

Transferrin-mediated Pulmonary GATA3 siRNA Delivery in a Murine Model of Allergic Asthma

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Abstract

Asthma continues to pose a major clinical burden despite the availability of modern biologic therapies. In eosinophilic asthma, the transcription factor GATA3 is a key regulator of Th₂ differentiation and type 2 cytokine production and therefore represents an attractive target for siRNA-based intervention. We previously established a transferrin-functionalized polyethyleneimine (Tf-PEI) polyplex system for targeted siRNA delivery to human T-cells. Here, we aimed to translate and adapt this platform to the murine system to enable therapeutic evaluation in a preclinical asthma model. Accordingly, a murine Tf-PEI polyplex system was developed for targeted delivery of siRNA to activated T-cells in the lung. Murine CD4⁺ T-cells were polarized toward a Th₂ phenotype and used to assess nanoparticle uptake and functional GATA3 silencing *in vitro*. The performance of the polyplexes was further evaluated in activated murine precision-cut lung slices and in an ovalbumin-induced murine asthma model. While non-targeted PEI showed high cellular uptake, this did not translate into efficient or sequence-specific gene silencing. In contrast, Tf-functionalized polyplexes enabled functional GATA3 knockdown, with partially functionalized Tf-PEI outperforming fully functionalized formulations. The optimized 50% Tf-PEI blend achieved the strongest silencing in primary Th₂ cells and lung slices and mediated an approximate 50% reduction in GATA3 mRNA levels *in vivo*. In the murine asthma model, this was accompanied by reduced inflammatory cell infiltration, improved lung histopathology, and the lack of pronounced inflammatory responses under the tested conditions.

Collectively, our results demonstrate that precise tuning of transferrin ligand density critically controls functional siRNA delivery to pulmonary T-cells *in vivo*, establishing a rational design framework for targeted RNA nanotherapeutics against type 2 airway inflammation.

1 Introduction

Asthma is one of the most prevalent chronic inflammatory airway diseases, affecting more than 260 million people worldwide [1]. Eosinophilic asthma, which is largely driven by type 2 immune responses, is strongly associated with severe asthma. Despite treatment with inhaled corticosteroids and biological therapies, a substantial proportion of patients remain insufficiently controlled, highlighting the need for additional therapeutic strategies [2].

GATA3 is a central transcription factor in CD4⁺ T-cells and plays a key role in Th₂ differentiation and type 2 cytokine production [3]. Its elevated expression in eosinophilic asthma makes GATA3 an attractive therapeutic target [4]. In contrast to therapies directed against individual cytokines, targeting GATA3 may allow intervention further upstream in the type 2 inflammatory cascade [5].

siRNA-based gene silencing offers a highly specific strategy to downregulate such disease-relevant genes [6]. However, unprotected siRNA is usually rapidly degraded by extracellular RNases, poorly internalized due to its negative charge, and inefficiently delivered to the cytosol [7]. In particular, endosomal escape remains one of the major bottlenecks in functional siRNA delivery [8]. This challenge is especially relevant for T-cells, which are generally difficult to transfect due to their low endocytic activity, sensitivity to transfection-induced stress, and efficient intracellular defense mechanisms against foreign nucleic acids [9–11]. These limitations emphasize the importance of delivery route in siRNA therapeutics. For respiratory diseases, the lung represents a convenient target, allowing local administration with reduced systemic exposure. At the same time, inhalation enables non-invasive delivery and high local concentrations [12]. In addition, the pulmonary environment is relatively low in nucleases and serum proteins, favoring siRNA stability [13]. However, multiple barriers limit effective delivery. In conducting airways, mucus can trap cationic carriers and promote rapid mucociliary clearance, while in the alveolar region, interactions with surfactants may destabilize delivery systems. Furthermore, clearance by alveolar macrophages and inefficient

cellular uptake restrict intracellular delivery [14]. These hurdles may be further exacerbated under inflammatory conditions [15].

Polymeric carriers such as polyethyleneimine (PEI) have shown considerable potential as nucleic acid delivery systems because of their ability to condense siRNA and promote cellular uptake. However, PEI-based systems are often limited by nonspecific interactions, excessive positive surface charge, and potential cytotoxic or pro-inflammatory effects [16]. In previous human *in vitro* and *ex vivo* models, our group demonstrated that conjugation of low molecular weight PEI with transferrin (Tf) improved uptake into activated T-cells, which showed increased expression of the transferrin-receptor (TfR; CD71), and enabled GATA3 knockdown [17, 18]. However, whether this targeting strategy can achieve therapeutically relevant siRNA delivery *in vivo* under inflammatory airway conditions remains unknown. Moreover, the impact of Tf ligand density on functional intracellular siRNA delivery has not yet been systematically investigated.

Therefore, this study aimed to establish a murine Tf-functionalized PEI-based siRNA delivery system for targeted GATA3 silencing in type 2 airway inflammation. Based on physicochemical screening, partially and fully Tf-functionalized polyplexes were compared to assess how Tf content affects cellular uptake and functional gene silencing. The optimized formulation was then evaluated in polarized murine Th₂ cells, activated murine precision-cut lung slices, and an OVA-induced murine asthma model to assess its potential for therapeutic pulmonary siRNA delivery.

2 Materials and Methods

2.1 Synthesis of mTf-PEI Conjugates and Polyplex Preparation

To assess the feasibility of TfR targeting in the murine system, murine holo-transferrin (mTf; Rockland Immunochemicals, Pottstown, PA, USA) was used to functionalize branched low-molecular-weight polyethyleneimine (PEI, 5 kDa). Branched PEI (Lupasol G100, BASF, Ludwigshafen, Germany) was diluted to 1 mg/mL in PBS containing 2 mM EDTA (Sigma-Aldrich, St. Louis, MO, USA) at pH 7.1. Thiolated PEI was generated using Traut's reagent, which preserves the effective charge density of PEI at physiological pH through the formation of amidinium groups during the ring-opening reaction. Briefly, Traut's reagent (2-iminothiolane hydrochloride, 14 mM in PBS containing 2 mM EDTA; Thermo Fisher Scientific, Waltham, MA, USA) was added to the PEI solution at a 2:1 molar ratio, and the mixture was stirred for 1 h at room temperature. To prevent thiol oxidation and undesired crosslinking, all reactions were performed in metal-free buffers supplemented with EDTA. The resulting PEI-thiol was purified using 3000 MWCO centrifugal filters (Amicon® Ultra, Merck Millipore, Darmstadt, Germany), and the degree of thiolation was determined using Ellman's assay.

mTf was concentrated in HEPES-buffered saline (HBS; 20 mM HEPES, 150 mM NaCl, pH 7.4) using 10,000 MWCO centrifugal filters (Vivaspin 6, Cytiva, Marlborough, MA, USA) to obtain a final concentration of 1 mg/mL. Subsequently, SPDP (16 µL, 20 mM in DMSO; Thermo Fisher Scientific) was added dropwise under stirring and incubated for 2 h at room temperature to obtain SPDP-activated mTf. The activated mTf was purified using 10,000 MWCO centrifugal filters and subsequently reacted with thiolated PEI under gentle stirring overnight at 4°C.

The resulting mTf-PEI conjugate was purified by ion-exchange chromatography using two HiTrap™ SP HP cation exchange columns on an ÄKTA purification system (Cytiva). Tf concentration was measured spectrophotometrically at 280 nm, while PEI content was quantified using a TNBS assay at 405 nm, as described previously [18].

Polyplexes were prepared in HBS. Defined amounts of mTf-PEI, PEI, or blends thereof at specified ratios were first diluted in HBS and then combined with an equal volume of siRNA in HBS to achieve the desired nitrogen-to-phosphate (N/P) ratios. The amount of polymer required to achieve each N/P ratio for a defined amount of siRNA was calculated using the following equation:

$$m_{polymer} (pg) = n_{siRNA} (pmol) \times M_{protonable\ unit} \left(\frac{g}{mol} \right) \times N/P \times number\ of\ nucleotides_{siRNA}$$

Complex formation was induced by turbulent pipette mixing followed by incubation for 30 min.

2.2 Particle Size and Zeta Potential

The particle size (hydrodynamic diameter), polydispersity index (PDI), and zeta potential (ζ -potential) were characterized using a Zetasizer Advance Ultra (Malvern Instruments, Malvern, UK). For determination of hydrodynamic diameter and PDI via dynamic light scattering (DLS), 100 μ L of each sample were transferred into ZEN0040 disposable cuvettes (Brand GmbH, Wertheim, Germany). The cuvettes were then placed in the instrument, which measures scattered light at a 173° backscatter angle.

To determine the ζ -potential, measurements were performed using Phase Analysis Light Scattering (PALS). Prior to analysis, 100 μ L of sample were diluted with 700 μ L of highly purified water and transferred into folded capillary zeta cells (DTS1070; Malvern Instruments). Each sample was measured three times. The refractive index of HBS was set to 1.33 and the viscosity parameter was defined as 0.891 mPa·s, while the dielectric constant was set to 79. The measurements were conducted at a temperature of 25°C. All data sets were processed using ZS Xplorer software (version 3.31).

Nanoparticle Tracking Analysis (NTA) was performed using a NanoSight NS300 (Malvern Instruments) equipped with a blue 488 nm laser and sCMOS camera. Samples were diluted in HBS to achieve an optimal concentration of 50–100 particles per frame. Five 60-second videos were captured at 25°C with a camera level of 16. Data was analyzed using NTA 3.4 Build 3.4.4 with a detection threshold of 4. Values are reported as the mean of five independent measurements.

2.3 Encapsulation Efficiency

The siRNA complexation efficiency was evaluated using a SYBR Gold Nucleic Acid Gel Stain (Thermo Fisher Scientific). A 10,000× SYBR Gold stock solution in DMSO was diluted 2,500-fold in formulation buffer to obtain a 4× working solution. Polyplexes were prepared as described above, and 20 μ L of each sample was loaded in triplicate into a 384-well black microplate. Controls included equal volumes of free siRNA (100%) and formulation buffer (blank). To each well, 6 μ L of the 4× SYBR Gold working solution was added. The plate was shielded from light with aluminum foil and incubated at room temperature for 5 min under orbital shaking (300 rpm). Fluorescence intensity was measured using a microplate reader (Tecan, Männedorf, Switzerland) at an excitation wavelength of 492 nm and an emission wavelength of 537 nm. The percentage of free siRNA was calculated by normalizing the fluorescence of the samples against the free siRNA and blank controls.

2.4 siRNA Release Assay

Samples, free siRNA solution and blanks were prepared in a 384-well black microplate. To 20 μ L of each sample, 2 μ L of heparin (2000 IU/mL; Thermo Fisher Scientific) solution and 6 μ L of the 4× SYBR Gold working solution were added using a multichannel pipette. The plate was protected from light with aluminum foil and incubated on a plate shaker at 300 rpm for 5 min at room temperature. Fluorescence intensity was subsequently measured at an

excitation wavelength of 492 nm and an emission wavelength of 537 nm. The amount of released siRNA was calculated based on the resulting fluorescence data.

2.5 Naïve CD4⁺ T cell Isolation from Spleen and Th₂ Polarization

All experiments involving animal organs or tissues were conducted according to the German law of animal protection and were approved by the Government of Upper Bavaria, Munich, Germany (5.1–231 5682/LMU/BMC/CAM). C57BL/6J mice (bred at Core facility animal models) aged 8–9 weeks were euthanized in accordance with institutional animal care guidelines. Immediately after euthanasia, spleens were harvested and placed in ice-cold PBS. To obtain single-cell suspensions, spleens were mechanically dissociated using a gentleMACS Dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany) and passed through a 70 µm cell strainer (Greiner Bio-One, Frickenhausen, Germany). Cells were centrifuged at 300 × g for 5 min at 4°C, followed by red blood cell lysis (Miltenyi Biotec). Naïve CD4⁺ T-cells were subsequently isolated using magnetic-activated cell sorting with the Naïve CD4⁺ T-cell Isolation Kit (Miltenyi Biotec), according to the manufacturer's instructions. Purified cells were resuspended in complete RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Sigma-Aldrich), and 1× penicillin/streptomycin (Sigma-Aldrich). Cells were counted and seeded at a density of 1 × 10⁶ cells/mL in 6-well tissue culture plates and incubated at 37°C with 5–10% CO₂.

For Th₂ polarization, naïve CD4⁺ T-cells were activated using Dynabeads™ Mouse T-Activator CD3/CD28 (Thermo Fisher Scientific) at a 1:1 bead-to-cell ratio. Cytokines IL-4 (10 ng/mL) and IL-2 (10 ng/mL), as well as anti-IFN-γ antibodies (10 µg/mL), were added to the culture medium according to the manufacturer's instructions (Miltenyi Biotec). Cells were cultured at 1 × 10⁶ cells/mL at 37°C and 5–10% CO₂ for six days, with medium replenishment and cell splitting performed as required based on cell proliferation. Successful Th₂ skewing was evaluated by flow cytometry using CD4⁺ and intracellular GATA3 staining, with analysis restricted to the lymphocyte gate based on forward and side scatter characteristics.

2.6 Transferrin Receptor Expression

Naïve CD4⁺ T-cells or activated Th₂ cells were prepared at appropriate concentrations and resuspended in PBS supplemented with 2 mM EDTA (Sigma-Aldrich) and 0.1% BSA (Miltenyi Biotec) (MACS buffer). To block Fc receptor-mediated nonspecific binding, cells were incubated with anti-mouse CD16/32 antibody (TruStain FcX™, BioLegend, San Diego, USA) at a final volume of 50 µL per sample. Specifically, 2 µL of Fc block reagent was added per sample and adjusted to volume with MACS buffer. Samples were incubated for 5–10 min on ice. Without a washing step, 50 µL of PE-conjugated staining antibody was added directly. Cells were stained with anti-mouse CD71-PE (clone R17217, Thermo Fisher Scientific) at a final volume of 100 µL per sample (1.25 µL antibody diluted in MACS buffer). Samples were incubated for 20–30 min on ice, protected from light. Following incubation, cells were washed three times with MACS buffer by centrifugation at 500 × g for 5 min at 4°C. After the final wash, cells were resuspended in MACS buffer for flow cytometric analysis. Unstained control samples were included to define background fluorescence and gating.

2.7 Cellular Uptake in Primary CD4⁺ T cells

To evaluate nanoparticle uptake, 1 × 10⁵ naïve CD4⁺ T-cells or differentiated Th₂ cells were seeded per well in a 96-well plate in a total volume of 200 µL. Naïve T-cells were allowed to rest overnight prior to treatment. Th₂ cells were reactivated with CD3/CD28 Dynabeads™ for 24 h before transfection. For cellular uptake experiments, nanoparticles were prepared in a total volume of 50 µL containing 50 pmol siRNA with a N/P ratio of 10. The siRNA mixture contained 20% Alexa Fluor 647 (AF647)-labeled and 80% unlabeled negative control siRNA (Sigma-Aldrich), with

labeling performed using AF647 NHS Ester (Thermo Fisher Scientific). Lipofectamine 2000 (Thermo Fisher Scientific) was prepared according to the manufacturer's instructions and used as a positive control, while free siRNA with the same composition served as a negative control. HBS was used as a blank control.

After transfection, cells were incubated for 1, 4, or 24 h. Subsequently, cells were transferred to 1.5 mL microcentrifuge tubes and washed twice with 400 μ L MACS buffer ($500 \times g$, 5 min, 21°C). Cells were then resuspended in 400 μ L of the same buffer for analysis. Flow cytometric analysis was performed using an Attune NxT cytometer (Thermo Fisher Scientific). A total of 10,000 events were recorded per sample using a 643 nm excitation laser and the RL1 detection filter. Nanoparticle uptake was quantified as median fluorescence intensity (MFI), calculated as the average of three technical replicates.

2.8 *In Vitro* GATA3 Silencing in Murine CD4⁺ T cells

A total of 200,000 murine Th₂ cells were seeded per well in 200 μ L of complete cell culture medium in a 96-well plate. They were then activated with CD3/CD28 Dynabeads™ in a 1:1 bead-to-cell ratio for 24 hours. Subsequently they were transfected with different formulations. Polyplexes were prepared using murine GATA3 siRNA (s201377; Thermo Fisher Scientific) or negative control siRNA, with a final amount of 50 pmol in 50 μ L HBS per well. Free siRNA and Lipofectamine 2000 (prepared according to the manufacturer's instructions) were used as negative and positive controls, respectively. After 48 h, RNA was isolated using High-Capacity RNA Mini Kits (Thermo Fisher Scientific) according to the manufacturer's protocol. Complementary DNA (cDNA) was synthesized from total RNA using a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). The resulting cDNA was diluted 1:10 and used for quantitative real-time PCR (RT-qPCR). Gene expression analysis was performed on a QuantStudio 3 Real-Time PCR system (Thermo Fisher Scientific) using SYBR™ Green PCR Master Mix (Thermo Fisher Scientific) and primers targeting murine GATA3 (Bio-Rad, Hilden, Germany) and β -actin (Qiagen, Hilden, Germany). Relative gene expression levels were calculated using the $\Delta\Delta\text{Ct}$ method with β -actin as the reference gene. Cycle threshold values were determined by the QuantStudio software.

2.9 Murine Precision Cut Lung Slices (mPCLS)

C57BL/6J mice were euthanized in accordance with institutional animal care guidelines as described in Section 2.5. After perfusion, the lungs were intratracheally inflated with 2 mL of 2% agarose to stabilize the pulmonary tissue. After an incubation period of approximately 15 min, the lungs were excised, transferred into culture medium, and placed at 4°C to allow complete solidification of the agarose.

Subsequently, lung tissue blocks were prepared and sectioned into slices with a thickness of 300 μ m using a vibration microtome (Hyrax V50, Carl Zeiss AG, Oberkochen, Germany). The resulting murine precision-cut lung slices (mPCLS) were cultured in DMEM/F-12 medium (Thermo Fisher Scientific) supplemented with 0.1% fetal bovine serum (FBS) and $1 \times$ penicillin/streptomycin. Prior to experimental procedures, mPCLS were further processed into circular tissue punches with a diameter of 4 mm.

2.10 *Ex Vivo* GATA3 Silencing in Activated mPCLS

To assess GATA3 knockdown, three murine precision-cut lung slice (mPCLS) punches were cultured per well in a 24-well plate containing 500 μ L of complete growth medium. Polyplexes were prepared using murine GATA3 siRNA or a negative control siRNA, with a final amount of 100 pmol per well. Free siRNA and Lipofectamine 2000 (prepared according to the manufacturer's instructions) were used as negative and positive controls, respectively.

To activate tissue-resident T-cells, mPCLS were stimulated with anti-CD3/CD28 magnetic beads at a bead-to-cell ratio of 1:1 for 3 days prior to transfection. To promote Th₂ polarization, IL-4 (10 ng/mL) and IL-2 (10 ng/mL), together with an anti-IFN- γ antibody (10 μ g/mL), were added to the culture medium according to the manufacturer's instructions (Miltenyi Biotec).

Subsequently, slices were treated with the respective nanoparticle formulations and incubated for 48 h at 37°C under 5% CO₂. All experiments were conducted in biological duplicates, each comprising technical replicates. For RNA isolation, lung slices were transferred into Lysing Matrix D tubes (M.P. Biomedicals, Eschwege, Germany) and homogenized in 1 mL TRIzol reagent (Thermo Fisher Scientific) using a FastPrep 24 Tissue Lyzer (M.P. Biomedicals). Homogenates were incubated for 5 min at room temperature before phase separation was initiated by the addition of 200 μ L chloroform. After mixing and a 3-min incubation, samples were centrifuged at 11,000 $\times$ g for 15 min at 4°C. The aqueous phase was transferred to a new tube, and RNA was precipitated with 500 μ L isopropanol for 10 min at room temperature, followed by centrifugation at 11,000 $\times$ g for 10 min at 4°C. The resulting RNA pellet was washed with ice-cold 75% RNase-free ethanol and centrifuged at 7,500 $\times$ g for 5 min at 4°C. After removal of the supernatant, RNA was dissolved in 30 μ L RNase-free water. RNA concentration was quantified using a NanoDrop One (Thermo Fisher Scientific) spectrophotometer. cDNA synthesis and RT-qPCR were performed as described in Section 2.8.

2.11 Animals and Asthma Induction Protocol

All animal experiments were conducted in accordance with the German Animal Welfare Act and approved by the Government of Upper Bavaria (ROB-55.2-2532.Vet_0220 – 171) and the Committee for Animal Experimentation of the Ludwig-Maximilians-Universität Munich, Germany. Female BALB/c mice (6–8 weeks old) were purchased from Charles River Laboratory (Sulzfeld, Germany) and used for all experiments. Animals were housed in a climate-controlled room in individually ventilated cages (Tecniplast Typ II long) under specified-pathogen-free conditions under a 12 h light/12 h dark cycle at a temperature of 22°C and a relative humidity of approximately 60%. Food and water were provided ad libitum. Mice were allowed to acclimatize to their new environment and to handling by animal care personnel for at least two weeks before being included in the experiments. For ovalbumin (OVA)-induced eosinophilic airway inflammation, mice were sensitized on days 0 and 14 by intraperitoneal injection of 10 μ g OVA (Grade V, Sigma-Aldrich) together with 2 mg aluminum hydroxide (Al(OH)₃; Sigma-Aldrich) suspended in 0.2 mL sterile isotonic PBS. The booster injection on day 14 was included to induce a robust Th₂-mediated immune response and to ensure the development of a pronounced asthma-like phenotype upon subsequent aerosol challenge.

On days 24, 25, and 26, mice were challenged in the awake state by inhalation of a 1% OVA aerosol in sterile PBS. For this purpose, animals were placed in nose-only inhalation chambers integrated into a Buxco Inhalation Exposure System (Data Sciences International, St. Paul, MN, USA) and allowed to acclimatize for 20 min prior to aerosol exposure. Incoming air was efficiently particle-filtered and dried to maintain a relative humidity between 30 and 70%. The system was operated at a pressure of -0.5 cmH₂O and a port flow rate of 0.3 L/min. The aerosol was generated using a nebulizer set to a speed of 0.05 mL/min. These settings were experimentally optimized in advance and adjusted according to the number of exposure chambers. Respiratory parameters, including respiratory rate and tidal volume, were continuously monitored using the integrated SmartStudy system. Based on the measured and calculated accumulated inhaled aerosol (AIA), aerosol delivery for each mouse was automatically terminated once an inhaled OVA dose of 120 μ g was reached, ensuring standardized allergen exposure.

2.12 Treatment Protocol

On days 36–38 following repeated OVA challenge (Section 2.11), mice were anesthetized with ketamine (80 mg/kg) and xylazine (16 mg/kg) and treated via intratracheal administration with polyplexes containing 750 pmol siGATA3 or AF647-labeled negative control siRNA in a total volume of 50 μ L. Fluorescent labeling enabled the simultaneous *in vivo* assessment of polyplex uptake and biodistribution. Control groups received either free siGATA3, AF647-labeled siRNA at the same dose, or HBS buffer only.

2.13 Serum and Bronchoalveolar Lavage Fluid (BALF) Collection

48 hours after treatment, mice were sacrificed under deep anesthesia by exsanguination. Briefly, an incision was made in a major artery, and the arterial blood was immediately collected using a capillary. Blood samples were allowed to clot for 90 min at room temperature. Samples were then centrifuged at $2,000 \times g$ for 15 min at room temperature, and the serum was carefully collected and stored at -80°C until further analysis.

Bronchoalveolar lavage (BAL) was then performed by inserting a cannula into the trachea. The lungs were lavaged twice with 500 μ L PBS supplemented with 2 mM EDTA and cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail (Roche, Basel, Switzerland). BALF cells were counted, and the lavage fluid was centrifuged at $500 \times g$ for 5 min at 4°C . The supernatant was collected and stored at -80°C for further analysis. Subsequently, the left ventricle was incised, and lung perfusion was performed by inserting a cannula into the right ventricle and flushing the pulmonary circulation with isotonic NaCl solution.

2.14 *In Vivo* Polyplex Biodistribution after Pulmonary Delivery

The heart, lungs, liver, spleen, and kidneys from groups treated with fluorescently labeled siRNA were dissected and subjected to imaging. Fluorescence intensity was measured using an IVIS Lumina III imaging system (PerkinElmer, Shelton, CT, USA) at an excitation wavelength of 635 nm and an emission wavelength of 668 nm.

2.15 *In Vivo* GATA3 Knockdown in CD4⁺ T cells

Lungs were homogenized to generate single-cell suspensions using the Mouse Lung Dissociation Kit (Miltenyi Biotec) according to the manufacturer's protocol. Cells were counted, and CD4⁺ T-cells were isolated by magnetic-activated cell sorting using the murine CD4⁺ T-cell Isolation Kit following the manufacturer's instructions. Equal numbers of CD4⁺ T-cells were used for RNA isolation using RNA Mini Kits (Thermo Fisher Scientific) according to the manufacturer's protocol. RNA concentration and purity were assessed spectrophotometrically. cDNA synthesis and qPCR were performed as described in Section 2.8. Relative gene expression levels were calculated using the $\Delta\Delta\text{Ct}$ method with β -actin as the reference gene and a designated control sample as calibrator. Ct values were determined automatically using QuantStudio software.

2.16 *In Vivo* Cellular Uptake in Lung Cells

To determine the relative uptake in different lung cell populations, cells were resuspended in MACS buffer. Live/dead discrimination was performed using Zombie UV viability dye (BioLegend), diluted 1:100 in PBS and incubated for 20 min at room temperature. Fc receptors were subsequently blocked using an anti-mouse Fc receptor blocking antibody (BD Biosciences, Franklin Lakes, NJ, USA). Cells were then stained for 30 min at 4°C with fluorochrome-conjugated antibodies targeting CD45 (FITC), CD8 (APC-Cy7), F4/80 (PE-Cy7), CD11c (BV605), CD326 (BV605), CD170 (PE/Dazzle 594), and CD19 (PerCP/Cy5.5) (all BioLegend), as well as CD3 (BUV395; BD Biosciences) and CD4 (VioBlue; Miltenyi Biotec). For intracellular staining, cells were fixed and permeabilized using the FOXP3/Transcription Factor Staining Buffer Set (Thermo Fisher Scientific) and stained with Pro-SPC-PE antibody (Santa Cruz Biotechnology, Dallas, USA) for 30 min at room temperature.

After washing, cells were filtered through a 70 μm cell strainer (Falcon, Corning Inc., Corning, NY, USA) and resuspended in MACS buffer. Samples were assessed on a Cytex Aurora spectral flow cytometer (Cytex Biosciences, Fremont, CA, USA) implemented with autofluorescence extraction for the detection of cellular uptake and analyzed using FlowJo software (BD Biosciences). Appropriate single-stained controls, fluorescence minus one (FMO) controls, and unstained controls were included for spectral unmixing and gating. Antibodies were used according to the manufacturers' recommendations.

2.17 Measurement of Cytokine Levels

Cytokine levels in BALF were measured to assess pro-inflammatory responses using the LEGENDplex™ Mouse Inflammation Cytokine Panel 2 (13-plex) (BioLegend) according to the manufacturer's instructions.

2.18 Histological Analysis

For histological analysis, the left lung lobe from all animals in the untreated group and one lung lobe from each treatment group were dissected, fixed in 4% paraformaldehyde (PFA) for at least 24 hours, and subsequently embedded in paraffin for hematoxylin and eosin (H&E) staining. Images were taken using an Axiovert 5 microscope connected to an AxioCam 205 camera system (Zeiss, Germany).

2.19 Statistical Analysis

All data are presented as mean $\pm$ standard deviation (SD). Statistical analyses were performed using one-way analysis of variance (ANOVA) in GraphPad Prism, unless otherwise stated. Statistical significance was defined as: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

3 Results and Discussion

3.1 Physicochemical Nanoparticle Characterization

Prior to nanoparticle formulation, successful purification of the mTf-PEI conjugate was confirmed by ion-exchange chromatography, with a representative ÄKTA chromatogram shown in **Supplementary Figure S1**. Nanoparticles were formulated at an N/P ratio of 10, which had previously been identified as optimal in human Tf-PEI systems by our group [17, 19]. In this study, the fraction of Tf-conjugated PEI relative to unmodified PEI was systematically varied to tune both physicochemical properties and ligand density. An optimal formulation was determined to exhibit a particle size of < 200 nm, a neutral to slightly negative zeta potential to minimize nonspecific interactions, and a narrow size distribution with a polydispersity index below 0.2 [20, 21]. Increasing the proportion of unmodified PEI resulted in a pronounced increase in hydrodynamic diameter (Fig. 1A). In neutral aqueous buffers such as HBS, PEI formed aggregates of approximately 500 nm, consistent with its pH-dependent protonation and the influence of ionic strength, as charge screening promotes interpolymer association and aggregation [22]. Incorporation of Tf markedly reduced particle size, with formulations containing $\geq 12.5\%$ Tf-PEI exhibiting diameters below 150 nm. This size reduction may be attributed to steric stabilization provided by the bulky and glycosylated structure of Tf, which can act as a physical barrier to particle-particle interactions [23, 24]. Additionally, the hydrophilic carbohydrate moieties may promote the formation of a hydration shell, further reducing aggregation [24]. Complementing the DLS measurements, nanoparticle tracking analysis (NTA) revealed a polydisperse size distribution for PEI-based formulations, with particles extending into higher size ranges, whereas mTf-containing formulations displayed narrower and more uniform size distributions (Fig. 1B).

As expected, pure PEI nanoparticles exhibited a strongly positive zeta potential due to their high density of protonated amines (Fig. 1C). With increasing Tf content, the zeta potential progressively decreased and shifted into the negative range at $\geq 25\%$ Tf-PEI. This shift is consistent with the surface exposure of negatively charged Tf, whose isoelectric point is reported to lie around 5.3–5.7, resulting in a net negative charge under physiological conditions [25]. Encapsulation efficiencies of 97–99%, as determined by SYBR Gold assay, confirmed that siRNA was effectively incorporated across all formulations, with minimal surface-accessible siRNA (Fig. 1D).

To assess functional performance, siRNA release was evaluated using a heparin displacement assay. Efficient release is essential for biological activity, as overly strong binding can hinder intracellular availability, whereas insufficient binding may lead to premature degradation [26]. Formulations with low Tf content or pure PEI released only ~ 50 – 60% of siRNA, indicating strong complexation. In contrast, formulations containing $\geq 25\%$ Tf-PEI released up to $\sim 80\%$ of siRNA (Fig. 1E). Taken together with the observed reductions in particle size and zeta potential, this behavior is consistent with decreased electrostatic compaction and altered complex structure in Tf-modified PEI systems, which may weaken siRNA binding and promote competitive displacement and release by heparin. These findings indicate a transition from tightly packed, charge-dominated complexes toward more accessible, sterically stabilized structures.

Based on these results, a formulation containing 50% murine Tf-PEI was selected for further studies, as it provided a favorable balance between particle stability and siRNA release. Pure PEI nanoparticles were included as a non-targeted control, while 100% Tf-PEI formulations were evaluated to assess the impact of high ligand density on biological performance. Collectively, these results show that varying Tf ligand content modulates both physicochemical properties and functional behavior, allowing the nanoparticle system to be tuned for optimal delivery performance.

3.2 GATA3 mRNA Expression and Transferrin Receptor Expression in Polarized CD4⁺ T Cells

To evaluate transfection efficiency *in vitro*, we aimed to establish target cells that reflect asthmatic type 2 conditions, alongside a non-target-cell population. Accordingly, primary naïve CD4⁺ T-cells were isolated from mice, a fraction of which was retained as a non-target control population, as uptake into these cells is undesirable to minimize off-target effects. The remaining naïve CD4⁺ T-cells were polarized toward a Th₂ phenotype. This was achieved by activation using CD3/CD28 magnetic beads, followed by culture and expansion in IL-4-containing medium, while simultaneously inhibiting type 1 differentiation through blockade of IFN- γ signaling. This approach is consistent with established protocols for Th₂ differentiation [27] and reflects the pathophysiology of eosinophilic asthma, which is predominantly driven by type 2 immune responses [2].

After six days of polarization, GATA3 mRNA expression was quantified. Compared to naïve CD4⁺ T-cells, which express GATA3 at basal levels [28], polarized cells exhibited a fourfold increase in GATA3 mRNA expression (Fig. 2A). Moreover, successful Th₂ skewing was confirmed at the protein level, with 69% of CD4⁺ T-cells staining positive for GATA3, whereas naïve CD4⁺ T-cells showed minimal GATA3 protein expression (Fig. 2A). As nanoparticle uptake is expected to occur via a TfR-mediated pathway, we next assessed surface expression of the TfR (CD71). Following Th₂ polarization, more than 70% of cells were CD71-positive, compared to naïve CD4⁺ T-cells. Upon restimulation, CD71 expression was maintained for up to two days (Fig. 2B), supporting the suitability of these cells as an *in vitro* type 2 inflammation model for transfection experiments.

3.3 Comparison of Cellular Uptake in Naïve CD4⁺ T Cells and Th₂ Cells

For particles composed solely of PEI, very high uptake was observed in both naïve CD4⁺ T-cells and Th₂ cells, reaching elevated levels after short incubation times and remaining consistently high over time (Fig. 3). This suggests a predominantly nonspecific, receptor-independent uptake mechanism, likely driven by the strong positive surface charge of the particles and the resulting electrostatic interactions with the negatively charged cell membrane [29]. For therapeutic applications, such nonspecific uptake is undesirable, as it may lead to off-target effects and associated side effects.

In naïve CD4⁺ T-cells (Fig. 3A), both Tf-conjugated formulations exhibited markedly lower uptake compared to PEI. This may be partially explained by reduced nonspecific electrostatic interactions, as the more neutral to negative surface charge of Tf-containing particles diminishes attractive interactions with the negatively charged cell membrane. In fact, electrostatic repulsion may further hinder receptor-independent uptake [30]. In addition, naïve T-cells reside in a quiescent state characterized by low metabolic activity including reduced endocytic and nutrient uptake processes compared to activated T-cells [31], along with lower availability of the TfR, as shown in the previous section. Together, these factors likely contribute to the reduced uptake observed for Tf-containing formulations in naïve cells.

In contrast, Tf-PEI polyplexes exhibited substantially higher uptake in polarized Th₂ cells compared to naïve T-cells (Fig. 3B). This is likely attributable to elevated TfR expression in activated Th₂ cells, promoting receptor-mediated internalization of Tf-conjugated particles. Tf-conjugated formulations exhibited relatively low uptake at 1 h, followed by a pronounced increase at later time points (4 h, 24 h), which is consistent with receptor-mediated processes that are typically slower than nonspecific uptake [32–34], but may provide increased selectivity due to their dependence on cell type and receptor expression, which is desirable for targeted delivery to activated T-cells. However, no significant difference between the lower and higher Tf-density polyplexes was observed. Nevertheless, overall uptake remained lower than that of PEI, which displayed the highest uptake already at early time points (1 h), followed by a decline in the number of internalized particles over time. The positive control, Lipofectamine, showed markedly high uptake already at early time points (1 h), indicating efficient early internalization, consistent with previous reports describing rapid endocytic uptake and trafficking of lipoplexes [35, 36].

3.4 GATA3 Knockdown in Murine Polarized Th₂ Cells

Although as shown above, Lipofectamine and particularly PEI exhibited the highest uptake in activated Th₂ cells, this did not translate into efficient functional gene knockdown (Fig. 4). Notably, in the case of PEI, treatment with control siRNA also resulted in reduced gene expression, indicating a lack of sequence-specific knockdown and instead suggesting vehicle-associated potential cytotoxic effects. These findings highlight that high cellular uptake alone is insufficient for effective siRNA delivery, as multiple intracellular barriers can limit functional activity. Inefficient endosomal escape and incomplete cytosolic release of siRNA may restrict its bioavailability, while additional limitations can arise at the level of incorporation into the RNA-induced silencing complex (RISC), which is required for gene silencing [37, 38]. In T-cells, cytosolic release of siRNA appears to be especially restrictive. Consistent with this, primary human T cells have been reported to maintain a comparatively higher endosomal pH than HeLa cells, with acidification remaining limited even several hours after uptake [39]. They exhibit a tightly regulated intracellular trafficking profile characterized by controlled endocytic trafficking and endosomal maturation [40, 41], which may

shorten the window for productive endosomal escape. In addition, siRNA can be recognized by endosomal RNA sensors such as TLR7 [42, 43]. Even after cytosolic entry, foreign RNA may be detected by cytosolic RNA-sensing pathways, including RIG-I-like receptors and the OAS family proteins [44], which can further reduce functional siRNA availability. This limitation is further illustrated by pure PEI-based delivery systems, for which endosomal escape has traditionally been attributed to the “proton sponge” effect [45]. However, this mechanism remains controversial and does not reliably result in efficient cytosolic release [46]. Moreover, the limited siRNA release observed in the heparin displacement assay may impair the availability of free siRNA required for efficient loading into RISC. Interestingly, the polyplex with the highest Tf density resulted in only a modest GATA3 knockdown of approximately 20%, whereas the 50% Tf-PEI formulation achieved a substantially stronger reduction of around 46%. This is particularly notable given that both formulations exhibited comparable cellular uptake kinetics, suggesting that differences in cellular internalization alone cannot account for the observed effects. Instead, differences in post-uptake processes such as intracellular trafficking, endosomal escape, and cytosolic release may contribute to the reduced silencing efficiency. Several studies have shown that even small variations in ligand density can alter receptor engagement, thereby influencing receptor-mediated internalization and downstream intracellular trafficking pathways [47, 48]. In this context, it is conceivable that a high Tf density enhances or accelerates recycling via the TfR, which is known to undergo rapid endosomal recycling [49, 50]. This may reduce the endosomal residence time available for siRNA dissociation and productive cytosolic release. In addition, the efficiency of endosomal escape in PEI-based systems is thought to depend, at least in part, on the availability of protonatable amines [46]. Conjugation with Tf may reduce the number of freely accessible amines, which could impair membrane interactions and consequently reduce endosomal escape efficiency. As endosomal escape has been widely recognized as a major bottleneck in siRNA delivery [51], even subtle differences in this step may significantly impact functional outcomes. Furthermore, differences in intracellular siRNA release cannot be excluded. Although both formulations displayed similar behavior in heparin displacement assays, these assays do not fully recapitulate the complex intracellular environment. In particular, the presence of comparatively large Tf molecules may alter polyplex architecture, as ligand conjugation and the extent of surface modification have been shown to influence the size, charge, and morphology of PEI-based nucleic acid complexes [52, 53]. Taken together, these findings suggest that in this context ligand density does not primarily influence cellular uptake, but rather affects downstream processes such as intracellular trafficking, endosomal escape, and potentially siRNA release. However, these mechanisms remain to be experimentally validated.

3.5 GATA3 Knockdown in Activated Murine Precision-Cut Lung Slices

To evaluate whether the observations obtained in primary Th₂ cells translate to a more complex and physiologically relevant environment, murine precision-cut lung slices (mPCLS) were employed as an *ex vivo* model. In contrast to isolated cell systems, mPCLS represent a structurally intact and heterogeneous tissue environment, introducing additional barriers such as limited diffusion, restricted access to cells within the tissue, and cell-type-specific uptake [54]. Analogous to the human model, resident T-cells within the slices were activated using a bead-based approach to mimic an inflammatory state. After three days of stimulation with murine CD3/CD28 activator Dynabeads in Th₂ cytokine-supplemented medium, GATA3 mRNA levels increased by more than two-fold (**Figure S2**), indicating the successful establishment of a Th₂-skewed inflammatory *ex vivo* model. This model therefore provides a suitable platform for evaluating siGATA3-containing formulations in a context relevant to Th₂-driven allergic asthma. Compared with the primary Th₂ cell model, the formulations generally exhibited improved GATA3 silencing efficiency in mPCLS, suggesting that siRNA-mediated knockdown can vary substantially depending on the biological

complexity of the model system. Free siRNA induced a moderate reduction in GATA3 mRNA levels of 32.47% compared to blank controls (Fig. 5), whereas Lipofectamine-mediated transfection resulted in a more pronounced knockdown of 41.72%. Despite the efficient cellular uptake of PEI-based polyplexes observed in Th₂-skewed T-cells, unmodified PEI polyplexes achieved only a moderate 23.20% reduction in GATA3 mRNA levels. Consistent with the findings in primary Th₂ cells, this indicates that cellular internalization alone is not sufficient to ensure functional gene silencing and that post-uptake barriers continue to limit effective siRNA delivery. Notably, polyplexes functionalized with the highest Tf density, 100% Tf-PEI, achieved a GATA3 mRNA reduction comparable to Lipofectamine. However, despite showing comparable uptake behavior to the 100% Tf-PEI formulation, the 50% Tf-PEI blend produced the most pronounced silencing effect, with approximately 81.63% GATA3 knockdown, making it the best-performing formulation in this *ex vivo* model. Given the comparable physicochemical properties and uptake profiles of both Tf-conjugated formulations, the observed differences in silencing efficiency are unlikely to be explained by cellular internalization alone. Instead, they point toward formulation-dependent differences in post-uptake processing. As discussed above, receptor-mediated uptake pathways are sensitive to ligand density, which can influence intracellular routing and thereby determine the fraction of siRNA that reaches the cytosol [48, 55]. In the context of a complex tissue environment, these effects may be further influenced by heterogeneous cell populations, differential receptor expression, spatial distribution within the tissue, and formulation-dependent tissue penetration [56–58].

Overall, while gene silencing could be achieved in primary Th₂ cells using both Tf-PEI systems, the mPCLS results highlight the increased complexity of siRNA delivery in tissue-based models. The superior performance of the 50% Tf-PEI formulation demonstrates that ligand density is a critical parameter for functional siRNA delivery under these conditions. However, the underlying mechanisms, particularly with respect to intracellular trafficking, endosomal escape, and tissue-level distribution, remain to be further investigated.

3.6 Murine Asthma Model

Following promising *in vitro* and *ex vivo* results, the therapeutic efficacy of the GATA3 siRNA polyplexes was evaluated in an asthmatic mouse model. Based on previous findings, the 50% Tf-PEI blend was selected as the Tf-conjugated polyplex formulation.

As mice do not naturally develop the full clinical features of human asthma, experimental models are required to mimic selected key aspects of the disease. An ovalbumin (OVA)-induced acute allergic airway inflammation model was employed, as OVA challenge models are widely used to reproduce key features of Th₂-biased eosinophilic airway inflammation. Female BALB/c mice were selected due to their tendency toward Th₂-skewed immune responses and robust eosinophilic airway inflammation in OVA-induced models [59]. Following establishment of the asthmatic phenotype, mice received three consecutive daily intratracheal administrations after inhalative OVA challenge and were sacrificed 48 h after the final dose.

3.7 *In Vivo* Biodistribution

Biodistribution analysis of Alexa Fluor 647-labeled siRNA after 48 h revealed predominant localization in the lungs across all groups, with only minimal detectable signal for unprotected siRNA (Fig. 6A; **Figure S3**). This was expected, as naked siRNA is susceptible to nuclease-mediated degradation and rapid clearance from the lung, whereas complexation within polyplexes can improve protection and local retention [60–62]. Interestingly, no significant differences in the overall accumulation of fluorescently labeled siRNA were observed between the PEI and Tf-PEI groups at the organ level (Fig. 6B). A slightly increased signal for Tf-PEI was detected in the liver, which may suggest

early systemic redistribution or metabolic processing. In addition, mouse hepatocytes express functional transferrin receptors and are capable of receptor-mediated transferrin uptake, which could further contribute to the hepatic accumulation of transferrin-functionalized particles [63]. Despite this comparable biodistribution, which reflects the combined contribution of extracellular, vascularly associated, and internalized particle fractions [64], distinct differences became apparent at the cellular level (Fig. 6C). As expected, free siRNA showed negligible uptake across lung cell populations. In contrast, both PEI and Tf-PEI polyplexes exhibited markedly enhanced uptake, as reflected by increased median fluorescence intensity. Uptake was particularly pronounced in eosinophils, macrophages and dendritic cells, which is consistent with their role as innate immune cells involved in airway inflammation [65]. Notably, a discrepancy between *in vitro* and *in vivo* findings was observed. While PEI demonstrated the highest uptake in the Th₂ cell culture model, this trend was not maintained in the *in vivo* asthma model. Instead, Tf-PEI showed a tendency toward higher internalization across all investigated cell types. One possible explanation is that positively charged PEI polyplexes may interact with negatively charged mucus components in the lung. Respiratory mucus represents an important extracellular barrier for pulmonary nucleic acid delivery, as particles retained within the mucus layer may show reduced penetration and can be removed by mucociliary clearance [66]. Such interactions could limit particle accessibility to target cells and thereby reduce cellular uptake. In contrast, Tf functionalization likely reduced nonspecific interactions with mucus due to altered surface properties, while promoting receptor-mediated uptake via the TfR, resulting in enhanced cellular internalization [18]. These findings highlight the importance of biological barriers present *in vivo*, which are not adequately represented in simplified *in vitro* systems [67]. In addition, the formation of a biomolecular corona *in vivo* may further modulate nanoparticle-cell interactions and shift ligand-mediated targeting efficiency [68]. Although no pronounced shift in overall cell-type specificity was observed, Tf-PEI demonstrated a tendency toward increased uptake in T-cell populations compared to non-functionalized PEI, although these differences did not reach statistical significance. This is consistent with a potential contribution of TfR-mediated internalization, particularly in activated T-cells [17].

3.8 *In Vivo* GATA3 Knockdown: Effects on GATA3 mRNA Expression, Lung Histopathology, and Inflammatory Cytokines

Subsequently, siRNA-mediated knockdown efficiency was evaluated (Fig. 7A). As GATA3 is predominantly expressed in CD4⁺ T-cells, this population was isolated from lung tissue, and GATA3 mRNA levels were quantified. As expected, unprotected siRNA-treated animals did not show reduced GATA3 mRNA levels compared with untreated asthmatic animals, likely because naked siRNA is rapidly degraded and cleared from the lung [61, 62]. This is in line with the low levels of siRNA delivery observed for this group in the biodistribution studies.

In contrast, treatment with PEI-based polyplexes led to a more than twofold increase in GATA3 mRNA levels compared to asthmatic controls. This suggests that PEI alone may trigger cellular stress or pro-inflammatory responses [69–71], thereby increasing GATA3 expression rather than enabling effective siRNA-mediated knockdown. This is consistent with previous reports describing the *in vivo* toxicity of PEI, which has been linked to its high cationic charge density and activation of immune and inflammatory pathways [72]. This may mask sequence-specific siRNA activity and potentially exacerbate inflammatory responses. In contrast, Tf-PEI polyplexes achieved an approximate 50% reduction in GATA3 mRNA levels compared to the asthmatic control group, demonstrating effective *in vivo* gene silencing in the murine asthma model. This suggests that the siRNA activity observed *in vitro* can translate to *in vivo* observations.

Interestingly, Tf-PEI loaded with control siRNA also led to a moderate reduction in GATA3 expression, although to a lesser extent. This may indicate additional biological effects of Tf itself, as Tf has been reported to exert

immunomodulatory effects and suppress innate immune signaling in certain contexts [73], which could contribute to the observed reduction. To evaluate the safety profile of our lead candidate Tf-PEI, pro-inflammatory cytokines in BALF were assessed following polyplex administration. Tf-PEI did not induce a pronounced increase in the measured pro-inflammatory cytokines, indicating good tolerability *in vivo* (Fig. 7B).

Next, the degree of inflammation and tissue morphology were assessed by H&E staining of lung sections across the different treatment groups (Fig. 7C). In the healthy group, a normal alveolar architecture was observed, characterized by thin and well-defined alveolar walls and the absence of inflammatory cell infiltration. In contrast, the asthmatic control group treated only with HBS buffer exhibited pronounced inflammatory cell infiltration, bronchial wall thickening, and airway narrowing. These histopathological changes support successful asthma induction in the murine model and are consistent with previously reported models [59]. A similar histological appearance was observed in the free siRNA group, indicating that unformulated siRNA had no detectable effect on the inflammatory status. Overall, lung structure in these groups appeared disorganized compared to healthy tissue. In contrast, treatment with Tf-PEI siGATA3 resulted in a noticeable reduction in inflammatory features, as evidenced by a more preserved lung architecture. These findings are consistent with partial GATA3 silencing and an associated improvement in lung pathology. However, inflammatory features remained clearly detectable, indicating that the treatment did not fully resolve the inflammatory response. The corresponding negative control (Tf-PEI siNC) showed little to no improvement. Conversely, the PEI-treated groups (PEI siGATA3 and PEI siNC) exhibited a similar or even more pronounced inflammatory phenotype compared to the asthmatic control. Dense cellular infiltration and thickened alveolar walls were observed, which may indicate pro-inflammatory effects of PEI itself, independent of siRNA activity.

4 Conclusion

In conclusion, this work establishes a murine Tf-functionalized PEI polyplex system for targeted siRNA delivery to lung-associated T-cells with an emphasis on the impact of Tf ligand density on functional intracellular siRNA delivery, building on previous studies with human Tf-based formulations. By systematically varying the degree of Tf functionalization, we identified ligand density as a critical formulation parameter that influences not only nanoparticle properties but also functional gene silencing. Notably, the 50% Tf-PEI blend outperformed both non-targeted PEI and fully Tf-functionalized polyplexes, suggesting that optimal silencing depends on a balance between receptor-mediated uptake, intracellular trafficking, endosomal escape, and siRNA release.

The optimized Tf-PEI formulation enabled effective GATA3 knockdown in polarized murine Th₂ cells, activated murine precision-cut lung slices, and an OVA-induced asthma model. *In vivo*, GATA3 siRNA Tf-PEI polyplexes reduced GATA3 expression, were associated with improved lung histopathology, and did not induce a pronounced increase in the measured pro-inflammatory cytokines under the tested conditions. In contrast, non-targeted PEI showed limited therapeutic benefit and appeared to contribute to an increased inflammatory phenotype. Overall, these findings support Tf-PEI blend polyplexes as a promising platform for targeted pulmonary siRNA delivery and suggest their potential as a therapeutic strategy for severe allergic asthma.

Declarations

Conflict of Interest

Olivia Merkel is a co-founder of RNhale GmbH. Olivia Merkel is a Scientific Advisory Board Member of Coriolis Pharma, AMW, and Corden Pharma as well as a consultant for PARI Pharma, AbbVie Deutschland, and Boehringer-

Competing Interests

Olivia Merkel is a co-founder of RNhale GmbH. Olivia Merkel is a Scientific Advisory Board Member of Coriolis Pharma, AMW, and Corden Pharma as well as a consultant for PARI Pharma, AbbVie Deutschland, and Boehringer-Ingelheim International.

Author Contribution

Anny Nguyen co-conceptualized the study, conducted the majority of the experimental work and data analysis, and wrote the original draft of the manuscript. Min Jiang, Joschka Müller, Muge Molbay, Simone Carneiro, Dana Matzek, and Adrian Kromer assisted with the in vivo experiments. Diana Leidy Porras-Gonzalez performed the sectioning of the murine PCLS. Gerald Burgstaller provided infrastructure and resources, Bastian Popper provided expert veterinary ethics and pathology support. Muge Molbay and Olivia Merkel reviewed and revised the manuscript. Olivia Merkel originally conceptualized the study, funded all work, provided resources and infrastructure and scientific administration and supervised the entire project.

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Data Availability

Data will be made available by the authors upon request.

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Figures

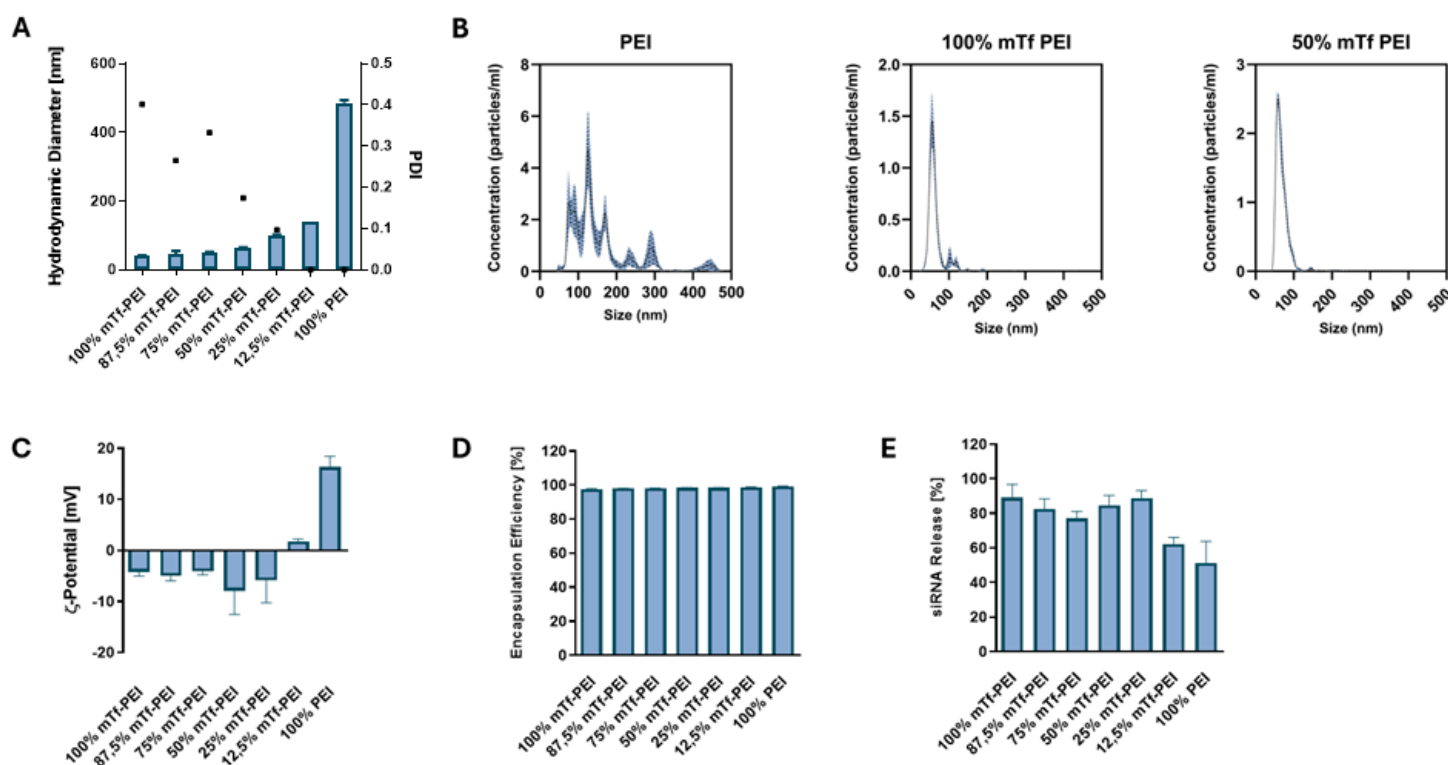


Figure 1

Physicochemical characterization of siRNA-loaded polyplexes containing varying ratios of Tf-conjugated PEI at N/P 10. (A) Hydrodynamic diameter and polydispersity index (PDI) of polyplexes determined by dynamic light scattering (DLS). (B) Particle size distribution and concentration were determined by nanoparticle tracking analysis (NTA). (C)

Zeta potential at pH 7.4 determined by phase analysis light scattering. **(D)** Encapsulation efficiency measured by SYBR Gold assay. **(E)** Release of siRNA after replacement with heparin. Data are presented as mean + SD, n = 3.

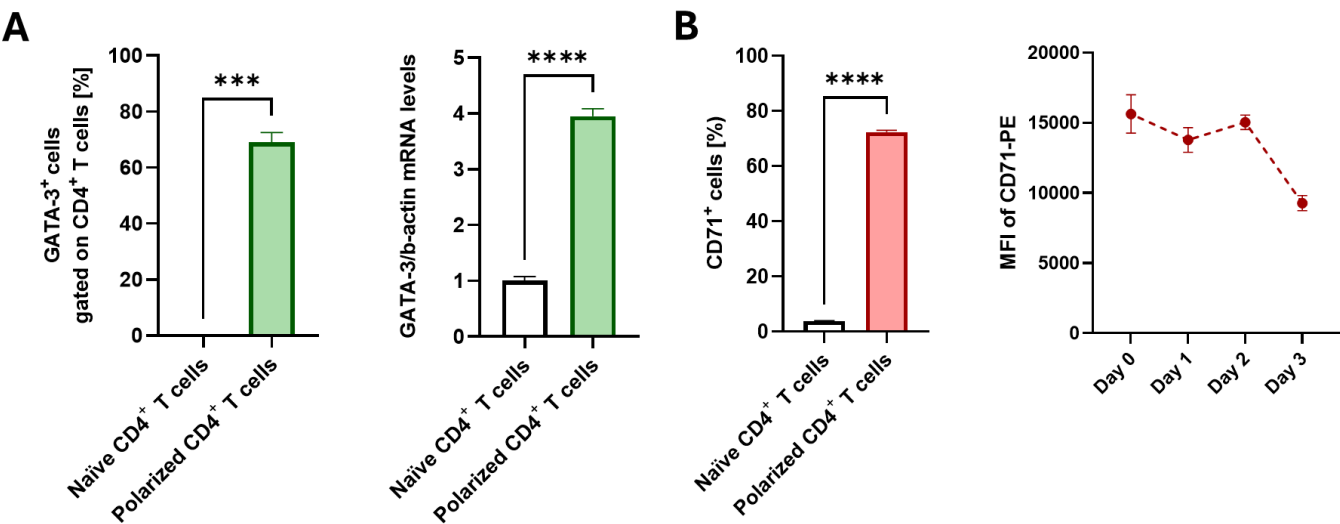


Figure 2

Characterization of polarized CD4⁺ T-cells. **(A)** Percentage of GATA3⁺ cells among gated CD4⁺ T-cells and relative GATA3/β-actin mRNA levels in naïve and polarized CD4⁺ T-cells. **(B)** Percentage of CD71⁺ cells in naïve and polarized CD4⁺ T-cells, and CD71-PE mean fluorescence intensity measured on days 0, 1, 2, and 3 after reactivation. Data are presented as mean + SD (n = 3). Statistical analysis was performed using unpaired t-test with Welch’s correction. ****p* < 0.001, *****p* < 0.0001.

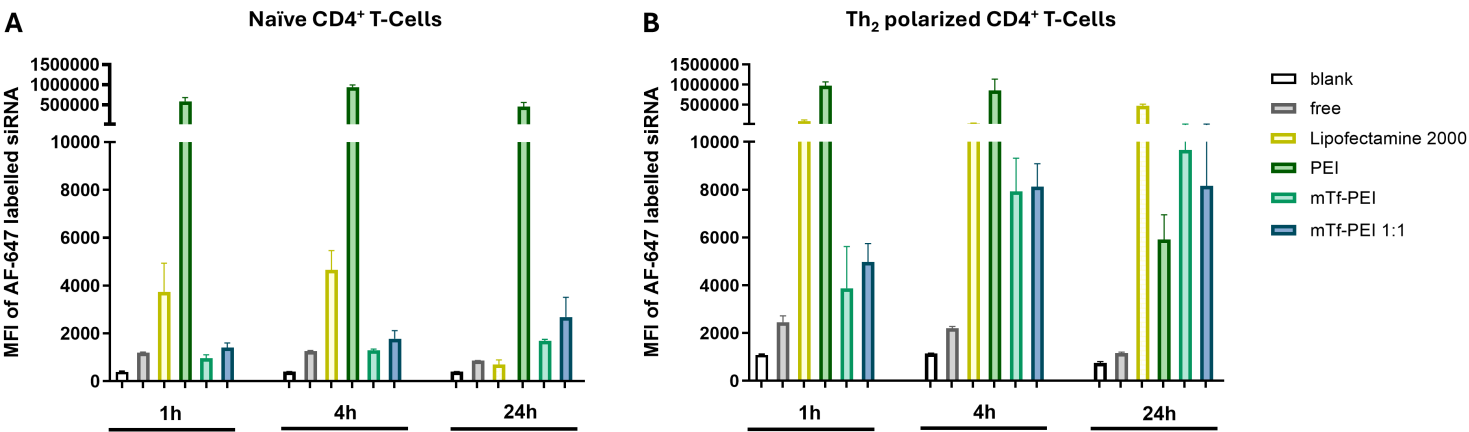


Figure 3

Cellular Uptake of Alexa Fluor 647 (AF647)-labeled siRNA delivered via different formulations in **(A)** Naïve CD4⁺ T-cells and **(B)** activated Th₂-polarized T-cells. Data represent mean + SD, n = 3.

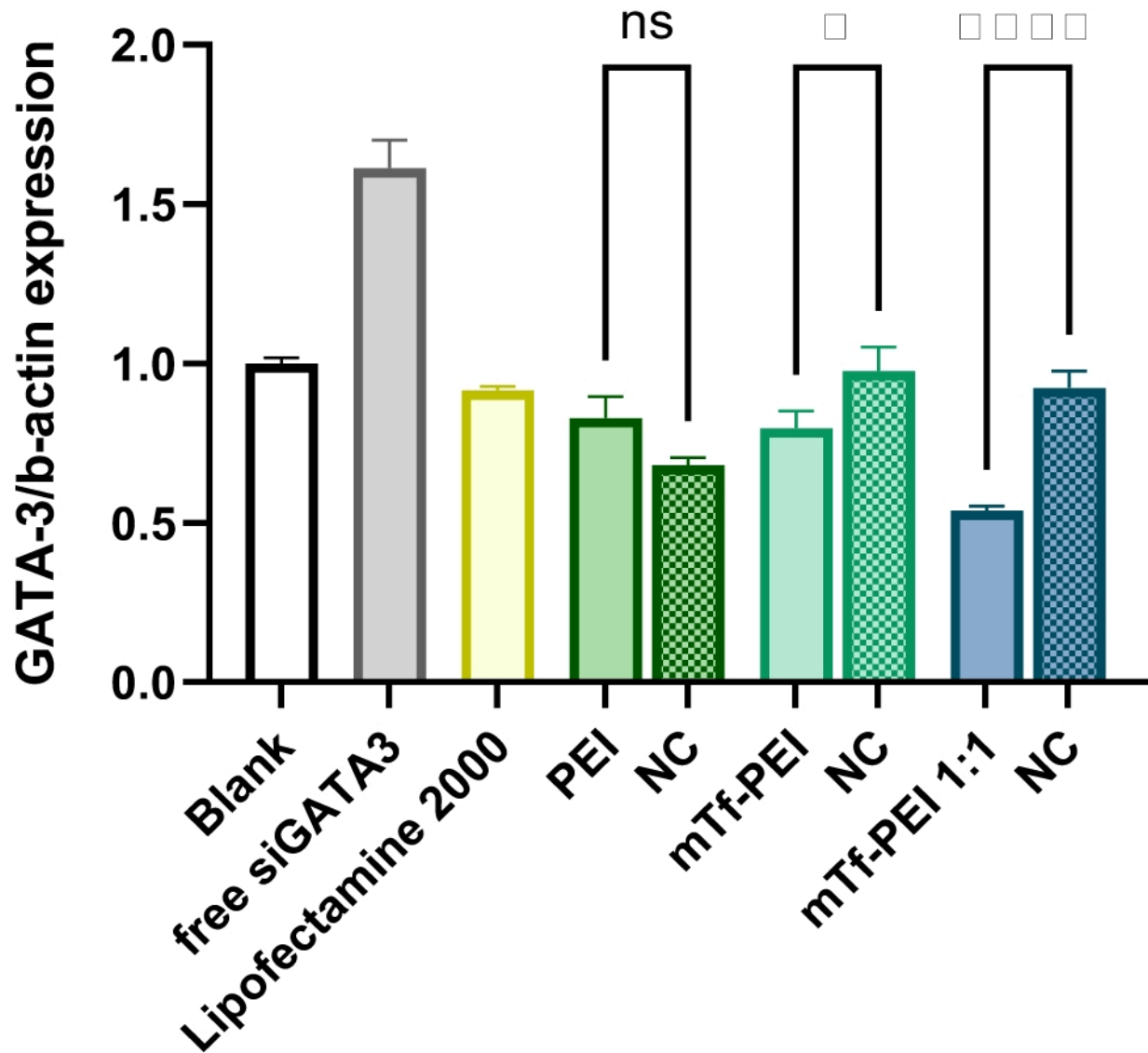


Figure 4

GATA3 knockdown in primary murine CD4⁺ Th₂ cells. GATA3 mRNA levels were determined via RT-qPCR and normalized to β -actin levels after 48 hours of incubation with 50 pmol of siGATA3-loaded polyplexes. Data points indicate mean + SD (n = 3). Statistical analysis was performed using one-way ANOVA with Tukey post hoc test; ns = not significant, * $p \leq 0.05$, **** $p \leq 0.0001$.

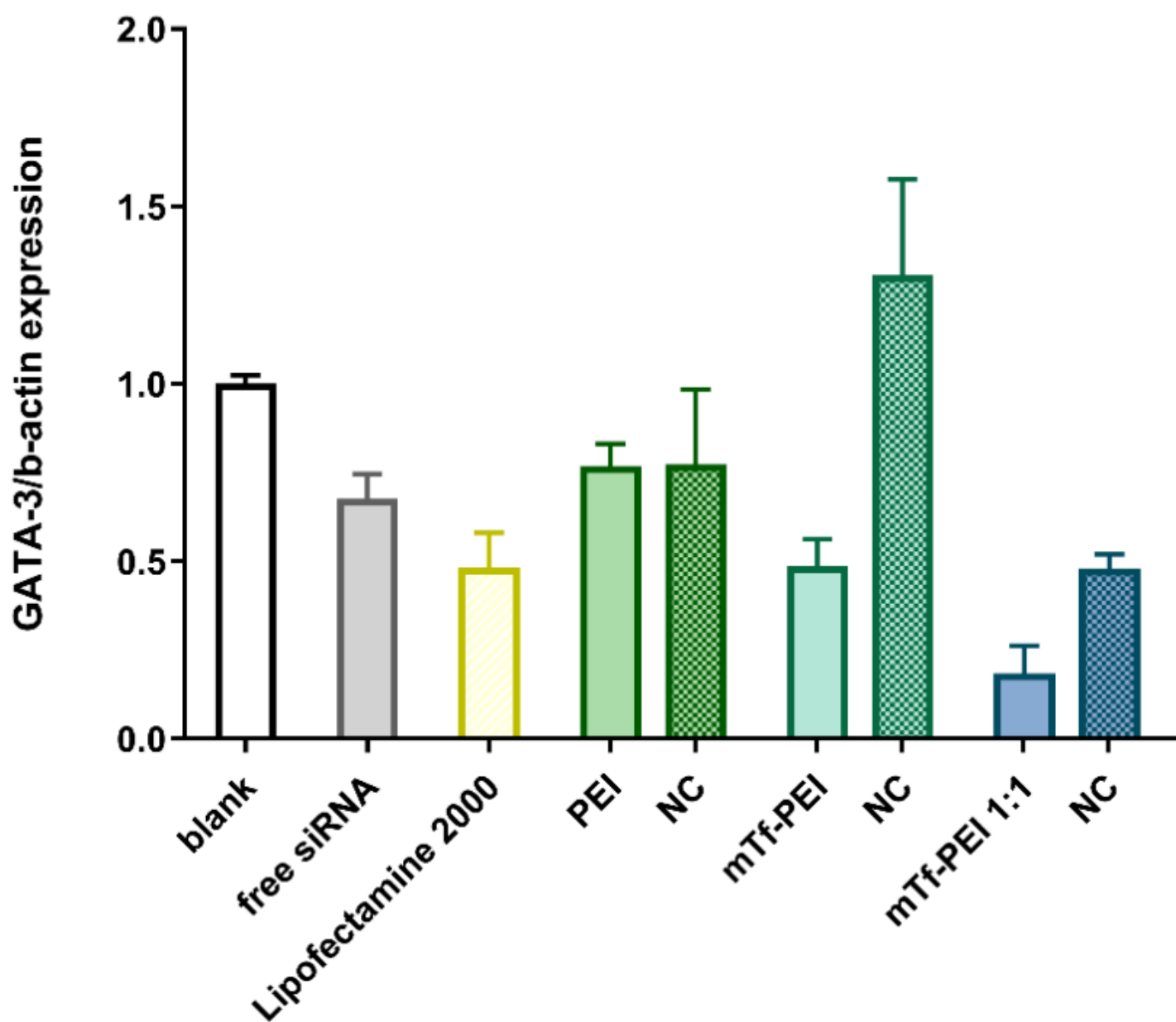


Figure 5

GATA3 knockdown in murine precision-cut lung slices. mPCLS were activated with Dynabeads for three days and subsequently transfected with different siRNA formulations containing 100 pmol siGATA3. GATA3 mRNA levels were quantified by RT-qPCR and normalized to β -actin. Data are presented as mean + SD (n = 2).

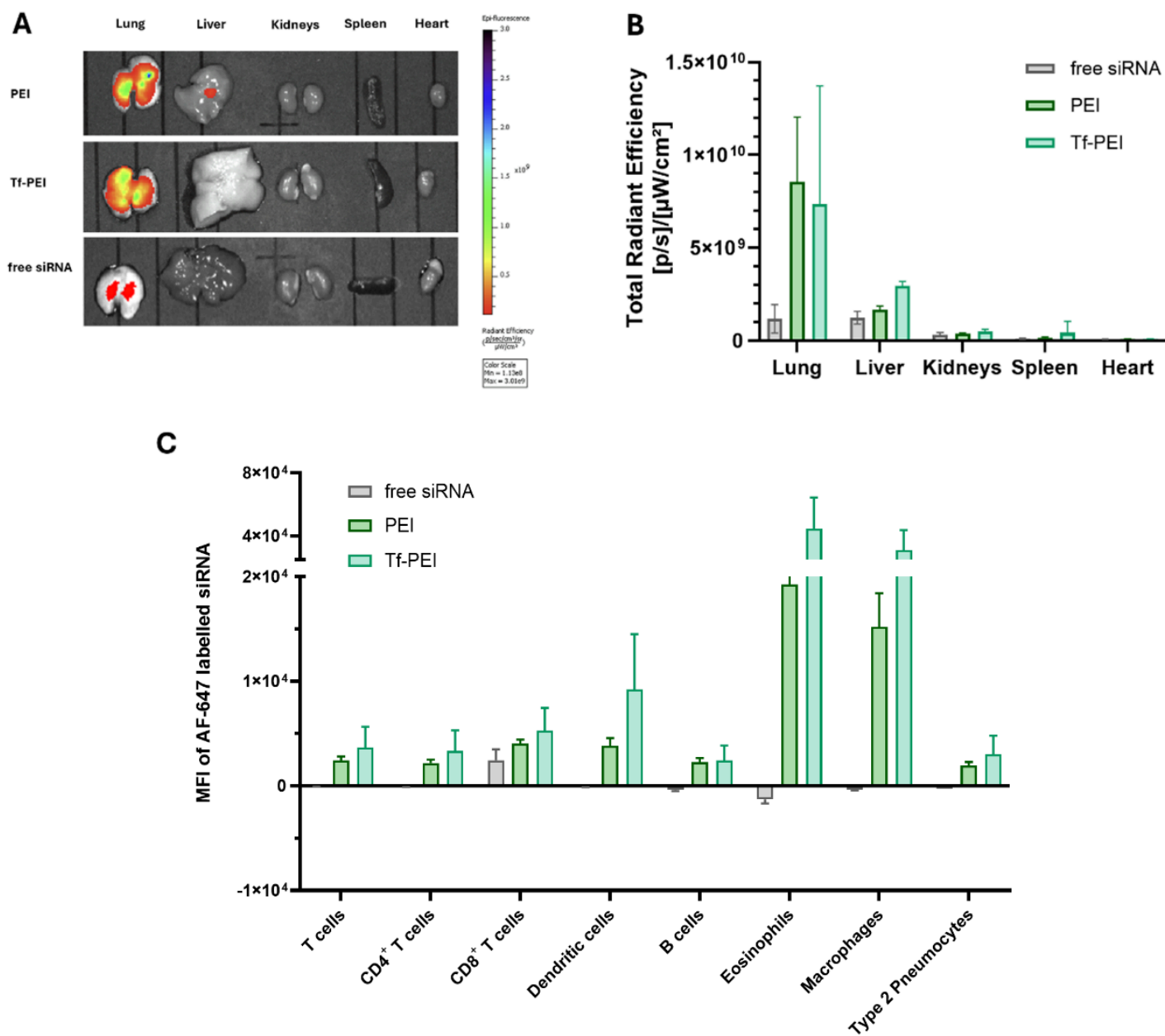


Figure 6

In vivobiodistribution and lung cellular uptake. **(A)** Representative organ distribution in mice following administration of AF647-labeled siRNA-loaded polyplexes. **(B)** Quantification of fluorescence intensity of Alexa Fluor 647-labeled siRNA in organs. Data are presented as mean + SD (n = 6 per group, except Tf-PEI group, n = 5). **(C)** Median fluorescence intensity of AF647-labeled siRNA across different lung cell populations. Data are presented as mean + SD (n = 6 for the PEI group, n = 5 for the Tf-PEI group, and n = 3 for the free siRNA group).

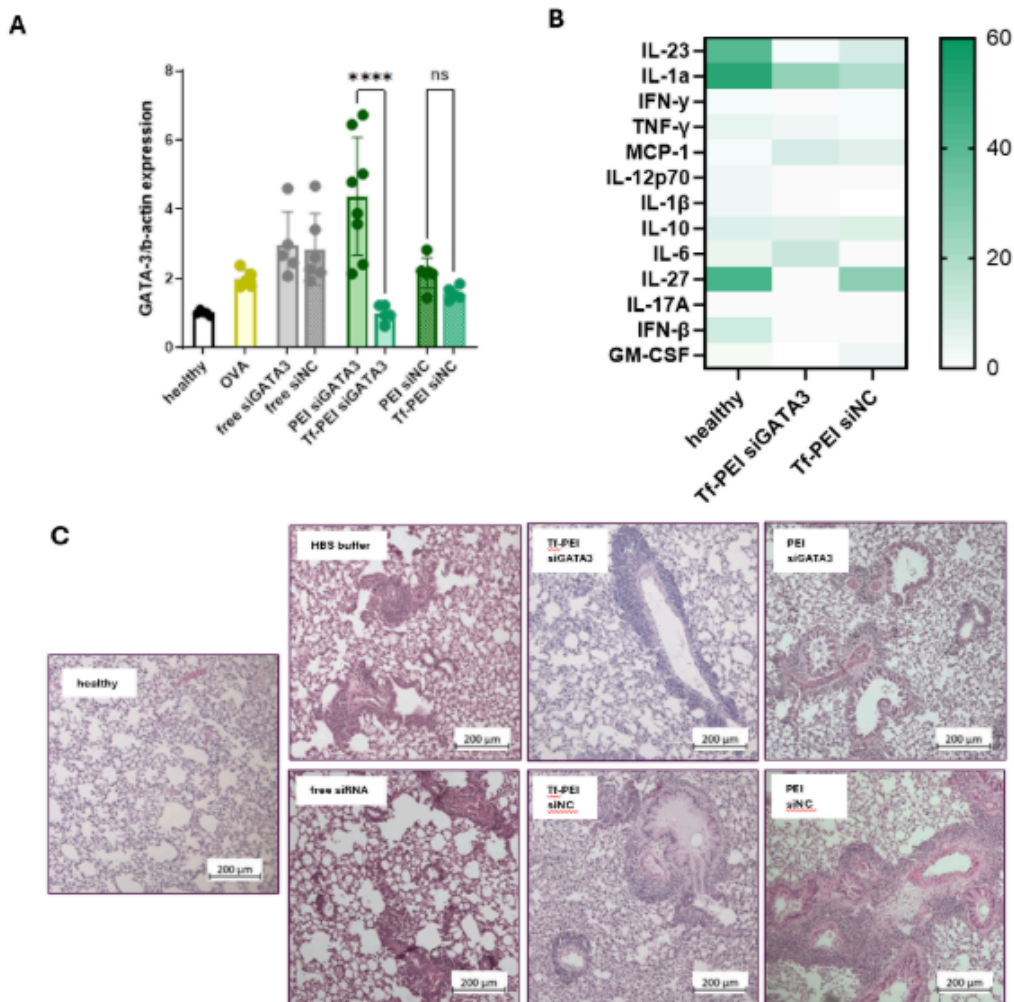


Figure 7

In vivo GATA3 Knockdown in pulmonary CD4⁺ T-cells. **(A)** GATA3 knockdown in OVA-induced asthmatic mice following polyplex treatment. Data are presented as mean $\pm$ SD ($n = 5$ per group, except $n = 6$ for the free siNC and PEI siNC groups, and $n = 8$ for the PEI siGATA3 group). **(B)** Pro-inflammatory cytokine levels in BALF following treatment with Tf-PEI. **(C)** H&E-stained lung sections collected from mice treated with different formulations. Scale bar: 200 μ m.

Supplementary Files

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