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Immunomodulatory Magnetic Microspheres for Augmenting Tumor-Specific Infiltration of Natural Killer (NK) Cells

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Abstract

The purpose of this research is to develop magnetic resonance imaging (MRI) visible immunomodulatory microspheres (IMM-MS) for efficient image guided cancer immunotherapy. IMM-MS composed of recombinant interferon gamma (IFN- γ), iron oxide nanocubes (IONC), and biodegradable poly(lactide-*co*-glycolide) (PLGA) were successfully prepared via a double-emulsion method. The prepared IMM-MS exhibited a sustained IFN- γ release and highly sensitive MR T_2 contrast effects. Finally, in an orthotopic liver tumor VX2 rabbit model, successful hepatic intra-arterial (IA) transcatheter delivery of IMM-MS to liver tumors was confirmed with MR images. The deposition of IMM-MS significantly increased NK-cell infiltration into the liver tumor site.

Graphical Abstract

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Notes

The authors declare no competing financial interest.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsami.7b02258.

Experimental details, particle size of IONC, MR R_2 relaxivity, ROI values results (PDF)

Keywords

cancer immunotherapy; natural killer (NK) cells infiltration; interferon-gamma (IFN- γ); microspheres; image-guided therapy

Hepatocellular carcinoma (HCC) is the most common form of primary liver cancer and the third leading cause of cancer-related death worldwide. HCC may be an ideal target for cytokine-targeted therapies. Patients with HCC present with unique anti- or pro-tumor responses during the development and progression of HCC.¹ Immunotherapy has been explored in the setting of liver cancer for decades.² Several reports have shown that cytokine-related adjuvant immunotherapy can significantly improve outcomes in HCC patients.^{3–5} Recently, adoptive immunotherapy (AIT) with natural killer (NK) lymphocytes has emerged as a potential HCC treatment strategy.⁶ NK cells can kill cancer cells through secretion of cytotoxic lymphokines and disruption of the tumor vascular.^{7,8} Intratumoral infiltration of NK cells correlates with a good prognosis in a broad range of tumor types.^{9–11} However, clinical responses following NK cell AIT has been limited in solid tumor when compared to responses observed following ATI in leukemia and blood-borne cancers.^{12,13} While the low therapeutic efficacy of NK cell ATI in solid tumors remains unclear, one of major reason could be insufficient homing NK cells to solid tumors.¹⁴ Fisher et al. demonstrated that only a minority (0.016% of injected cells) of adoptively transferred ¹¹¹In-labeled lymphocytes were observed in the tumors of treated patients.¹⁵ In this regard, the infiltration efficacy of endogenous and/or infused NK cells to tumor sites may be a key factor that is influencing the antitumor effects.

Recently, the IFN- γ -inducible chemokine C-X-C motif ligand (CXCL9, 10 and 11) was reported as a predictive factor for intratumoral infiltration of NK cells.^{16,17} Wennerberg et al. showed enhanced migratory capacity of NK cells toward CXCL10-transfected melanoma tumors compared to a CXCL10-negative tumor, with NK cell infiltration resulting in significantly increased therapeutic efficacy.¹⁴ Inspired by this phenomenon, we demonstrate here transcatheter intra-arterial (IA) local delivery of chemoattractant encapsulated in polymeric microspheres to induce efficient NK cells infiltration to tumor sites for the targeted treatment of liver cancer. Additionally, iron oxide nanocubes (IONC) were incorporated into the polymeric microsphere for magnetic resonance imaging (MRI)-guided transcatheter delivery. The imageguided delivery is important in three aspects of clinical practice. First, it is possible to confirm whether the drug-loaded microspheres are properly delivered to disease site after injection. Second, an amount of the injected microspheres can be quantitatively analyzed to determine a dose of postinfusion. Finally, distribution of the injected microspheres in the body can be monitored for a long-term period. In the design,

biocompatible poly(lactide-*co*-glycolide) (PLGA) and synthesized IONP were used as base materials, and IFN- γ was encapsulated in the microspheres through a double-emulsion (Water in Oil in Water, W/O/W) method. In the manufacturing process, the hydrophilic IFN- γ is contained in the inner water phase and the hydrophobic IONP is encapsulated with PLGA on the oil phase. Finally, the MRI-visible IFN- γ and IONP coencapsulated PLGA microspheres were selectively delivered to liver tumors via MRI-monitored transcatheter IA local infusion in an orthotopic VX2 rabbit model. We hypothesized (Scheme 1) that release of IFN- γ from the microspheres could effectively induce the migration of the cytotoxic NK cells into tumor site through CXCL-10 signaling induction.

In this study, we designed immunomodulatory microspheres (IMM-MS) composed of PLGA as a biocompatible polymer, IONC as an MR contrast agent, and IFN- γ as an immunomodulatory protein agent (Scheme 1). First, the IONC was synthesized through a heap-up method.¹⁸ Cube-shaped iron oxide nanoparticles were successfully synthesized (Figure 1a) with size of approximately 23 nm (Figure S1); these nanoparticles demonstrated superior MR imaging properties compared to clinically available iron agent Ferumoxytol (Figure S2). The shape and the physicochemical properties were consistent with our previous paper.¹⁹ Next, the as-synthesized iron oxide nanocubes and IFN- γ were coencapsulated in PLGA microspheres (i.e., IMM-MS) via a double emulsion method. Spherical-shaped microparticles were clearly observed with optical microscopy (Figure 1b). The size of the IMM-MS was determined to be approximately 10 μ m (Figure 1c), as measured using ImageJ software. To investigate the surface morphology of IMM-MS, we used scanning electron microscopy (SEM). As shown in Figure 1d, a smooth surface morphology was observed for IMM-MS. Relaxivity studies were next performed to demonstrate the potential to use MRI to visualize IMM-MS delivery (Figure 1e). Strong signal decay proportional to particle concentration was observed in T_2 -weighted phantom images. The amount of IONC to be contained in the IMM-MS was optimized in a preliminary study (Figure S3). The optimal composition of IMM-MS, that with highest R_2 relaxivity, was selected for further studies. The R_2 relaxivity value of the optimal IMM-MS was determined to be 1588 mM⁻¹ S¹⁻ (in 1 wt % agar phantom at a field strength of 7 T at 300 K), which is similar to our previously reported microspheres system containing IONC.¹⁹ These results indicate the feasibility of using an MRI to image in vivo delivery of our IMM-MS during the IA infusion procedure.

To investigate drug release behavior of the IMM-MS, we performed in vitro drug release tests using an ELISA. As an additional control group, microspheres encapsulating only IFN- γ (i.e., control MS without IONC) were also prepared before the experiment. Prior to the drug release test, the loading efficiency and contents for IONC and IFN- γ were determined by ICP-OES and ELISA, respectively (Table 1). Although a slight enhancement of IFN- γ loading efficacy was observed for the IMM-MS compared to control MS, both exhibited efficacies >90%. The IONC loading efficacy within the IMM-MS was roughly 65%, which was consistent with our previous report.¹⁹ As shown in Figure 2, IMM-MS exhibited much slower initial burst compared to control MS, whereas ~75% of IFN- γ was released from control MS in 1 h, IMM-MS released only ~27% of IFN- γ in 1 h. Furthermore, IMM-MS showed sustained drug release behavior over the entire test period. The sustained drug release behavior could be attributed to the water channel blocking by the addition of the iron

oxide nanoparticles. It is well-known that the drug encapsulated in PLGA microspheres is released through water-channels inside of PLGA microsphere.^{20,21} As shown in Scheme S1, when iron oxide nanoparticles (e.g., IONC) are coencapsulated with IFN- γ in the preparation process of PLGA microspheres, water exchange via the water-channels of the PLGA polymeric matrix may be blocked by the nanoparticles, resulting in a sustained drug release from these polymeric microspheres with minimalized initial burst. To verify this hypothesis, we plan to measure the release rates of protein drugs at various PLGA to IONP ratio conditions in a future study.

To confirm CXCL10 chemokine production induced by the released IFN- γ , the CXCL10 level was measured in two different in vitro cultured cell lines including MCA-RH7777 (rat hepatocellular carcinoma²²) and DSL-6A/C1 (rat pancreatic carcinoma²³). According to previous reports,^{14,24,25} CXCL10 is an important chemokine for NK cell recruitment through IFN- γ triggered signaling pathway. As we expected, when the cells were exposed to IFN- γ released from IMM-MS (equivalent to IFN- γ exposure dose of 100 ng/mL), substantial increases in CXCL10 production levels were observed for both MCA-RH7777 and DSA-6A/C1 cell lines, compared to that of nontreated cells (Figure 3). This result suggests that IFN- γ released from our IMM-MS is successfully able to induce CXCL10 secretion from tumor cells, which should ultimately aid in the recruitment of NK cells into the targeted tumor regions.

To investigate the potential for in vivo MRI-guided delivery to liver tumors, transcatheter hepatic IA infusions of the IMMMS were performed in the rabbit VX2 model.²⁶ For this animal study, we used a recombinant rabbit IFN- γ for induction of biological response in the rabbit model. VX2 tumor tissue was implanted in the liver using percutaneous ultrasound guided injection procedures as previously described;²⁶ tumor growth was monitored with follow-up MR imaging. Once tumor sizes reached approximately 1 cm in diameter, roughly 14 days postimplantation, IMM-MS were infused via IA catheter placed within the tumor feeding branches. X-ray digital subtraction angiographic (DSA) was used to guide catheter placement and confirm appropriate positioning for selective delivery of IMM-MS to the tumor site (Figure 4a). To demonstrate potential for MRI-monitored delivery of the IMM-MS, we also performed MR imaging on a 7T MRI system. As shown in Figure 4b, significant changes in the T_2^* map were observed at the tumor site after transcatheter infusion of IMM-MS. ROI measurements at the tumor site before infusion of IMM-MS was 215 ± 7 , it was substantially increased to 1292 ± 7 (Figure S4), indicating efficient accumulation of IMM-MS containing MRI visible IONC at the tumor site. Next, to confirm the deposition of IMM-MS within the liver, histological staining of the tissue for iron was carried out using a Prussian blue staining kit (Figure 4c). A strong blue signal was clearly observed in the intratumoral blood vessels. Confirmation of successful delivery of the IMM-MS to the tumor sites was achieved in vivo with MRI and histologically postnecropsy.

To investigate IFN- γ -induced NK cell infiltration after IMM-MS infusion in the VX2 rabbit liver tumor model, we sacrificed IMM-MS administered rabbits 12 h after infusion, and then stained the harvested liver tumor tissues with rabbit anti-CD56 for immunohistochemical analysis. The CD56 is a well-known cell surface marker for NK cells.^{27,28} As shown in Figure 5, a significantly greater amount of infiltrated NK cells were observed in the IMM-

MS treated group, compared to control groups (i.e., nontreated or receiving PLGA MS not loaded with IFN- γ). These results suggest that the released IFN- γ cytokines from IMM-MS can successfully increase the NK cell infiltration with potential mechanism being the previously described IFN- γ triggered signaling pathway inducing CXCL10-mediated migration.¹⁴ In this study, we tested with nontransgenic animals, but a future study using transgenic animal models (e.g., CXCL 10 knockout models) would be helpful in demonstrating the CXCL10-mediated infiltration of NK cells. Given the promising results of these initial in vitro and in vivo studies, additional longitudinal studies are now clearly warranted to rigorously examine the potential antitumor therapeutic efficacy of these IMM-MS.

In this study, we have demonstrated new immunomodulatory microspheres (IMM-MS) that enable MRI-guided transcatheter IA delivery to liver tumor for liver tumor immunotherapy. The IMM-MS was successfully prepared with biocompatible PLGA polymer, immunomodulatory cytokine (IFN- γ), and IONC. During in vitro tests, the released IFN- γ from IMM-MS could induce chemotactic cytokine (e.g., CXCL10) release from tumors cell that should elicit NK cell recruitment. In the VX2 rabbit model, IMM-MS were successfully infused under X-ray DSA guidance and MRI confirmed selective transcatheter delivery of the IMM-MS to the targeted liver tumors. Finally, using immunohistochemistry analyses, a significant enhancement of NK cell infiltration was observed in the IMM-MS treated group. We believe this new design of an image-guided immunomodulatory microsphere system should be promising for various biomedical research fields including cancer immunology, image-guided interventional oncology, and drug delivery.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Physicochemical characterization of immunomodulatory microspheres (IMM-MS). (a) TEM image of iron oxide nanocubes (Scale bar: 200 nm), inset: magnified TEM image (Scale bar: 50 nm). (b) Optical microscopy image of IMM-MS: the color was converted to gray (Scale bar: 50 μm). (c) Size distribution of IMM-MS (analyzed by ImageJ software). (d) SEM image of IMM-MS (Scale bar: 10 μm). (e) T_2 -weighted MR images and plot of R_2 value of IMM-MS in 1% agar phantoms at various concentrations of IMM-MS (mg/mL) at 7 T MRI (analyzed by Jim software).

Figure 2.

In vitro IFN- γ release measurement from control MS (without IONC) and IMM-MS in PBS (150 mM, pH 7.4) at 37 °C ($n = 3$).

Figure 3.

In vitro IFN- γ induced CXCL10 chemokine production of (a) McA-RH7777 and (b) DSL-6A/C1 cell lines ($n = 3$, * $p < 0.001$).

Figure 4. Magnetic resonance imaging (MRI)-monitored transcatheter hepatic intra-arterial (IA) local infusion of immunomodulatory microspheres (IMM-MS) in orthotopic VX2 rabbit liver tumor model. (a) Hepatic X-ray angiography image of VX2 liver tumor model after IA injection of IMM-MS (black circle: tumor site). (b) In vivo T_2^* map images acquired before and after transcatheter IA infusion of IMM-MS (red dotted line: tumor site). Intrahepatic deposition of IMM-MS is depicted as regions of signal loss within the T_2^* map images postinfusion (yellow dotted line), and (c) Prussian blue staining of treated tumor-bearing liver tissues 12 h post-IA injection.

Figure 5. Immunohistochemical analysis (Anti-CD56 staining) for infiltration of NK-cells in tumor region. (a–c) Representative optical image of the anti-CD56 stained tissue from (a) untreated, (b) empty-microspheres (MS) (without IFN- γ), and (c) immunomodulatory microspheres (IMM-MS) treatment group. (d) Quantitative analysis of the CD56 positive NK cells in the tumor tissues ($n = 4$, $*p < 0.05$).

Scheme 1.

Schematic Representation of Immunomodulatory Microspheres (IMM-MS): (a) Schematic Illustration of Structural Composition of the IMM-MS and (b) Transcatheter Intra-arterial (IA) Local Infusion of IMM-MS and Released IFN- γ Inducing NK Cell Infiltration

Table 1

Quantitative Characterization of Immunomodulatory Microspheres (IMM-MS)

sample	feed		IONC		IFN- γ	
	IONC (mg) ^a	IFN- γ (μ g) ^b	loading efficiency (%) ^c	loading contents (wt %) ^d	loading efficiency (%) ^c	loading contents (wt %) ^d
control MS		100	64.8 $\pm$ 3.9	0.65 $\pm$ 0.04	91.9 $\pm$ 3.0	0.091 $\pm$ 0.003
IMM-MS	1.0	100	65.3 $\pm$ 2.9	0.65 $\pm$ 0.03	93.3 $\pm$ 5.4	0.093 $\pm$ 0.005

^aWeight of feed iron oxide nanocubes (IONC) per 100 mg of PLGA.

^bWeight of feed IFN- γ per 100 mg of PLGA.

^cLoading efficiency = (actual mass of IONC or IFN- γ) $\times$ 100, as determined by ICP-OES and ELISA, respectively.

^dLoading contents = (actual mass of IONC or IFN- γ /actual total mass of IMM-MS) $\times$ 100, as determined by ICP-OES and ELISA, respectively ($n = 3$).