PHARMACOLOGY | SHORT COMMUNICATION

In vitro characterization of chemokine-loaded liposomes
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Abstract: The recent emergence of immunotherapies is transforming cancer treatments. Although many cancer immunotherapies are finding enormous success for treating hematologic tumors, a major obstacle for the treatment of solid tumors is localizing immune cells to the tumor site. Therefore, we have developed a technology that is capable of directing immune cell migration. Specifically, we have packaged chemokines, signaling molecules that promote immune cell migration, inside polyethylene glycol decorated-liposomes. The release profiles of chemokines and other large molecules from the liposomes have been examined in serum-containing media. We have demonstrated that the liposomes are able to release chemokines to induce immune cell migration. Additionally, these liposomes have been shown in vitro to limit cancer cell growth through increased immune cell recruitment. This strategy of encapsulating chemokines within liposomes paves the way for additional cancer immunotherapies and chemokine-based therapies.

Subjects: Biomaterials; Nanoscience & Nanotechnology; Nanobiotechnology

Keywords: nanotechnology; liposomes; peptide; chemokine delivery; immunotherapy

1. Introduction
Cancer is the second leading cause of death in the United States with an estimated over 600,000 deaths and 1.7 million new diagnoses expected in 2019 (American Cancer Society, 2019).

ABOUT THE AUTHOR
My area of research focuses on developing innovative nanoparticles for cancer immunotherapy. My research is joint collaboration between the Porter and Zhong labs. The Porter lab primarily focuses on designing nanoparticles and utilizing medical acoustics for diagnostic and therapeutic applications. The Zhong lab’s central focus is on B cell biology, specifically a subpopulation called B1 B cells, and defining their functions and role with respect to cancer immunology. The interdisciplinary research detailed in this article demonstrates that large biomolecules can be loaded and released for delivery which sets the framework for the creation of additional innovative nanoparticles. This research also paves the way further cancer immunotherapy researches which attempts to leverage the anticancer immunological properties of B cells, an area of continuing development. Overall, it demonstrates the utility of employing nanotechnology for cancer immunotherapy.

PUBLIC INTEREST STATEMENT
Cancer is a devastating disease that impacts millions of individuals each year. Newer treatments which use the body’s immune system to fight cancer have been showing miraculous results in many cancer types. However, cancer immunotherapies still possess a number of limitations such as getting enough immune cells to bolster an attack on cancer, so we have turned to utilizing innovative nanoparticles to potentially improve their efficacy. In this article, we have designed and evaluated nanoparticles that are capable of releasing biological signals which recruit immune cells. The particles were successfully shown to combat cancer growth by increasing immune cell recruitment to the cancer cells. This work may potentially be combined with existing cancer immunotherapies or conventional cancer therapies to create more effective cancer treatments.
Furthermore, many individuals battling with cancer have treatment options limited to surgical resection, radiation therapy, and conventional chemotherapy. Although these treatment options can be beneficial for improving short-term survival, they are often associated with high morbidity and mortality due to adverse side effects on normal tissue and organs (Steichen, Caldorera-Moore, & Peppas, 2013). Additionally, conventional chemotherapies are rarely curative and, in many cases, patient survival time is only marginally improved at the cost of a reduced quality of life. Therefore, there is a significant demand for improved cancer therapeutics.

Cancer immunotherapy is a rapidly expanding field that is revolutionizing the cancer treatment paradigm. Cancer immunotherapies are able to employ the immune system to recognize and combat cancer. Notably, cell-based immunotherapies have demonstrated significant success for the treatment of hematologic cancers (Androulla & Lefkothea, 2018). However, this therapeutic approach has faced challenges for the treatment of solid tumors (D’Aloia, Zizzari, Sacchetti, Pierelli, & Alimandi, 2018). One major limitation is attaining sufficient homing of activated immune cells to the tumor site (Pockaj et al., 1994). Solid tumors lack the same degree of accessibility as hematologic cancers; therefore, immune cells have greater difficulty localizing to the tumor sites which results in lower therapeutic success (Slaney, Kershaw, & Darcy, 2014).

As a preliminary step towards addressing the obstacle of insufficient immune cell tumor localization, we have designed a delivery vehicle for chemokines, signaling proteins that induce immune cell migration, in order to promote immune cell recruitment. We have encapsulated chemokines inside polyethylene glycol (PEG) decorated-liposomes that contain a small percentage of lysolipid to facilitate the release of entrapped molecules over several hours. There are multiple advantages of utilizing liposomes over other types of nanocarriers for the delivery of chemokines. For instance, their large aqueous core provides an opportunity for sufficient entrapment of chemokines. Also, their amphipathic nature allows encapsulation of both hydrophobic and hydrophilic molecules potentially opening the path for unique combinatorial therapies. Additionally, liposomes are biocompatible, associated with minimal toxicity, and highly tunable allowing for modulation of their charge, size, and release characteristics. Their surface can be modified with ligands or polymers such as PEG which has been shown to be beneficial for increasing circulation time and improving tumor accumulation (Jokerst, Lobovkina, Zare, & Gambhir, 2011). Also, PEGylated liposomes have been utilized successfully for the delivery of conventional chemotherapies less than 1 kilodalton (kDa), such as doxorubicin to tumor sites in xenograft tumor models (Needham & Dewhirst, 2001). Chemokines are orders of magnitude larger than conventional chemotherapeutic agents and consequently loading large biomolecules into liposomes can be challenging. Therefore, in this study, chemokines were sequestered in PEGylated liposomes using reverse-phase evaporation, a method that has been previously employed for loading oligonucleotides into cationic liposomes (Ko & Bickel, 2012). The chemokine selection as well as the liposome formulation is equally important for designing chemokine-loaded liposomes that are capable of directing immune cells to cancer cells.

After comparing several chemokines, 10 kDa sized CXCL13 was selected based on its ability to attract B cells, especially the B-1 cell subpopulation (Ansel, Harris, & Cyster, 2002). B-1 B cells assist with several antitumor immunological processes. For instance, B-1 B cells express high T cell activation molecules, CD80 and CD86, and have been reported to promote potent antigen presentation and T cell stimulation (Papi, Longo-Maugéri, & Mariano, 2016; Restifo, Dudley, & Rosenberg, 2012). Additionally, B-1 B cells produce immunoglobulin M (IgM) which can help the immune system identify cancer cells (Brändlein et al., 2003; Lei et al., 2015; Tsiantoulas, Diehl, Witztum, & Binder, 2014; Vollmers & Brandlein, 2005). Furthermore, a recent study analyzing the immune landscape in colorectal cancers revealed that CXCL13 plays an important role in patient survival (Bindea et al., 2013). The study examined the gene expression of patients’ tumor cells as well as immune cells in the tumor microenvironment to gain insight into the interaction between the two. Higher levels of CXCL13 expression in tumor cells were correlated with greater intratumoral B cells, helper T cells and cytotoxic T cells, as well as prolonged disease-free survival time. Additional experiments with mouse colorectal cancer models demonstrated that administration of
CXCL13 resulted in decreased tumor growth. These findings suggest that CXCL13 within the tumor microenvironment improves immune cell tumor localization and that its administration helps inhibit tumor growth. Encapsulation of CXCL13 into PEGylated liposomes will be beneficial for its delivery by improving its circulation time which will greatly expand its potential as a therapeutic (Jokerst et al., 2011).

In this study, CXCL13 or fluorescein isothiocyanate (FITC) dextran of comparable molecular weight was encapsulated in PEGylated liposomes. 10 kDa FITC dextran was used as a surrogate for CXCL13 while optimizing the liposome formulation in serum-containing media. We demonstrate that the liposomes gradually release CXCL13 to induce immune cell migration. Additionally, we provide in vitro evidence that cancer cell growth can be limited by leveraging CXCL13-loaded liposomes to promote immune cell recruitment.

2. Material and methods

2.1. Liposome preparation

1, 2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine (MPPC), and 1,2-distearoyl-sn-glycero-3-phosphatidylethanolamine-N-[methoxy(polyethylene glycol)-2000 (DSPE-PEG-2000)] were purchased from Avanti Polar Lipids (Alabaster, AL). The modified lysolecithin-containing thermosensitive liposome (LTSL) molar percentage used for studying the loading and release of CXCL13 consisted of 80% DPPC, 10% MPPC, and 10% DSPE-PEG-2000. The total mass of lipid to solute ratio for the formulation was 40:1. Recombinant mouse CXCL13 and 10 kilodalton FITC dextran were purchased from Biolegend (San Diego, CA) and Sigma-Aldrich (Natick, MA), respectively. Loading of CXCL13 and 10 kDa FITC dextran into the liposomes was performed by reverse-phase evaporation as detailed (Ko & Bickel, 2012). Liposome sizing was determined by dynamic light scattering (Brookhaven 90Plus). Non-encapsulated CXCL13 or 10 kDa FITC dextran were separated from liposomes by extensive dialysis with 300 kDa membrane pore size dialysis tubing (Billerica, MA). Quantification of CXCL13 encapsulated with the liposomes was determined using CBQCA Protein Quantitation Kit purchased from Thermo Fisher Scientific (Waltham, MA).

2.2. Liposomal release studies

Fluorescent-labeled CXCL13 and 10 kDa FITC dextran were used to study solute release from liposomes. For these studies, CXCL13 was conjugated with Alexa Fluor 488 C5 Maleimide (Thermo Fisher Scientific, Waltham, MA) via thiol maleimide reaction. Unconjugated Alexa Fluor 488 C5 Maleimide was separated by extensive dialysis with 1 kDa membrane pore dialysis tubing. Thin layer chromatography was utilized with methanol and dichloromethane at a 90:10 ratio to confirm successful conjugation. The release of 10 kDa FITC dextran and Alexa Fluor 488-conjugated CXCL13 was determined by measurements via a spectrofluorophotometer (Shimadzu RF 5301). Fluorescence was measured at excitation and emission wavelengths of 497 nm and 518 nm, respectively. Liposomes were incubated at 37°C for various durations then dialyzed at 4°C overnight. Fluorescence measurements of the liposomes were recorded pre-dialysis and post-dialysis to determine the change in fluorescence signal. This change in signal was utilized to determine the amount of released 10 kDa FITC dextran and Alexa Fluor 488-conjugated CXCL13.

2.3. Transwell migration assay and co-culture assay

Mouse immune cells, peritoneal cavity washout (PCW) cells, were collected by injecting 10 milliliters of Hank’s Balanced Salt Solution (HBSS) with 2% fetal bovine serum (FBS) into the peritoneal cavity as previously reported (Ray & Dittel, 2010). Murine colon adenocarcinoma (MC38) cells were engineered to express TdTomato (Tdt) fluorescent protein and were maintained in Dulbecco’s Modified Eagle’s Medium with 10% FBS, and 1% penicillin/streptomycin. Transwell migration assays with PCW cells were performed as previously described with a concentration of 0.3 μg/ml of CXCL13 as this has been established to be the optimal concentration for attracting B cells (Moratz & Kehrl, 2004). The PCW cell population is made up of B-1 B cells and additional immune cell subtypes (Ray & Dittel, 2010). 5-μm Corning Costar transwell chambers were used for this assay (Cambridge, MA).
The incubation period for the assay was 6 hours as this was the optimal duration for recording immune cell migration. Migrated cells were counted using the Nexcelom Celigo microwell plate imager. For the co-culture assay, MC38-Tdt cells were seeded on the bottom transwells for 24 hours prior to performing the migration assay. On the day of the experiment, the media was replaced with each treatment condition and the transwell inserts containing PCW cells were placed on top of the transwell plate. After incubating for 6 hours, the transwell inserts were removed and the combination of transmigrated PCW cells and MC38-Tdt cells were co-cultured for an additional 24 hours. Images were taken with Keyence Fluorescence Microscope BZ-X800 using 4X and 10X objective lens before migration, immediately after migration, and 24 hours after migration. The number of MC38-Tdt cells in each of the images was counted using the Keyence BZ-X Analyzer.

2.4. Statistical analysis
All data are presented as mean ± standard deviation of experiments in triplicates. The data was analyzed with Welch’s t-test (p-value ≤ 0.05).

3. Results

3.1. Liposome formulation and evaluation
In order to design a chemokine-loaded liposome that was both relatively stable in serum-containing media and able to gradually release large biomolecules, we modified the LTSL formulation originally reported (Needham, Anyarambhatla, Kong, & Dewhirst, 2000). The molar percentage of PEGylated lipid was increased and the molar percentage of DPPC was decreased relative to the reported LTSL composition. We sought to examine the ability of the liposomes to gradually release over time when incubated at 37°C in serum-containing media. 10 kDa FITC dextran was utilized to mimic CXCL13 because of its similar size and fluorescence. As shown in Figure 1, the release of both 10 kDa FITC dextran and Alexa Fluor 488-conjugated CXCL13 was gradual over the course of several hours. This was expected due to their similar size and suggests that additional biomolecules at this molecular weight would have comparable release. After the course of 6 hours, the majority of encapsulated Alexa Fluor 488-conjugated CXCL13 and 10 kDa FITC dextran was released from the liposomes. The liposomes’ loading efficiency on average was approximately 25% across batches. DLS data for the CXCL13-loaded liposomes is shown in Table 1.

3.2. Chemokine-loaded liposomes induce cell migration
Furthermore, we were interested in determining whether the CXCL13-loaded liposomes were able to direct immune cell migration. Transwell migration assays using freshly collected mouse PCW cells were performed to evaluate the ability of the liposomes to release chemokine and promote immune cell migration. An equivalent amount of CXCL13 was administered as free CXCL13 or loaded into liposomes. Empty liposomes served as a control to determine whether immune cells were attracted to the particles alone. Images of the transmigrated immune cells were taken after...
incubation (Figure 2(a)). As seen in Figure 2(b), there was significant immune cell migration among wells containing CXCL13-loaded liposomes or free CXCL13 compared to media alone or empty liposomes. The increase in transmigrated immune cells among wells incubated with CXCL13-loaded liposomes compared to empty liposomes suggests that the loaded liposomes were able to release a sufficient amount of CXCL13 to generate a chemokine gradient and promote immune cell recruitment. The few transmigrated immune cells among wells incubated with media alone or empty liposomes are likely less than five microns in diameter, the pore size of the transwell inserts, which allow them to fall through the inserts and settle to the bottom well. Although an equal amount of CXCL13 was administered as free CXCL13 or loaded into liposomes and at the end of the 6-hour incubation period both conditions have essentially the same amount of released CXCL13, the CXCL13-loaded liposomes did not induce as much immune cell migration compared to free CXCL13. This is likely due to the fact that the free CXCL13 is accessible to the immune cells throughout the entire incubation period, while the CXCL13 loaded into the liposomes is gradually released during this time.

3.3. Cancer cell growth reduced by chemokine-loaded liposomes

The transwell migration assay with the addition of cancer cells on the bottom of the transwells was performed to assess the impact of recruited immune cells via CXCL13-loaded liposomes on cancer cell growth. Initially, MC38-Tdt cells were incubated solely with empty liposomes, CXCL13-loaded liposomes, or free CXCL13, without the addition of PCW cells to the upper transwell insert, and monitored over several days. There was no significant difference in cancer cell growth over time between all conditions suggesting that the liposomes and CXCL13 alone do not directly impact cancer cell growth (Supplementary Figure 1). The experiment was repeated with the addition of PCW cells to the upper transwell insert (Figure 3(a)) and microscope images were taken to track the growth of the MC38-Tdt cells (Figure 3(b)). The Tdtomato expression allowed for distinguishing the MC38 cells in the transwells from the recruited transmigrated immune cells. There was less cancer cell growth over time among wells incubated with CXCL13-loaded liposomes or free CXCL13 compared to media alone or empty liposomes (Figure 3(c)). This reduction in cancer cell growth in wells administered with CXCL13-loaded liposomes or free CXCL13 is likely due to the increased number of transmigrated immune cells that are recruited via CXCL13. This in vitro experimental setup exemplifies the potential of the CXCL13-loaded liposomes as a strategy for limiting cancer growth. These results are in good agreement with the findings in the previous report on CXCL13’s importance within the tumor microenvironment and its role in promoting immune cell tumor recruitment and decreased cancer growth (Bindea et al., 2013).

4. Discussion

In this study, we have successfully designed and evaluated a chemokine-loaded liposome. We have also examined the release profile of 10 kDa sized molecules from the liposomes which can be utilized for evaluating other molecules of similar size. The liposomes were shown to be able to release chemokines and induce immune cell migration. We have demonstrated in vitro that chemokine-loaded liposomes may be utilized as a potential strategy for recruiting immune cells to cancer cells. In previous studies, CXCL13 was administered directly into the colonic submucosa of mice with syngeneic colorectal cancer and resulted in decreased tumor growth (Bindea et al., 2013). The advantage of packaging CXCL13 within PEGylated liposomes is that it potentially allows for intravenous administration and increased protection from degradation during circulation (Jokerst et al., 2011). Additionally, gradual liposomal release of CXCL13 is beneficial to ensure

| Table 1. Liposome characteristics |
|----------------------------------|
|                                | Size (nm) | Polydispersity index | Zeta Potential (mV) |
| CXCL13-loaded Liposomes          | 169.3 ± 1.4 | 0.196 ± 0.011       | −0.22 ± 5.22        |

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that a chemokine gradient is maintained over time and immune cells are consistently being recruited to the tumor. In future studies, the efficiency of the CXCL13-loaded liposomes to localize to the tumor space and effectiveness in limiting tumor can be assessed in a syngeneic mouse tumor model. Also, the impact of the liposome on CXCL13 half-life can be evaluated.

5. Conclusions
These findings make significant advancements in both nanotechnology and immunotherapy. With respect to nanotechnology, although encapsulating large biomolecules such as chemokines into liposomes is challenging, we have demonstrated that it can be done successfully. By developing the first CXCL13-loaded nanoparticle, which is not only important for CXCL13 delivery, but also for chemokine delivery, we have demonstrated that chemokines can be packaged and delivered using PEGylated liposomes. Moreover, a multitude of chemokine-based therapies are being pursued as stand-alone strategies or combination therapies for cancer treatment (Mohit & Rafati, 2012). For instance, administration of CCL21 for lung cancer resulted in enhanced leukocyte tumor infiltration and reduced immunosuppressive cells (Kar et al., 2011). Additionally, studies have looked at the

Figure 2. Transwell migration of immune cells induced by CXCL13. PCW cells were plated in the transwell insert and incubated with media alone, empty liposomes, CXCL13-loaded liposomes, free CXCL13 for 6 hours. (A) Microscopic images of the transmigrated cells in wells administered with media alone, empty liposomes, CXCL13-loaded liposomes, or free CXCL13. The images are representative of triplicate wells. (B) The number of transmigrated cells was recorded. The CXCL13-loaded liposomes and free CXCL13 induced greater immune cell migration compared to media alone or empty liposomes. (*p < 0.05).
Figure 3. Growth inhibition of MC38-Tdt cells by transmigrated immune cells. PCW cells were plated in the transwell inserts and incubated on top of transwell chambers, containing MC38-Tdt cells, with media alone, empty liposomes, CXCL 13-loaded liposomes, or free CXCL 13 for 6 hours. MC38-Tdt cells were co-incubated with transmigrated immune cells for 24 hours after the transwell migration assay.

(a) A schematic of the experimental set-up for the co-incubation assay. (b) Microscopic images of MC38-Tdt cells from wells administered with media alone, empty liposomes, CXCL 13-loaded liposomes, or free CXCL 13, 24 hours after the transwell migration assay. The images are representative of triplicate wells. (c) The number of MC38-Tdt cells after 24 hour co-culture with transmigrated immune cells was recorded. Wells administered with CXCL 13-loaded liposomes or free CXCL13 showed reduced cancer cell growth compared to media alone or empty liposomes. (*p < 0.05)
combinatorial effects of administering chemokines and cytotoxic agents such as CXCL10 and cisplatin for the treatment of solid tumors (Li et al., 2005). However, a major obstacle for chemokine-based therapies is efficient delivery methods (Mohit & Rafati, 2012). The encapsulation procedure that we have employed in this study can be used for additional chemokines and cytokines. Although in this study we analyzed the impact of general immune cell migration towards cancer cells, chemokine-loaded liposomes can potentially be loaded with a diverse array of immunomodulatory agents and small anticancer molecules to improve the capabilities of chemokine-based therapies. In future studies, CXCL13-loaded liposomes in combination with conventional chemotherapeutics can be evaluated for synergistic effects. Also, chemokine-loaded liposomes can be used in combination with other immunotherapies to mobilize activated immune cells to the tumor site. This study highlights the utility of nanotechnology for cancer immunotherapy applications, opening the path for further interdisciplinary research which incorporates these two rapidly expanding fields.

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Competing Interests
The authors declares no competing interests.

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Declaration of interest
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