with F-sIrf5-CRV at 23.2 μmol/kg lipid, corresponding to 69 μg/kg siRNA, and 0.3 mg/kg pSi in 100 μl PBS. After 24 h circulation, the mice were killed under deep isoﬂuorane anesthesia (no response to toe pinch) by cardiac perfusion, and the heart, lungs, liver, kidneys, and spleen were collected. Organs were ﬁxed immediately in 4% PFA and sent to the University of California, San Diego (UCSD)’s histology core to be parafﬁnized and sectioned for H&E staining. The stained slides were histopathologically evaluated by Dr Kent Osborn (Associate Director, Animal Care Program, UCSD).

In vivo infection model. After 16 h incubation in brain heart infusion broth, 10 μl of S. aureus were sub-cultured in 5 ml of fresh broth for 2 h to reach growth phase. The optical density at 600 nm was measured using a spectrophotometer and the broth set as the blank. Five milliliters of bacterial culture at OD600 ≈ 0.32 was centrifuged, the bacteria were washed by centrifugation in PBS three times, and re-suspended in 200 μl of PBS for inoculation. The S. aureus pneumonia animal model was established in 6- to 8-week-old male Balb/C mice by intratracheal catheter injection of ~1 × 106 CFU of bacteria in 10 μl of PBS. All treatment injections were performed 24 h after inoculation of the bacteria.

Biodistribution of CRV. Three healthy and infected (24 h post infection) mice were intravenously injected with CRV tagged with 5′-FAM dye in 100 μl PBS at a concentration of 1 mg/ml. Organs were harvested after 1 h circulation and ﬁxed in 4% PFA. Organs were sent to the UCSD histology core to be parafﬁnized, sectioned, stained with H&E, and analyzed using the Zeiss LSM 710 NLO confocal microscope with single photon laser (for excitation of Dil, calcein, LysoTracker Red) and Mai-Tai Laser HB (690-1020 nm) (for two-photon excitation of DAPI).

NP biodistribution. Eight-week-old male Balb/C mice were intratracheally infected as described. Twenty-four hours post infection, infected and healthy mice were intravenously injected with siIrf5-loaded fusogenic pSiNPs with or without CRV conjugation, at 23.2 μmol/kg lipid, corresponding to ~69 μg/kg siRNA, and 0.3 mg/kg pSi 100 μl in PBS. The Dil-loaded particle localization was visualized using the IVIS 200 (Perkin Elmer) with 0.12 s exposure time on the DiR-excitation and emission ﬁlters. Both healthy and infected animals were killed and collected for organs, lungs, and other organs, with additional 1 h post-treatment analyses for infected animals injected with the fusogenic NP formulations. ImageJ was used to quantify the ﬂuorescence of each organ, and averaged over the three mice per group. Infected lung homing was further validated using FACS. Twenty-four hours post infection, mice were intravenously injected with calcine-loaded NP particles with CRV, fusogenic particles without CRV, and fusogenic particles with CRV at 23.2 μmol/kg lipid, corresponding to 69 μg/kg siRNA, and 0.3 mg/kg pSi in 100 μl PBS. One hour post infection, the mice were killed by cardiac perfusion with PBS. The collected lungs were homogenized and the homogenates were processed with the LSR Fortessa FACS instrument, and analyzed using the FlowJo software (FlowJo, LLC). The microscopy of infected lungs, mice were intravenously injected 24 h post infection with Dil-loaded formulations of NF and fusogenic particles (without siRNA) conjugated with CRV, and were killed for lung collection and ﬁxation in 4% PFA at 24 h post injection. The ﬁxed lungs were parafﬁnized and sectioned, and stained with FITC-labelled F4/80 macrophage marker. The sections were observed under Zeiss LSM 710 NLO confocal microscope for DiL and FITC localizations.

In vivo therapeutic efﬁcacy of fusogenic NPs. Eight-week-old male Balb/C mice were intratracheally infected as described. Twenty-four hours post infection, infected mice were intravenously injected with 100 μl of PBS or siIrf5-loaded fusogenic pSiNPs with or without CRV conjugation, at 23.2 μmol/kg lipid, corresponding to 69 μg/kg siRNA, and 0.3 mg/kg pSi 100 μl PBS. Infected mice were intravenously injected with 100 μl of PBS or siIrf5-loaded fusogenic and NP-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, mice were killed for BAL processing. Before qRT-PCR processing, the cell pellets were kept dry at –80 °C. Collected lungs were weighed and homogenized, 30 mg of the homogenates were isolated for qRT-PCR processing, and stored at –80 °C.

The in vivo knockdown of Irf5 was quantiﬁed using two-step qRT-PCR (Roche LightCycler 96). The cell pellets from infected mice were intravenously injected with 100 μl of PBS or siIrf5-loaded fusogenic and NP-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, mice were killed for BAL processing. Before qRT-PCR processing, the cell pellets were kept dry at –80 °C. Collected lungs were weighed and homogenized, 30 mg of the homogenates were isolated for qRT-PCR processing, and stored at –80 °C.

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

Data availability. The data that support the ﬁndings of this study are available from the corresponding author upon request.

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**Author contributions**
B.K. designed, synthesized, and characterized NPs, and performed in vitro and in vivo experiments. H.P. performed phage display screening and identified the macrophage-binding peptide. J.K. synthesized NPs and performed in vitro experiments. B.K., H.P., J.K., J.P., E.R. and M.J.S. discussed and analyzed data. B.K., H.P., J.P., E.R. and M.J.S. conceived the project and wrote the manuscript. All authors read and approved the manuscript.

**Additional information**

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**Competing interests:** M.J.S. is a scientific founder of Spinnaker Biosciences, Inc., and has an equity interest in the company. Although one of the grants that supported this research has been identified for conflict of interest management based on the overall scope of the project and its potential benefit to Spinnaker Biosciences, Inc., the research findings included in this particular publication may not necessarily relate to the interests of Spinnaker Biosciences, Inc. The terms of this arrangement have been reviewed and approved by the University of California, San Diego in accordance with its conflict of interest policies. The remaining authors declare no competing interests.

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