Targeted Delivery of siRNA Lipoplexes to Cancer Cells Using Macrophage Transient Horizontal Gene Transfer

Elizabeth C. Wayne,* Christian Long, Matthew J. Haney, Elena V. Batrakova, Tina M. Leisner, Leslie V. Parise, and Alexander V. Kabanov*

Delivery of nucleic acids into solid tumor environments remains a pressing challenge. This study examines the ability of macrophages to horizontally transfer small interfering RNA (siRNA) lipoplexes to cancer cells. Macrophages are a natural candidate for a drug carrier because of their ability to accumulate at high densities in many cancer types, including breast, prostate, brain, and colon cancer. Here, it is demonstrated that macrophages can horizontally transfer siRNA to cancer cells during in vitro coculture. The amount of transfer can be dosed depending on the amount of siRNA loaded and total number of macrophages delivered. Macrophages loaded with calcium integrin binding protein-1 (CIB1)-siRNA result in decreased tumorsphere growth and decreased mRNA expression of CIB1 and KI67 in MDA-MB-468 human breast cancer cells. Adoptive transfer of macrophages transfected with CIB1-siRNA localizes to the orthotopic MDA-MB-468 tumor. Furthermore, it is reported that macrophage activation can modulate this transfer process as well as intracellular trafficking protein Rab27a. As macrophages are heavily involved in tumor progression, understanding how to use macrophages for drug delivery can substantially benefit the treatment of tumors.

1. Introduction

Nanotechnology has been transformative for the efficacy of cancer treatment. Through the precise engineering of nanoparticles, one can increase drug circulation half-life, synergize combinatorial therapies, and deliver insoluble drugs. However, nanoparticles can have relatively poor tumor distribution, poor endosomal escape. The development of new particles alone may not always overcome the barrier to achieving efficient drug delivery into solid tumors. This is especially true for nucleic acid-based therapeutics, where tumor delivery and endosomal escape of oligonucleotides are particularly challenging. The enhanced permeability and retention (EPR) effect, which the promise of cancer nanotechnology has partially relied on, has proved more heterogeneous and unpredictable than anticipated. In contrast to the limitations of nanoparticles, immune cells are an optimal drug delivery vehicle due to their ability to cross-navigate the blood and lymphatic circulatory system, penetrate the blood–brain barrier, the fibrotic exterior of a tumor, or other barriers, and actively accumulate in diseased regions. Combining the benefits of nanoparticle encapsulation and stability with the intrinsic trafficking, phagocytosis, and secretion activity of immune cells may be the best combination to achieve therapeutic doses.

Targeted delivery of RNA interference (RNAi) therapeutics to the tumor microenvironment has been aggressively pursued but is technically challenging. Small interfering RNA (siRNA) therapeutics are highly effective but are currently limited clinically by poor pharmacokinetics and can benefit from cell carriers. siRNA therapies can target with high precision previously “undruggable” mutations because it can knockdown genes post-transcription/pre-translation stage. Further, siRNA therapy avoids the possible genomic mutations associated with DNA therapies. Because naked siRNA has a short half-life in circulation, strategies to overcome this...
limitation involve nanoparticle delivery or structural modification of the siRNA itself. However, these limitations of siRNA pharmacokinetics do not impede their direct delivery to the eye, lung, and liver. One recent example is Patisiran (Alnylam), an RNAi therapeutic that targets a protein produced in the liver, has completed Phase 3 trials and is the first RNAi therapeutic to receive FDA approval. Still, many cancer-related RNAi therapies are limited by effective transport into solid tumors and endosomal escape once inside the cell.

Cationic siRNA lipoplexes are beneficial packaging systems for nonviral siRNA delivery. Cationic lipids form complexes with negatively charged siRNA via electrostatic interaction. Optimization of the complex formation parameters (such as lipid composition, ratio to siRNA, mixing temperature, etc.) has led to efficient delivery into the cell and subsequent endosomal escape in vitro. However, due to cationic lipid-induced toxicity, inflammation, and poor distribution profiles, in vivo success of lipoplexes has been limited.

Using immune cells as drug delivery vehicles has been well published. Immune cells have been used as broad functional carriers, where the surface is coated with therapeutic nanoparticles. This method has been employed to target white blood cells within the bloodstream, T lymphocytes, natural killer cells among others. Red blood cells have been used to carry drugs and have been very useful in delivering drugs to blood clots and injury sites because of their long lifespan and large quantity. The methods previously described involve incorporation of nanoparticles to the surface of the immune cells and rely passively on the migratory properties of the immune cells. However, another type of packaging which requires a functional interaction between the nanoparticle cargo and the immune cells. This describes cells such as macrophages that can internalize their delivery cargo and release at the disease site. Macrophages in other contexts have been shown to transfer oligonucleotides to surrounding cells. Our previous work has shown that macrophages can horizontally transfer proteins and DNA into neurons and even immune cells in distal organs.

There are two central reasons why macrophages are ideal carriers for delivery into solid tumors. First, macrophages penetrate and accumulate in tumors at significant numbers throughout tumor progression. This accumulation can account for 30–50% of the mass of a solid tumor. Many immunotherapies centralize T cells; however, there are several reasons why this strategy is limiting. It has been well documented that T-cells either do not localize or cannot survive the tumor suppressive environment. While T-cell therapies have shown success in blood cancers such as leukemia and non-Hodgkin’s lymphoma, these therapies leave out 80% of the patient population who present with solid tumors. Even within blood cancers, only subset of those patients have sufficient numbers of T cells that can be harvested. Since macrophages participate in antigen presentation, targeting macrophages may also have the benefit of priming the solid tumor environment for T-cell infiltration. Second, macrophages phagocytose and secrete oligonucleotides and proteins into the surrounding environment. Intercellular communication can modulate the micro or local environment via secretion of cytokines and nucleic acids. However, it is less well appreciated that macrophages can transfer exogenously administered proteins and oligonucleotides. Considered together, macrophages pose an advantageous and efficient delivery carrier for the tumor microenvironment.

In this study we demonstrate that macrophages horizontally transfer siRNA to surrounding cells in both 2D and 3D tumoursphere coculture models. Macrophages that were adoptively transferred into mice were also able to penetrate the tumor microenvironment and transmit pharmacologically active siRNA to cancer cells in an orthotopic MDA-MB-468 breast cancer model. This work propels the general use of macrophages for therapeutic delivery and provides a new model for drug delivery into solid tumor masses that have been notoriously difficult for nanoparticle and other immunotherapies to penetrate.

2. Results and Discussion

2.1. Characterization of siRNA Lipoplex Loading into Macrophages

To demonstrate proof of transient horizontal transfer, commercially available cationic transfection lipoplexes (GeneSilencer; Genlantis) were used to load scrambled, nonhomologous siRNA labeled with Cy5.5 (Dharmacon) into the macrophages. Negative Stain TEM imaging and nanoparticle tracking analysis (NTA) revealed condensed siRNA lipoplexes with average sizes of 177.2 nm ± 6.6 nm (Figure S1, Supporting Information). Imaging flow cytometry (Imagestream, Millipore) analysis confirmed that the siRNA lipoplexes were found in intracellular vesicles (Figure 1a). There was a heterogeneous distribution in the uptake amount of siRNA lipoplexes (Figure 1b). The siRNA lipoplexes were fully internalized with less at 0.01% of siRNA lipoplex signal colocalizing with the cellular membrane (Figure 1c). Following transfection, macrophages were rinsed with 1 mg mL−1 heparin sulfate to dissolve noninternalized lipoplexes. Measurements of macrophages before and after heparin sulfate wash reveal significant differences in the mean intensity (Figure 1d). The amount of Cy5.5-labeled-siRNA found within the cell was proportional to the initial loading concentrations (Figure 1e). The mean intensity data was fit to an exponential decay curve to calculate the half-life of siRNA within the macrophage populations. The half-life of the siRNA in the macrophages increased with higher loading concentrations from 1.3 to 2.1 days for 0.2 to 4 μg respectively. This value encapsulates population levels dynamics of siRNA transfer, degradation, and cell division—how these factors individually affect the half-life of siRNA within macrophages should be further studied.

Cytotoxicity due to siRNA loading was measured using the CCK-8 assay. The absorbance was higher in the nonloaded macrophages but similar across all three siRNA constructs tested (Figure 1f). To observe the effect of siRNA loading on the proliferation, macrophage cell growth was measured over 7 days and found no significant differences in growth rate between Luciferase siRNA (Luc siRNA) and scrambled siRNA loaded macrophages (Figure 1g). Thus, the macrophage proliferation and survival behavior were unaffected by the loading with siRNA lipoplexes. In addition, we characterized siRNA loading...
into RAW 264.7 cells and found similar trends with regards to dosing and cytotoxicity (Figure S2, Supporting Information).

2.2. Luc siRNA Horizontal Transfer from Macrophages Results in Knockdown of Luciferase in the Cancer Cells

The transfer of Luc siRNA from macrophages to the cancer cells was examined by measuring the reduction of luciferase bioluminescence activity in the cancer cells following coculture with Balb/c RAW 264.7 macrophages transfected with Luc siRNA (Figure 2a). As the number of macrophages increased, the knockdown also increased (Figure 2b), demonstrating evidence of siRNA transfer that was dependent on the ratio of macrophages and the duration of coculture. As a negative control, macrophages were also transfected with an equal amount of scrambled, nonhomologous siRNA (control-siRNA). There was no reduction in luciferase activity when cancer cells were cocultured with control-siRNA which suggests cancer cell death is due to coculture is not likely occurring. Furthermore, no difference in luciferase activity (Figure 2c) was noted in cancer cells incubated in media harvested from macrophages 24 h after transfection with either control siRNA or Luc siRNA. Interestingly, under conditions of this experiment, the maximal knockdown was observed when macrophage to cancer cell ratio was higher than 50% (Figure 2d). Since tumor associated macrophages make up to 50% of tumor mass,\(^{[37,38]}\) this suggests a real potential for macrophages as delivery carriers in cancer treatments.

Macrophage horizontal transfer of siRNA was also tested using other macrophage cell lines. Knockdown of luciferase expression in 3LL Lewis Lung carcinoma cells was replicated in human THP-1 macrophages (Figure S3, Supporting Information). THP-1 did not monocytes show effective transfer, and this may be due to their poor siRNA transfection (Figure S4, Supporting Information). Optimization of culture conditions and siRNA transfection parameters will be needed to fully test monocyte gene transfer. Overall, these results using several
macrophage and cancer cell models support the finding that the siRNA is being transferred from the macrophage to the cancer cells and suppresses gene expression.

2.3. siRNA Horizontal Transfer Dynamics

The Luc siRNA assay provided a sensitive tool for demonstration of siRNA transfer from a macrophage donor to a cancer cell recipient; however, it was difficult to understand the kinetics of this transfer without quantifying cell population numbers and the amount of siRNA within each group. Therefore, a fluorescent coculture model was developed (Figure 3a). IC21 macrophages were stably transduced with a nuclear localizing red fluorescent protein, Nuclight Red (Essen Bioscience) lentivirus (IC21-NR) and MDA-MB-231 cancer cells were stably transduced to express GFP (MDA-MB-231-GFP). The two cell populations remain distinct during coculture evidenced by flow cytometry analysis (Figure 3b).

We further characterized our coculture model by investigating the effect of coculture on cell viability on MDA-MB-231 cells. Using time-lapse fluorescent microscopy, we cocultured the human cells with either human THP-1 macrophages, C57Bl6 IC-21 macrophages, or Balb/c RAW 264.7 macrophages. As a control MDA-MB-231 GFP cells incubated alone. Viability was assessed by the increase in GFP signal over time. To determine whether cytokine signaling from macrophages affected the cancer cells growth, MDA-MB-231 GFP cells were incubated with media from the macrophage cells lines. We found that while the MDA-MB-231 GFP cells grew best in the control group, there was no significant difference in proliferation among the macrophage conditioned media treated cancer cells (Figure S5a, Supporting Information).

MDA-MB-231 GFP cells were also cocultured with human with macrophages at a 1:2 ratio comparable to experiments within the paper (Figure S5b, Supporting Information). There was a statistically significant difference in proliferation in the coculture experiment between the monoculture control and the macrophages cocultures. However, there was no significant difference between human or mouse media or coculture groups. The difference in proliferation between the cancer cell monoculture control and cocultured with macrophages is likely a result of limited surface area upon which to grow and nutrient resources—the cocultures had more cells (Figure S5c, Supporting Information). We conclude that there is an effect on cancer cell growth due resource competition as a result of coculturing. Importantly, there are no distinguishable effect on viability of culturing human cancer cells with mouse macrophages in comparison to culturing with human macrophages.
IC21-NR macrophages were transfected with two different concentrations of a scrambled siRNA tagged with a Cy5.5 fluorescent dye (scrambled siRNA-Cy5.5). Immediately after transfection, the macrophages were rinsed and mixed with MDA-MB-231-GFP cells in rounded, ultralow attachment 96 wells at a 50:50 cell ratio. Then, the seeded 96-well plates were centrifuged at 1500 × g for 15 min to initiate tumorsphere formation before placing into a 37 °C incubator. At different timepoints, the tumorspheres were collected, disaggregated, and analyzed via flow cytometry. The average percentage of measured cells that were macrophages (black) or cancer cells (green) in the coculture at different timepoints. f–g) IC21-NR macrophages (1.0 × 10⁶) are transfected 2 µg of GFP-siRNA and cocultured with MDA-MB-231-GFP cells at a ratio of f) 1:3 or g) 1:10. h) IC21-NR macrophages (1.0 × 10⁶) are transfected with 2, 4, or 6 µg of GFP-siRNA and cocultured with MDA-231-GFP cells. h) The percentage of GFP+ expressing cells were analyzed 48 h after coculture using flow cytometry. Statistical analysis is done by unpaired t-test using Prism software: **p < 0.005; ***p < 0.001; n = 3 for each measurement.
decrease in GFP+ positive cells (Figure 3g). Interestingly, the macrophage:cancer cell ratio and the percentage knockdown fits within the relationship proposed in (Figure 2d) even though the macrophage cell line and target cancer cells are different.

As a point of comparison, MDA-MB-231 cancer cells were directly transfected with GFP-siRNA using geneSilencer lipoplexes (Figure S6, Supporting Information). The percentage of GFP+ cells decreased by 72% using similar loaded conditions to the macrophage transfer experiment (2 µg GFP-siRNA, measured 48 h post transfection). While direct transfection was more effective, their capacity for in vivo application is limited due to toxicity. The ability of macrophages to home to regions of inflammation, carry and transfer function siRNA is a notable combination.

To test the role of initial loading, we kept the coculture ratio at 1:10 but varied the initial amount from 2 to 6 µg of GFP-siRNA (Figure 3h). While there were decreases in the percentage of GFP+ cells (13% and 6% for 4 and 6 µg, respectively), they did not perform significantly better than 2 µg. This may be due to the saturation of the macrophage loading capacity which plateaus at 2 µg using the geneSilencer transfection system (Figure S7, Supporting Information). In the future we might specifically consider technologies that can increase loading in macrophages such as using mannose-receptor targeted polyplexes.

2.4. Macrophages Loaded with CIB1-siRNA Inhibit Growth of MDA-MB-468 Cells

Next, we examined whether the transfer of siRNA from macrophage to cancer cells could result in a therapeutic anticancer effect. Towards this goal, we delivered siRNA to knockdown calcium integrin binding protein-1 (CIB1) that is known to promote survival and proliferation in triple negative breast cancer (TNBC) cells via regulation of AKT and EKT activation.[39,40] Consistent with previous reports, the human TNBC MDA-MB-468 cells displayed a dose-dependent decrease in the cell survival after transfection with CIB1-siRNA (Figure 4a). In contrast, mouse IC21 macrophages were insensitive to CIB1-siRNA (Figure 4b). Moreover, quantitative real-time polymerase chain reaction (qPCR) analysis of the macrophages did not show a measurable amount of CIB1 RNA, which made them perfectly suitable as a vehicle for the delivery of CIB1-siRNA to the TNBC cells. IC21 macrophages were transfected with CIB1-siRNA and then cocultured MDA-MB-468 cells at a 1:2 ratio (one macrophage for every 2 cancer cells) in a tumoursphere as described above to recapitulate the 3D contact of macrophages and cancer cells as seen in vivo. The growth of tumourspheres was analyzed by live cell imaging and expressed as percentage of growth change (Figure 4c). There was a significant reduction in the tumourspheres growth kinetics for the groups containing macrophages transfected with CIB1-siRNA and those transfected with control siRNA. During the 4 days of coculture the control tumourspheres increased up to 70% in confluency whereas the CIB1-siRNA treated tumourspheres experienced only a 20% or 30% change in growth (Figure 4d). There was no significant difference between the two initial loading concentrations of CIB1-siRNA (2 or 4 µg) on the effect on tumoursphere growth, albeit the higher CIB1-siRNA concentration trended toward increased growth inhibition. Overall however, both CIB1-siRNA groups show smaller and more compact tumourspheres than control siRNA groups 4 days post cocultivation.

In a separate study, tumourspheres were analyzed for changes in mRNA expression of CIB1 and KI67, a marker of human cell proliferation. Coculture of MDA-MB-468 breast cancer cells with IC21 macrophages transfected with varying amounts of CIB1-siRNA resulted in significant decreases in CIB1 and KI67 mRNA expression (Figure 4e). In contrast, MDA-MB-231 cells were not sensitive to CIB1 signaling.[39,40] Coculture of MDA-MB-231 cells with IC21 macrophages transfected with CIB1-siRNA did not significantly reduce expression of CIB1 or KI67 (Figure 4f). To test the effect of gene transfer on normal cells, MCF10a (human epithelial breast cells) were also cocultured with IC21 macrophages transfected with 2 µg CIB1-siRNA. No significant differences in CIB1 or KI67 mRNA expression were found (Figure S8, Supporting Information).

2.5. Macrophage Activation Facilitates Horizontal Transfer of siRNA

Because macrophages change their functionality based on local external stimuli, it was important to investigate whether activation informs macrophage horizontal gene transfer. Macrophages were activated into three phenotypes, proinflammatory (M1; 1µg LPS), anti-inflammatory (M2; 20 ng mL⁻¹ IL-4), or tumor associated macrophages (TAM; cancer conditioned media (CCM)). Naïve, unconditioned macrophages (M0) were used as a control for all experiments. The activation phenotype (Table 1) was characterized by qPCR measurement of mRNA expression of relevant genes (Figure S9, Supporting Information). Macrophages activated 24 h prior to transfection with scrambled siRNA Cy5.5 lipoplexes were found to have differential uptake activity (Figure 5a). M0 and M1 activated macrophages contained comparable percentages of siRNA Cy5.5+ cells, however M1 macrophages had a higher mean intensity indicating that M1 macrophages endocytosed a larger amount of siRNA lipoplexes (Figure S6, Supporting Information). In contrast, both M2 and CCM activated macrophages endocytosed fewer siRNA lipoplexes than M0 and M1.

The effect of macrophage activation on intracellular trafficking of siRNA lipoplexes by transfecting M0 macrophages with siRNA lipoplexes and exposing to activation conditions for 24 h before analysis. The M1 macrophages contained 56% less siRNA lipoplexes in comparison to naïve, M0 macrophages (Figure 5b). Interestingly, M2 activated macrophages contained 67% less while CCM macrophages contained 82% less (Figure 5b). To elucidate the fate of the siRNA lipoplexes, lysosomal activity was measured. Colocalization studies using imaging flow cytometry revealed that siRNA lipoplexes M1 activated macrophages had higher colocalization with LysoTracker Green (Invitrogen) (Figure 5i) than CCM activated macrophages. Quantified over 10 000 cells for each condition (Figure 5h), M1 macrophages had on average a colocalization score of 2.5 in comparison to CCM (=1.2). Moreover, CCM activated macrophages secreted higher amounts of exosomes in...
comparison to M1 activated macrophages (Figure 5g). Finally, macrophages activated into an M2 phenotype and loaded with siRNA lipoplexes exhibited a higher degree of siRNA transfer than M1 macrophages to both MDA-MB-231-Luc human breast cancer cells (Figure 5d) and 3LL-Luc murine Lewis lung carcinoma cells (Figure 5e).

To test the effect of the lipoplex in gene transfer, macrophages were transfected via electroporation. IC21 macrophages were electroporated with 2 µg of scrambled siRNA Cy5.5 and exposed to activating media for 24 h before analysis with flow cytometry (Figure 5c). Results show a similar trend to lipoplex-transfected macrophages (Figure 5b) although there was a higher percentage of cells containing siRNA Cy5.5 in all groups. In addition, IC21 macrophages electroporated with 2 µg of Luc siRNA and cocultured with 3LL-Luc cancer cells demonstrated similar trends in knockdown of luciferase activity (Figure 5f). This suggests that the lipoplex may affect intracellular trafficking but that macrophage horizontal gene transfer is also linked to the activation phenotype of the macrophage.

2.6. Horizontal Transfer Facilitated via Rab27a Recycling Pathway

Because of the finding of enhanced exosomal secretion in M2-activated macrophages, exosomal trafficking pathways were investigated. Rab27a is a trafficking protein that regulates the intracellular exosome secretion pathway (Figure 6a).[41,42] We stably transduced IC21 and RAW 264.7 macrophages to express Rab27a-shRNA. Western blot analysis confirms knockdown of Rab27a protein expression in the knockout cell line.
Table 1. Primers used in qPCR experiments. Chart of primers used in qPCR reactions (M denotes a mouse specific gene; H denotes a human specific gene; F forward; R reverse).

| Primer | Sequence |
|--------|----------|
| 18s F  | TGGCCGCGCTTAGGTTGAAATT |
| 18 R   | TGGCAATGCTTTGCGATT |
| M Arg1 F | CTCAAGGCAAAGTCCTCTAGG |
| M Arg1 R | GCAGGCTGCAATGACCATCA |
| M Mr2 F | TCTCCCCAGACCCACTTTC |
| M Mr2 R | AACTGTTCCCTACTGTAAGC |
| M Nos2 F | ACATGACCCGTCACAGAT |
| M Nos2 R | CAGAGGGGATCCTGCTC |
| M CD163 F | GTCGCCACAGAATGTTTC |
| M CD163 R | CCAGAGCTTTACTGACAG |
| M TNFA F | GCTTCTCGAACCCTACAGCA |
| M TNFA R | GGTATCCGCATCTACCCCTG |
| H CIB1 F | ACATGAAGCCTCCATAGCTCC |
| H CIB1 R | CACGCCATAACCGCTG |
| H K67 F | GGGCATTCTTGTCCTTAAAT |
| H K67 R | GTATGCCCTTTCCGAAAA |

(Worksheet 6b,c). IC21 and RAW 264.7 cell lines were transfected with 2 μg of scrambled siRNA Cy5.5 lipoplexes and measured for the mean intensity 24 h later. In both cell types, Rab27a- macrophages retained more siRNA than wt macrophages (Figure 6d,e) and exhibited a narrower spectral distribution of the mean intensity macrophage (Figure 6f). Moreover, there were fewer exosomes isolated over a 24 h period from RAW 264.7 (Figure 6h) and IC 21 Rab27a- (Figure 6g) macrophages in comparison to IC21 wt. Rab27a- macrophages transfected with 2 μg of Luc siRNA and cocultured with 3LL-Luc (Figure 6i). Neither cell type cancer cells had a significant knockdown in luciferase activity after 48 h.

In Figure 5, it was demonstrated that macrophage activation enhanced siRNA transfer. A possible mechanism is through enhanced exosomal secretion under M2, anti-inflammatory macrophage activation, particularly when exposed to cancer conditioned media. To test this hypothesis, RAW 264.7 wt macrophages were transfected with Luc siRNA and rinsed with heparin sulfate to remove remaining extracellular lipoplexes. Macrophages were then incubated in either nonconditioned serum free media (SFM) or cancer conditioned serum free media (CCM-SFM) for 24 h. As a control, nontransfected macrophages were incubated in nonconditioned serum free media. Exosomes were isolated from the media via polyethylene glycol (PEG) precipitation, collected in 100 μL of DMEM. Twenty microliters of exosomal solution was incubated with 3LL-Luc cells. After incubation, we found that there was decreased luciferase activity in the SFM and CCM-SFM exosome treated groups in comparison to the control group (Figure 6k). Moreover, the CCM-SFM group had 23% lower luciferase activity than the SFM group, suggesting higher efficacy. Quantification of the exosomes reveal that CCM-SFM had a higher concentration of exosomes than the SFM or control groups (Figure 6l). Taken altogether, this strongly suggests that exosomal secretion via M2 activation is involved with gene transfer.

2.7. CIB1-siRNA Loaded IC21 Macrophages Infiltrate and Transfer siRNA into MDA-MB-468 Tumors

Finally, macrophage horizontal transfer of siRNA was tested in an in vivo mammary tumor model. IC21 macrophages were used in the in vivo model because the kinetics of siRNA transfer has already been well characterized in the previous in vitro models. There is no evidence that the macrophages themselves cause cytotoxicity to cells in our coculture experiments. In addition, the CIB1-siRNA construct has already been validated and characterized to decrease MDA-MB-468 tumor growth in nude animal models in previous literature.[39, 40] Moreover, previous preclinical studies have delivered mouse macrophages in conjunction with nude mouse models inoculated with human cancer cells.[41, 44] We used the macrophage and human cancer cell lines used in the in vitro tumorsphere assays in (Figure 4) in the paper.

MDA-MB-468 cells were orthotopically implanted into nude mice and monitored weekly. At 5 weeks, mice were injected with naïve, non-activated IC21 macrophages labeled with NuLight Red (IC21-NR), a nuclear localizing red protein that allows distinction between native and adaptively transferred macrophages. Mice were sacrificed 24 h after injection and the primary tumors were resected and weighed before digestion. Flow cytometry analysis of digested tumor cells positively confirms an infiltration of IC21-NR macrophage into the tumor (Figure 7a). Further analysis shows a correlation between the size of the tumor and the percentage of IC21-NR macrophage infiltration. Similar to the experimental scheme in (Figure 2), the percentage of CIB1-siRNA-Cy5.5 remaining within both the infiltrated IC21-NR macrophages and the host tumor was calculated. Impressively, 2% of the tumor cells were CIB1-siRNA-Cy5.5+ (Figure 7b). Of the tumor infiltrated IC21-NR macrophages 4% retained CIB1-siRNA-Cy5.5+, which is slightly lower than the 24 h time point in vitro tumorsphere models (Figure 3d).

3. Conclusions

In this paper, we demonstrated that macrophages can horizontally transfer siRNA to tumor cells. We developed two reporter-gene based models to characterize the pharmacodynamic siRNA transfer between macrophage and cancer cell populations. In addition, we confirmed macrophages horizontal transfer of siRNA can result in therapeutic effect as evidenced by decreased tumorsphere growth via delivery of CIB1-siRNA to MDA-MB-468 breast cancer cells. Altogether, our data suggests that macrophages can deliver siRNA and that the delivery can be titrated through the initial amount loaded into macrophages and the ratio of macrophages. These models can be used in future studies to optimize the therapeutic effect of macrophage delivery systems.

There are several reasons that make macrophage horizontal gene transfer very worthy for exploring as a strategy in cancer gene therapy. First, the high ratio of macrophages might be achievable in the tumor environment where macrophages reportedly reside in high ratios.[35] Second, while this technique may not be beneficial for delivering a therapeutic that required
near universal transfer for effect (i.e., p53, KRAS), it would be useful for delivering therapeutics that can alter the tumor microenvironment (i.e., matrix metalloproteinases, TGF-beta). Third, the number of macrophages needed can be reduced via optimization of the macrophage transfection conditions. If it is possible to load more siRNA into the macrophage and control its release, then macrophage horizontal gene transfer becomes a very valuable delivery mechanism. Finally, the mere finding of gene transfers presents a revolutionarily new strategy for gene delivery into solid tumors, an environment that is difficult...
to infiltrate. It could also be a unique future strategy for drug delivery to metastatic sites, as inflamed monocytes and macrophages form premetastatic niche in the metastatic process.[46] Our study found that M2 macrophages were better at transferring siRNA to cancer cells, due to the role macrophage activation plays a significant role in siRNA uptake and release.
Macrophages polarized into an M1, proinflammatory phenotype exhibited higher uptake of siRNA lipoplexes and high lysosomal activity, which directly correlates with the known behavior of these macrophages. In contrast, M2, anti-inflammatory activated macrophages exhibited lower uptake rates of siRNA lipoplexes and lower lysosomal activity. M2 macrophages produced more exosomes than M1 macrophages. Taken together, this evidence suggests that M2 activated macrophage are more efficient at horizontal gene transfer because of lower lysosomal degradation and higher exosomal secretion activity. The activation dependent role for gene transfer has large implications for gene delivery into the solid tumor. Macrophages and monocytes recruited to the tumors become alternatively polarized to immunosuppressive M2 type.\textsuperscript{[47]} Our findings suggest that this may become an additional trigger-release mechanism for the horizontal gene transfer from the siRNA preloaded macrophages migrating to the tumors ("Trojan horses") to the surrounding tumor cells. Several studies demonstrate that nanoparticle size, shape, and composition can modify macrophage activation.\textsuperscript{[48]} Future studies should explore how the design of nanoparticles can modulate macrophage activation in vivo.

Today, most clinical approaches to tumor gene delivery are limited to direct injections of the nuclei acid to the tumor.\textsuperscript{[49,50]} Recently, extracellular vesicles (EVs) or exosomes have attracted considerable attention due their ability to target many cancers.\textsuperscript{[51–53]} There are several advantages to using macrophages as a delivery vehicle in comparison to exosomes. Exosomes can be loaded relatively well with proteins,\textsuperscript{[54–56]} but loading with nucleic acids is considerably more difficult due to aggregation and charge. Purification and isolation of exosomes from pretransfected cells is also challenging due to low yield and quality control issues.\textsuperscript{[53,57]} But perhaps the best selling advantage is that use macrophages can allow for the site-specific localization and sustained release of exosomes, which additionally may be triggered in tumor environment.

Future work should explore the potential of translating gene delivery using macrophages in a clinical setting. Macrophages reside in the tissue, however, monocytes, their bloodborne precursors are an ideal cell type for cell therapy because they are easily harvested from the bloodstream. Monocytes/macrophages present a competitive advantage for delivery into the tumor environment because of the high recruitment and proliferation that occurs during tumorogenesis.\textsuperscript{[2,46,47]} Furthermore, monocyte based cell therapies already exist in other disease contexts and have shown some efficacy.\textsuperscript{[58,59]} Immunogenicity must be considered; however it is likely to be similar to other types of autologous cell therapies.\textsuperscript{[60–62]}

The implications of macrophage horizontal gene transfer are valuable for the macrophage biology community and the gene delivery field alike. An appreciation for the contribution of macrophages to disease progression and regeneration processes has grown over the last decade. Several studies have shown that macrophages can act as drug release “depots.” In as early as 2000, liver macrophages (Kupfer cells) were reported to act as drug reservoirs for docetaxel nanoparticles.\textsuperscript{[63]} More recent studies have shown macrophage slow release with drug from nanoparticle engulfment.\textsuperscript{[64,65]} Our study corroborates previous reports that macrophages can release engulfed materials but also adds to this literature by demonstrating that macrophages can also release genes that were artificially loaded. The data presented focuses on siRNA, however, it is likely that other nucleic acids (i.e., pDNA, mRNA, microRNA) can also be delivered in this fashion.\textsuperscript{[59]} Moreover, the finding that macrophage polarization modulates this release of siRNA inspires the design of nanoparticle carriers that can take advantage of this mechanism. This establishes a framework for macrophages as a cellular theranostic: macrophage activity can be used to study...
pharmacokinetics and pharmacodynamics of drug–tumor interactions as well as promote the specificity of gene delivery.

4. Experimental Section

Cell Culture: IC21 macrophages were maintained in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% fetal bovine serum (FBS). RAW 264.7 macrophages were maintained in Dulbecco’s modified Eagle’s medium (DMEM) media supplemented with 10% FBS. Human THP-1 monocytes were cultured in RPMI 10% FBS. THP-1 monocytes were incubated with 20 ng mL\(^{-1}\) phorbol 12-myristate 13-acetate (PMA; Sigma) in RPMI 10% FBS media overnight to different into macrophages.

MDA-MB-231 breast cancer cells were lentivirally transduced to express green fluorescent protein (MDA-MB-231-GFP). 4T1 breast cancer cells were lentivirally transduced to express renilla luciferase (4T1-RLuc). 3LL lung cancer cells were lentivirally transduced to express firefly luciferase (3LL-Fluc). All cancer cell lines (MDA-MB-231-GFP/Fluc, 4T1-RLuc, MDA-MB-468, 3LL-Fluc) were maintained in DMEM media supplemented with 10% FBS. All cells were cultured under standard cell culture conditions (37°C, 5% CO\(_2\)).

CCK-8 Assay: CCK-8 assay (Dojindo) was used to measure viability. Forty-eight hours after transfection, media was replaced with a solution containing 90 µL of DMEM and 10 µL of CCK8 solution. CCK8 solution was allowed to incubate for 4 h to allow time for reaction to occur. Absorbance was measured at 540 nm using Spectromax M5 (Molecular Devices).

siRNA Sequences: Silencer Firefly Luciferase (GL2 + GL3) siRNA is directly purchased from Thermofisher (AM4629). Silencer Negative Control siRNA (referred to as control siRNA) is additionally purchased from Thermofisher and used as control in the luciferase and time-lapse imaging experiments. All other siRNA sequences were custom synthesized by Drimaco Inc. (GE Drimaco). The nonhomologous scrambled siRNA (referred to as siRNA-Cy5.5) sequence sense is 5’-U.U.U.C.U.C.C.A.G.C.U.U.G.U.C.U.C.G.U.C.G.U.C.G.U.C.G.U.C.G.U.C.G.U.C.G.U.C.G.U.C.G.U.C.G.U.C.G.U.C.G.D-3’. The CIB1-siRNA sequence sense is 5’-C.A.G.C.U.C.U.C.U.G.A.G.C.U.G.A.G.C.U.G.A.G.C.U.G.A.G.C.U.G.A.G.C.D-3’. The recovery of exosomes was quantified by NTA.

Electroporation siRNA Transfection: Macrophages grown in T175 flasks were incubated with serum-depleted media for 24 h. The exosomal media is collected centrifuged at 1500 × g for 10 min to remove cells and large cell debris. The pellet was discarded, and the supernatant was centrifuged at 20 000 × g to remove larger debris and intact organelles. The resulting supernatant is then centrifuged at 150 000 × g for 30 min. Supernatant was further filtered using 0.2 µm syringe filters and mixed with 10% PEG overnight. Next, the media/PEG mixture was centrifuged at 4600 × g for 1 h to pellet the exosomes. The media was removed, the pellet was resuspended in 100 µL 1× PBS buffer, and stored at −20°C until analysis. The recovery of exosomes was quantified by NTA.

Imaging: ImageStreamX Imaging Flow Cytometry: Flow cytometry analytics and fluorescent images of cells were collected using ImageStreamX (Millipore). Images were collected at 60× magnification. Ten thousand cells minimum were analyzed for each sample. Analysis of cellular fluorescence was performed using IDEAS (Millipore) software. Specifically, colocalization analysis was done using the bright similarity detail algorithm and the number of compartments in individual cells was analyzed using the Spot Count algorithm.

Negative Stain TEM: siRNA lipoplex samples were applied onto negatively glow-discharged carbon-coated grids (400 mesh, copper grid) for 1 min, and excess liquid was removed by blotting with filter paper. Freshly prepared 1.5% uranyl formate (pH 5) was added (5 µL) for 1 min and then blotted. Digital micrographs were collected using JEOL JEM 1230 Transmission Electron Microscope operated between 40 and 120 kV and equipped with 5-axis goniometer stage in X, Y, Z, +z− 45° tilt, 360° rotation Gatan Orius SC1000 CCD camera. The images were recorded using Gatan Microscopy Suite 3.0 software.

Bioluminescence Assays: After transfection, transfected macrophages were cocultured in 96 well plates with either 4T1-RLuc, MDA-231-Fluc, or 3LL-Fluc cancer cell lines. At various time points, the media was removed and 30 µL of cell lysis buffer (Promega) was added to the plate. The plates were wrapped in parafilm and stored in the freezer until measurement. Ten microliter sample lysis mixture was analyzed for bioluminescent activity (Clomax 20/20 Luminometer) using either QunatiLuc (Inovogen) for renilla luciferase or Luciferin (Promega) for firefly luciferase. Luminescence measurements were normalized using measurements of total protein concentration using the BCA Protein assay (Thermo Fisher Scientific).

Macrophage Activation: Balb/c RAW 264.7 and C57B16 IC21 macrophages were plated at a density of 1.0 × 10\(^4\) mL\(^{-1}\) in a 6 well plate
in 2 mL of activation media for 24 h. The activation conditions were: M0 (naive, RPMI +10% FBS media only), M1 (1 µg mL⁻¹ LPS and 20 ng mL⁻¹ IFN-γ in RPMI supplemented with 10% FBS), and M2 (20 ng mL⁻¹ IL-4 and 20 ng mL⁻¹ IL-13 in RPMI supplemented with 10% FBS). CCM is media collected from MDA-MB-468 human breast cancer cells. After activation, media was changed and replaced with serum-free RPMI in preparation for experimentation. Macrophage polarization was validated by measuring mRNA expression of genes commonly upregulated during activation. M2 activation levels were assessed using CD206 mannose receptor and Arg1 while M1 activation levels were assessed using iNOS, TNFα, and CD86 (Figure S8, Supporting Information). In all other experiments if not specified differently the naïve unconditioned macrophages were used.

**Real-Time PCR:** mRNA gene expression was determined using SYBR green quantitative real-time PCR (qPCR) on cDNA template. cDNA was generated from 1000 ng RNA per sample using oligo(dT)₁₂-₁₈ primers and iScript CDNA Synthesis Kit (Biorad), according to manufacturer’s instructions. Product was amplified with 20 × 10⁻⁶ M forward and reverse primers of gene of interest and SybGreen Mastermix (Life Technologies) on an Applied Biosystems 7900 real-time PCR. The primer sequences for SybGreen primer sets were listed in the Supporting Information (Table 1).

**Live Cell Imaging:** Tumorsphere time-lapse images were collected using the Incucyte (Essen Bioscience), a microscope and incubation system that allows for long-term monitoring of live cells. Brightfield images were taken at 10x objective every 2 h. For the tumorsphere images were taken at 10× objective every 2 h. For the tumorsphere using the Incucyte (Essen Bioscience), a microscope and incubation system that allows for long-term monitoring of live cells. Brightfield images were taken at 10x objective every 2 h. For the tumorsphere growth studies the growth of the tumorspheres was monitored using Incucyte (Essen Bioscience), an incubator and built-in microscope live-cell imaging system. Incucyte software was used to calculate the confluence, or percentage of the image covered by the tumorsphere. The confluence was used to calculate the percentage growth change over time using the following equation

\[
\text{Percentage growth change} = \frac{\text{Confluence}(t) - \text{Confluence}(t = 0)}{\text{Confluence}(t = 0)} \times 100 (1)
\]

**Supporting Information**

Supporting Information is available from the Wiley Online Library or from the author.

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**Conflict of Interest**

The authors declare no conflict of interest.

**Keywords**

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[1] J. Shi, P. Kantoff, R. Wooster, O. Farokhzad, Nat. Rev. Cancer 2017, 17, 20.

[2] A. Dwyer, L. Ellies, A. Holme, F. Pixley, J. Biol. Methods 2016, 3, 49.

[3] J. Escamilla, S. Schokrpur, C. Liu, S. Priceman, D. Moughon, Z. Jiang, F. Pouliot, C. Magyar, J. Sung, J. Xu, G. Deng, B. West, G. Bollag, Y. Fradet, L. Lacroix, M. Jung, J. Huang, L. Wu, Lily Cancer Res. 2015, 75, 6.

[4] E. Harrison, S. Azam, C. Pecot, Front. Pharmacol. 2018, 9, 307.

[5] K. Tatiparti, S. Saju, S. Kashaw, A. Iyer, Nanomaterials 2017, 7, 77.

[6] P. Resnier, T. Montier, V. Mathieu, J. Benoit, C. Passirani, Biomaterials 2013, 34, 6429.

[7] J. Park, J. Park, Y. Pei, J. Xu, Y. Yeo, Adv. Drug Delivery Rev. 2016, 104.

[8] B. Xiao, L. Ma, D. Merlin, Expert Opin. Drug Deliv. 2016, 14.

[9] S. Ku, S. Jo, Y. Lee, K. Kim, Adv. Drug Delivery Rev. 2016, 104.

[10] M. Perepelyuk, C. Thangavel, Y. Liu, R. Den, B. Lu, A. Snook, S. Shoyele, Mol. Ther. - Nucleic Acids 2016, 5, e282.

[11] D. Adams, O. Suhr, P. Dyck, W. Litchy, R. Leahy, J. Chen, J. Gollob, T. Coelho, BMC Neuro. 2017, 17, 1.

[12] T. Allen, P. Cullis, Adv. Drug Delivery Rev. 2013, 65, 36.

[13] T. Ganbold, G. Gerile, H. Xiao, H. Haigide, RSC Adv. 2017, 7, 8823.

[14] B. Ozpolat, A. Sood, G. Lopez-Berestein, Adv. Drug Delivery Rev. 2014, 66, 110.

[15] A. Cardoso, S. Trabulo, J. Moreira, N. Düzgün, M. de Lima, Methods Enzymol. 2009, 465, 267.

[16] N. Khatri, D. Baradat, I. Vhora, I. M. Rathi, A. Misra, AAPS PharmSciTech 2014, 15, 822.

[17] S. Spagnou, A. Miller, M. Keller, Biochemistry 2004, 43, 13348.

[18] S. Zhang, B. Zhao, H. Jiang, B. Wang, B. Ma, J. Controlled Release 2007, 123, 1.

[19] M. Mitchell, E. Wayne, K. Rana, C. Schaffer, M. King, Proc. Natl. Acad. Sci. USA 2014, 111, 930.

[20] E. Wayne, S. Chandrasenkar, M. Mitchell, M. Chan, R. Lee, C. Schaffer, M. King, J. Controlled Release 2016, 223, 215.

[21] M. Stephan, J. Moon, S. Um, A. Bershteyn, D. Irvine, Nat. Med. 2010, 16, 1035.

[22] M. King, Front. Oncol. 2012, 2, 184.

[23] B. Huang, W. Abraham, Y. Zheng, S. Bustamante López, S. Luo, D. Irvine, Sci. Transl. Med. 2015, 7, 291.

[24] S. Chandrasenkar, M. Chan, J. Li, M. King, Biomaterials 2016, 77, 66.

[25] A. Anselmo, S. Mitragotri, J. Controlled Release 2014, 190, 15.

[26] A. Aucher, D. Rudnicka, D. Davis, J. Immunol. 2013, 191, 6250.

[27] J. Zhou, X. Li, X. Wu, T. Zhang, Q. Zhu, X. Wang, H. Wang, K. Wang, Y. Lin, X. Wang, Cancer Immunol. Res. 2018, 6, 1578.

[28] M. Haney, Y. Zhao, E. Harrison, V. Mahajan, S. Ahmed, Z. He, P. Suresh, S. Hingten, N. Klyachko, R. Mosley, H. Gendelman, A. Kabanov, E. Batrakova, PLoS One 2013, 8, 4.

[29] V. Mahajan, Z. Gaymalov, D. Alakhoova, R. Gupta, I. Zucker, A. Kabanov, Biomaterials 2016, 75, 58.

[30] Z. Gaymalov, Z. Yang, V. Pisarev, V. Alakhoov, A. Kabanov, Biomaterials 2009, 30, 1232.

[31] J. Joyce, D. Fearon, Science 2015, 348, 6230.

[32] M. D’Alloia, I. Zizzari, B. Sacchetti, L. Pierelli, M. Alimandi, Cell Death Dis. 2018, 9, 3.

[33] M. Casucci, C. Bonini, A. Bondanizia, Biochim. Biophys. Acta, Rev. Cancer 2016, 1865, 1.
