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Local delivery of siRNA-loaded calcium phosphate nanoparticles abates pulmonary inflammation

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Abstract

The local interference of cytokine signaling mediated by siRNA-loaded nanoparticles might be a promising new therapeutic approach to dampen inflammation during pulmonary diseases. For the local therapeutic treatment of pulmonary inflammation, we produced multi-shell nanoparticles consisting of a calcium phosphate core, coated with siRNAs directed against pro-inflammatory mediators, encapsulated into poly(lactic-co-glycolic acid), and coated with a final outer layer of polyethylenimine. Nasal instillation of nanoparticles loaded with a mixture of siRNAs directed against different cytokines to mice suffering from TH1 cell-mediated lung inflammation, or of siRNA directed against NS-1 in an influenza infection model led to a significant reduction of target gene expression which was accompanied by distinct amelioration of lung inflammation in both models. Thus, this study provides strong evidence that the specific and local modulation of the inflammatory response by CaP/PLGA nanoparticle-mediated siRNA delivery could be a promising approach for the treatment of inflammatory disorders of the lung.

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Pulmonary inflammatory diseases, such as chronic obstructive pulmonary disease (COPD) or interstitial lung disease (ILD), have a high prevalence around the world and are characterized by mostly chronic inflammation and irreversible airway destruction. In addition to clinical symptoms, including coughing and dyspnea, systemic effects have been reported which are the main cause of death for patients suffering from COPD.1 The etiology of these diseases is not fully understood, but environmental factors, such as the inhalation of air pollutants, and genetic susceptibility contribute to the pathogenesis. The inhalation of irritants is followed by activation of alveolar macrophages leading to the secretion of chemokines and cytokines and thereby the continuous influx of more immune cells into the lung resulting in an inflammatory response of the airways.2 Despite ongoing research, available therapies only aim to minimize symptoms, and to date no therapy that interferes with the underlying mechanisms of inflammation has reached clinical admission. Recent advances in the use of biological treatment for other inflammatory conditions led to new approaches for the therapy of chronic pulmonary inflammation. As an altered cytokine and chemokine profile is found in patients with chronic pulmonary inflammation, therapies aimed at the neutralization of cytokines or blocking of chemokine receptors are regarded as promising approaches, and several medications are under development and reached clinical trials.3 So far, no trial has reached clinical significance and it is suggested that drugs which affect the synthesis of several cytokines

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may be more successful than medications targeting only one specific cytokine or its receptor. One new way to interfere in the cytokine signaling is the use of gene silencing by RNA interference. RNA interference, mediated by small interfering RNAs (siRNAs) of 19–23 base pairs, is a natural occurring mechanism for post-transcriptional gene silencing. The main limitation of RNA interference for therapeutic purposes is a successful local delivery into the target cells. The transport into the cytoplasm is difficult due to instability and susceptibility to degradation by nucleases. Among other delivery vehicles, calcium phosphate (CaP) nanoparticles have been shown to be highly efficient as carriers of small molecules into the cell. As mineral in human hard tissue, like teeth or bones, calcium phosphate is a biocompatible and degradable compound. In our previous study, CaP/poly(lactic-co-glycolic acid) (PLGA) nanoparticles were designed for the successful therapeutic delivery of siRNA to the gut. Following these promising results of siRNA delivery, we investigated the potential of these biodegradable, non-toxic siRNA-loaded CaP/PLGA nanoparticles for the therapeutic interference of pulmonary inflammation. We analyzed the uptake of these nanoparticles by cells present in the lung in vivo and examined the therapeutic efficiency of siRNA-loaded CaP/PLGA nanoparticles in mice suffering from TH1 cell induced lung inflammation or viral infection after nasal instillation.

Methods

Preparation of CaP/PLGA nanoparticles

The nanoparticles were prepared and functionalized as described previously. In brief, single shell CaP/siRNA nanoparticles were synthesized by mixing equal amounts of an aqueous solution of calcium-L-lactate (6.25 mM) and diammonium hydrogen phosphate (3.74 mM). After mixing, the calcium phosphate dispersion was mixed with solutions of either functional siRNA, scrambled siRNA (GE healthcare life sciences, Chalfont St. Giles, UK), or fluorescence-labeled oligonucleotides (Life Technologies) (Harlan Winkelmann GmbH, Borchen, Germany). SPC-HA Mice (Malvern, laser wavelength 2395–2403) were purchased from Harlan Laboratories (Harlan Winkelmann GmbH, Borchen, Germany). 5 × 10⁵ HA-specific T Helper 1 (TH1) cells were transfected into SPC-HA transgenic mice using autolysis (BD Bioscience, Heidelberg, Germany). BALB/c mice were sacrificed, the ripcage was removed, the trachea exposed, and a 22G catheter was inserted carefully in the lower trachea; 400 μL PBS was slowly infused into the lung via the catheter and the fluid was taken out again. This procedure was repeated three times and the fluid was collected for later Luminex assay analysis.

Isolation of cells and flow cytometric cell sorting of lymphocytes

Single cell suspensions from the lung and liver were obtained by digestion in media containing 5% FCS, and 100 U/mL collagenase IV of clostridium histolyticum (Sigma-Aldrich, Bonn, Germany) or 200 μg/ml collagenase D (Roche, Mannheim, Germany) respectively, at 37 °C for 45 minutes. Samples were pipetted up and down every 5–10 minutes. Afterwards, the remaining tissue was squashed through 70 μm cell strainers and washed with PBS containing 2% FCS and 2 mM EDTA or erythrocyte lysis buffer, respectively. Spleens and lymph nodes were squashed through 70 μm cell strainers, washed with erythrocyte lysis buffer or PBS containing 2% FCS and 2 mM EDTA, respectively. CD4+ T cells were enriched from spleens of TCR-CA transgenic mice using autolysis technology (Miltenyi Biotech, Bergisch-Gladbach, Germany) according to manufacturer’s instructions. HA-specific CD4+ T cells were obtained by staining cells with α-CD4 and an antibody against the HA-specific TCR [11] and sorting them by flow cytometry using an ARIA II Sorter (BD Bioscience, Heidelberg, Germany).

Induction of inflammation in SPC-HA transgenic mice and treatment with nanoparticles

HA-specific CD4+ T cells were sorted, activated in vitro with plate-bound α-CD3 and soluble α-CD28 antibodies (both BD Bioscience), and were polarized into IFN-γ producing TH1 cells by adding recombinant mouse IL-12 (20 ng/μL; R&D Systems, Wiesbaden, Germany) and α-IL-4 antibody (200 ng/μL; eBioscience, San Diego, CA) to the media. At day 6, TH1 polarization was confirmed by flow cytometric staining for IFN-γ.

Bronchoalveolar lavage

Mice were sacrificed, the ripcage was removed, the trachea exposed, and a 22G catheter was inserted carefully in the lower trachea; 400 μL PBS was slowly infused into the lung via the catheter and the fluid was taken out again. This procedure was repeated three times and the fluid was collected for later Luminex assay analysis.
Flow cytometric analysis

Flow cytometric analysis was performed on a Canto II flow cytometer using FACS DIVA software (BD Bioscience). Oligonucleotides were labeled with AlexaFluor647 (Life Technologies), and dead cells were excluded by staining with 7-AAD (eBioscience, San Diego, California, USA) or the fixable viability dye eFluor780 (eBioscience). Analysis was performed using α-mouse antibodies against CD45 (30-F11), MHCII (M5/114.15.2), CD11c (N418, all Biolegend, San Diego, CA), F4/80 (BM8), CD16/CD32 (93, both eBioscience, San Diego, CA), CD11b (M1/70), B220 (RA3-6B2), CD4 (RM4–5), CD8 (53–67, all BD Bioscience, Heidelberg, Germany), conjugated with fluorescein isothiocyanate, phycoerythrin, phycoerythrin-cyanine7, pacific blue, or brilliant violet 510. For uptake analysis in the lung cells were divided into DCs (F4/80 'MHCII' 'CD11c' 'CD11b'), macrophages (F4/80 'CD11cmed'), alveolar macrophages (F4/80 'CD11cmed' high auto fluorescence), epithelial cells (negative for CD45, F4/80, CD11c, CD11b, CD16/32), alveolar epithelial cells type I (as epithelial cells and T11e) and alveolar epithelial cells type II (as epithelial cells and high in the side scatter).

RNA isolation, quantitative real-time PCR and luminex assay

Isolation of RNA from biopsy samples from the lung was performed using the RNeasy Fibrous Tissue Mini Kit (Qiagen, Hilden, Germany). Subsequently, 1 μg of RNA underwent reverse transcription using M-MLV (H-) point mutant reverse transcriptase (Promega, Mannheim, Germany). Quantitative real-time analysis was performed with a 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA), using the Fast SYBR Green Master Mix (Life Technologies) and specific primers for IFN-γ (5′-AGG AAC TGG CAA AAG GAT GGT GA-3′ and 5′-TGT TGC TGA TGG CCT GAT TGT CTT-3′), TNF-α (5′ CAA TGG ACA GGC TTC CTC ACA G-3′ and 5′-TAC ATA AAT AAA CCT TCC GGC CC-3′), IP-10 (Interferon gamma-induced protein 10; 5′-CTC TCC ATCA CTC CCC TTT ACC C-3′ and 5′-GCT TCG GCA GTT ACT TTT GTC TCA-3′), KC (Keratinocyte-derived cytokine; 5′-CAT GGC TGG GAT TCA CCT C-3′ and 5′-CAG ACC ACT TGC GAC CGA A-3′), CCL-2 (5′-CTG TGC TGG CCC CAA GAA GG-3′ and 5′-TAA GGC ATC ATA GTC GCA GTC ACA G-3′), NS-1 (5′-CAG GAC ACA ATG ATG AGG ATG-3′ and 5′-GTT TCA GAG ACT GCA ACT GTC-3′) and RPS9 (5′-CTG GAC GAG GGC AAG ATG AAG-3′ and 5′-TGA CTG TGG CGG ATG AGC ACA-3′). Relative mRNA levels were calculated with included standard curves for each individual gene and further normalization to the housekeeping gene RPS9. Cytokines from the bronchoalveolar lavage fluid were quantified using a Procarta Cytokine assay kit (Panomics, Santa Clara, CA, USA) according to the manufacturer’s guidelines. The assay was run with a Luminex 200 system using Luminex IS software (Luminex Corporation, Austin, TX, USA).

Results

Characterization of calcium phosphate nanoparticles

CaP/PLGA nanoparticles were prepared in a multistep process as described previously. The CaP core was coated with siRNA or fluorescence-labeled oligonucleotides, encapsulated into PLGA, and finally coated with polyethylenimine (PEI). This composition was previously found by our group being especially potent for the successful delivery and release of nucleic acids to cells at mucosal surfaces. The successful synthesis was evaluated by scanning electron microscopy (Figure 1, A) and dynamic light scattering measurements (Figure 1, B). The final nanoparticles were homogenous and spherical in morphology and exhibited an average diameter of ~145 nm and a zetapotential of +23 mV (Figure 1, C) confirming the colloidal stability in water.

To further analyze the general distribution, the uptake of nanoparticles between different animals was low, indicating a comparable and reproducible uptake in this experimental setting (Figure 2, A). To address if siRNA-loaded CaP/PLGA nanoparticles can be used as local delivery vehicle for pulmonary diseases in vivo, the delivery of these nanoparticles to the lung after nasal instillation was evaluated. Fluorescence-labeled CaP/PLGA nanoparticles were administered to BALB/c mice once, and 1 hour post-administration, a fluorescent signal was detectable in the lungs of mice treated with nanoparticles as demonstrated by in vivo life imaging (Figure 2, A) as well as fluorescence microscopy of tissue sections (Figure 2, B). Furthermore, the variability of the uptake of nanoparticles between different animals was low, indicating a comparable and reproducible uptake in this experimental setting (Figure 2, A).
systemic distribution. Further flow cytometric analysis of the lung demonstrated that macrophages and dendritic cells were the main cell populations to internalize the functionalized CaP/PLGA nanoparticles, while only very few epithelial cells and lymphocytes endocytosed the nanoparticles (Figure 2, C). These results confirm the local uptake of functionalized CaP/PLGA nanoparticles by the lung in vivo after nasal instillation, diminishing the possibility of side effects.

Establishment of TH1-induced lung inflammation

To analyze the therapeutic approach using siRNA-loaded CaP/PLGA nanoparticles and determine potential target genes, a TH1-mediated model of pulmonary inflammation was used. T cell receptor (TCR) transgenic HA-specific CD4+ T cells from TCR-HA transgenic mice were sorted and differentiated into TH1 cells in vitro. The differentiation efficiency was determined on
day 6 by analysis of the IFN-γ production (Figure 3, A). Approximately 35–45% of the CD4+ T cells produced IFN-γ confirming a TH1 phenotype. After the differentiation, $5 \times 10^5$ HA-specific TH1 cells were adoptively transferred into SPC-HA transgenic mice and non-transgenic littermates. Severity of inflammation was assessed by weight loss (B) and histology on day 6 (C). Expression of pro-inflammatory cytokines and chemokines was assessed by real-time PCR (D) and Luminex analysis (E). Statistical analysis was performed using Student’s t-test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; n.d., not detectable).

Cytokine and chemokine signaling plays an important role during pulmonary infection and inflammation. For instance, during COPD, several cytokines have been reported to be elevated in the fluid obtained from bronchoalveolar lavages or biopsies from the lung. To determine target genes for the siRNA treatment in this disease model, biopsies from the lung were analyzed for the expression of several cytokines. Diseased SPC-HA transgenic mice expressed significantly increased levels of IFN-γ, IP-10, KC, CCL-2 and TNF-α compared to healthy littermates after transfer of TH1 cells (Figure 3, D). This elevated expression was confirmed on protein level in the fluid obtained from bronchoalveolar lavages (Figure 3, E). The strongest increase compared to non-transgenic mice was detectable in the expression of IP-10 on mRNA and protein level, but also CCL-2, TNF-α, KC and IFN-γ were significantly elevated in mice suffering from TH1-induced pulmonary inflammation. The most prominent expression on mRNA level was found for IP-10, CCL-2, and IFN-γ, therefore, those genes were chosen as therapeutic targets in this disease model.
Treatment with siRNA-loaded CaP/PLGA nanoparticles abrogates pulmonary inflammation

To examine the therapeutic potential of siRNA-loaded CaP/PLGA nanoparticles, SPC-HA mice were treated with a mixture of siRNAs or scrambled siRNA-loaded nanoparticles on days 1, 3 and 5 after T11 cell transfer (Figure 4, A). During the treatment, the weight loss was monitored daily. While non-transgenic mice did not lose any weight after the cell transfer, SPC-HA transgenic mice which were treated with scrambled siRNA-loaded nanoparticles or PBS exhibited a weight loss of 7% by day 6. Interestingly, the transgenic mice treated with specific siRNAs lost less weight than the animals of the control groups (Figure 4, B). Mice were sacrificed on day 6 and further analyzed. Importantly, mice treated...
with siRNA-loaded CaP/PLGA nanoparticles had a significantly reduced number of cells within the BAL fluid compared to scrambled control or PBS treated mice, indicating a less severe inflammation, as patients suffering from pulmonary inflammation often exhibit an influx of immune cells due to increased chemokine production14 (Figure 4, C). Furthermore, the lung tissue of mice treated with siRNA against CCL-2, IP-10 and IFN-γ was less severely inflamed than tissue from control animals. Less alveolar epithelial cell type II hyperplasia, a reduced accumulation of alveolar macrophages and a decreased number of neutrophils in the interstitium were found in siRNA-treated mice. This is also reflected in the significantly reduced histological score of the lung tissue (Figure 4, D). Finally, to validate if the potential beneficial effect of the siRNA-loaded CaP/PLGA nanoparticle treatment is due to the decreased expression of the target genes, biopsies of the lung and fluid from the BAL were analyzed for gene expression. Indeed, the expression of all three target genes was significantly decreased on mRNA and protein level (Figure 4, E). The expression of CCL-2 was reduced by 40% compared to the scrambled control treated animals on both, mRNA and protein level. The IP-10 expression in lung tissue was decreased by 50% on mRNA level while the down-regulation on protein level was less prominent but nevertheless reduced by 25%. The IFN-γ production was also significantly diminished by 55% on protein level. In summary, these results indicate a beneficial therapeutic effect of siRNA-loaded CaP/PLGA nanoparticles for inflammation of the lung.

Treatment with siRNA-loaded CaP/PLGA nanoparticles reduces viral lung infection

In addition to inflammatory diseases the lung is prone to viral infections. While for some viral infections vaccines are available, other diseases like the severe acute respiratory syndrome caused by a coronavirus are not curable and a vaccine does not exist.15 A therapeutic rather than a prophylactic approach to treat viral infections is the use of siRNA to interfere in the reproduction of the virus and inhibit its spreading. To verify if this interference is a possibility to reduce the viral load, CaP/PLGA nanoparticles were loaded with siRNA directed against the non-structural protein 1 (NS1) of the influenza virus. BALB/c mice were infected with the influenza virus and treated with siRNA-loaded nanoparticles two days post infection and analyzed for signs of sickness on day 5 (Figure 5, A). To verify that the viral protein expression is altered due to the treatment with siRNA-loaded nanoparticles, the NS-1 expression was analyzed in the lung tissue. Indeed, the expression of NS-1 in the lung of infected mice which received siRNA-loaded CaP/PLGA nanoparticle was significantly decreased (Figure 5, B). In consequence, also the viral load was significantly reduced in the lung on day 5 (Figure 5, C). The histopathological analysis of the lung tissue revealed less structural damages and less infiltrated cells in mice treated with siRNA against NS1 compared to mice treated with scrambled siRNA-loaded CaP/PLGA nanoparticles or PBS. The histological score was significantly decreased by almost half in...
mice which received NS1 siRNA treatment (Figure 5, D). In summary, these results illustrate a promising therapeutic approach against viral infections.

Discussion

Inflammatory conditions of the lung are widespread and heterogeneous, including interstitial lung disease (ILD), chronic obstructive pulmonary disease (COPD) and asthma. They are characterized by airflow limitation, emphysema, fibrosis or bronchitis. Despite ongoing research, currently available therapies, mainly inhalers, aim to minimize symptoms and aggravations but are not curative. Inhalers open the airway muscles by relaxation or interfere in the activation of the immune system but are unspecific. Therefore, medications to treat the underlying inflammation or infection might be an improved and promising treatment strategy. Here, we have used an inflammatory model based on CD4+ TH1 lymphocytes which are described to be important effectors of pulmonary injury, especially in ILD. Many diseases, including ILD and COPD, are caused by excessive, incorrect activity of genes; the ability of siRNA to modulate gene expression specifically on a molecular level offers a suitable approach for interference. Here, we used a sterile model of lung inflammation to analyze the potency of local modification of gene expression by siRNA. siRNA directed against CCL-2, IP-10 and IFN-γ was used to reduce gene expression in inflamed lung tissue, and siRNA directed against NS-1 of the influenza virus itself to diminish viral replication. To exert its function, siRNA must be delivered to the target tissue; here, we demonstrated that CaP/PLGA nanoparticles can successfully be used as delivery vehicle to the lung tissue. The nanoparticles protect the siRNA from the environment and can be used to decrease gene expression and thereby abrogate inflammatory responses in the lung.

Calcium phosphate has previously been described as a safe and biocompatible biomedical carrier due to its natural occurrence in mammalian hard tissues. In this study, we have used CaP nanoparticles encapsulated within PLGA, a safe and biodegradable polymer used as pharmaceutical delivery vehicle, to deliver siRNA safely into target cells. PEI, a different polymer, was applied to the surface for an increased cellular uptake and endosomal escape. Alongside an increased uptake efficiency, PEI was reported to induce inflammatory responses in the lung; however, in this study, no inflammatory effects were detected. This might be due to the highest uptake rate by macrophages in this study, while a study by Beyerle and colleagues demonstrated that PEI mainly affects alveolar epithelial cells. Other reports highlighted that the drug delivery to alveolar macrophages has been shown to be most efficient with particles in the low micrometer range, while smaller particles have been suggested to have a toxic effect. This effect might be due to the distribution of the nanoparticles beyond the lung into the blood and brain. However, not all particles in the nanoscale behave the same, but their properties depend on the composition. Other publications noticed only a minimal translocation of nanoparticles applied to the lung. Furthermore, compared to larger particles, particles in the nanoscale can target a variety of cells and not only macrophages, resulting in an increased uptake and delivery of active compound. In previous publications, we have shown that this composition of nanoparticles is highly efficient to achieve an increased ability of gene silencing per nanoparticle as well as an efficient uptake and down-regulation of target genes at mucosal surfaces.

Patients suffering from acute or chronic lung disorders have increased levels of several chemokines and cytokines in sputum samples, blood or the fluid obtained from BALs. In our model, we regulated the expression of IFN-γ, CCL-2 and IP-10 to achieve a decreased inflammation of the lung. IFN-γ was found to be elevated in lung biopsies of patients suffering from COPD, and Wang and colleagues demonstrated the importance of IFN-γ in lung related diseases, as IFN-γ overexpressing mice develop pulmonary emphysema. In response to IFN-γ, IP-10 is secreted by several cell types and functions as chemoattractant for natural killer cells and macrophages. IP-10 is elevated in blood and sputum samples of COPD patients, and increased levels were found to correlate with disease severity. Similar to IP-10, CCL-2 functions as chemoattractant and is elevated in sputum samples of patients with inflammatory lung conditions. Studies regarding the function of CCL-2 or its receptor, CCR2, hardly exist. However, a murine antagonist against CCR2 was successfully applied to reduce influenza virus induced lung pathology. The treatment with CaP/PLGA nanoparticles loaded with siRNA against these three target genes led to decreased levels of gene expression in the TLR1-induced model of lung inflammation, thus we have indirectly shown the successful delivery of siRNA and its release to the cytoplasm into cells from the lung, after nasal instillation. The knockdown of all three mRNAs was sufficient to decrease protein levels of the target genes and most importantly, to restrict the pulmonary inflammation. So far, biological agents have not proven successful in clinical trials for inflammatory conditions of the lung; one reason might be the different phenotypes and varying symptoms between patients. Furthermore, the inflammatory response is likely not driven by only one mediator but may be attributed to several dysregulated genes. The siRNA-loaded CaP/PLGA nanoparticles have turned out as a promising approach, as they are easily adaptable to individual patients and can target several overexpressed genes at once.

A further interesting field of application for siRNA-loaded CaP/PLGA nanoparticles is the application against lung infections by viruses. While flu caused by the influenza virus is rarely dangerous due to effective immunization, several viral infections exist with no potent vaccine available. A therapeutic rather than a prophylactic approach against viral infections might be the knockdown of viral gene expression themselves. As proof-of-principle experiment, we used siRNA-loaded CaP/PLGA nanoparticles directed against NS-1 of the influenza virus and demonstrated a reduced viral load and pathology. To our knowledge, this is the first study to use siRNA against NS-1 as a therapeutic treatment and to apply the nanoparticles locally to the lung. A previous study only exhibited the beneficial effect of intravenous, prophylactic treatment with siRNA against influenza viral infection. In summary, we were able to use siRNA-loaded CaP/PLGA nanoparticles covered with PEI directed against IFN-γ, IP-10...
and CCL-2 to achieve a specific knockdown of the target gene expression in vivo by RNA interference. By multiple nasal instillations, we were able to diminish pulmonary inflammation caused by TH1 cells. Additionally, we demonstrated the beneficial effect of RNA interference against viral genes as therapeutic treatment. In summary, our data provide a promising approach for the specific delivery of siRNA to the lung and the treatment of pulmonary inflammation.

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