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# Exploitation of Synthetic mRNA To Drive Immune Effector Cell Recruitment and Functional Reprogramming In Vivo

**Yitian Xu,*,† Lu Huang,† Jonathan L. Kirschman,‡ Daryll A. Vanover,‡ Pooja M. Tiwari,‡ Philip J. Santangelo,‡ Xiling Shen,*,§,¶ and David G. Russell†**

**Therapeutic strategies based on in vitro–transcribed mRNA (IVT) are attractive because they avoid the permanent signature of genomic integration that is associated with DNA-based therapy and result in the transient production of proteins of interest. To date, IVT has mainly been used in vaccination protocols to generate immune responses to foreign Ags. In this "proof-of-principle" study, we explore a strategy of combinatorial IVT to recruit and reprogram immune effector cells to acquire divergent biological functions in mice in vivo. First, we demonstrate that synthetic mRNA encoding CCL3 is able to recruit murine monocytes in a nonprogrammed state, exhibiting neither bactericidal nor tissue-repairing properties. However, upon addition of either *Ifn-γ* mRNA or *Il-4* mRNA, we successfully polarized these cells to adopt either M1 or M2 macrophage activation phenotypes. This cellular reprogramming was demonstrated through increased expression of known surface markers and through the differential modulation of NADPH oxidase activity, or the superoxide burst. Our study demonstrates how IVT strategies can be combined to recruit and reprogram immune effector cells that have the capacity to fulfill complex biological tasks in vivo. *The Journal of Immunology*, 2019, 202: 000–000.**

Synthetic mRNA therapy has been gaining interest in recent years, thanks to recent advances in enhanced stability and a reduction in inflammatory response (1–9). Because of its nonreplicative nature, synthetic mRNA acts simply as a transient carrier of information that will be degraded via metabolic pathways within a few days (2, 10). Therefore, synthetic mRNA poses minimal risk of genomic insertional mutagenesis, an obvious concern for DNA-based therapy (1, 2, 10, 11). Moreover, synthetic mRNA therapy does not require nuclear delivery for transcription, unlike DNA-based therapy (1, 2, 10, 11). Finally, synthetic mRNA can maintain protein expression at a controllable level for several days (2, 10), which compares favorably with recombinant protein that is delivered as a bolus with relatively short-lived $t_{1/2}$ (12, 13). Lastly, recent data indicate that appropriate chemical modification of synthetic mRNA can provide a localized and intense expression of desired proteins in selected tissues or organs (1, 2, 13).

Preclinical studies using synthetic mRNA have been conducted in various fields, including cancer immunotherapy (14–17), infectious disease vaccine delivery (18–20), immune-inflammatory diseases (21, 22) and protein-replacement therapy (23, 24). Clinical trials involved HIV vaccine design (25, 26) and treatment of cancers such as prostate cancer (27, 28), pancreatic cancer (29), colon cancer (30), melanoma (30), and acute myeloid leukemia (31, 32). These studies emphasize the expression of proteins that can recognize and eliminate malignant targets (virus, microbe, or cancer cells) (16, 17, 23, 24, 28), or pathogen-derived proteins that trigger Ag-specific immune recognition (14, 15, 18, 19, 27, 29, 30).

One area of application for synthetic mRNA that we feel has been underexploited is in the controlled expression of immunomodulatory proteins capable of recruiting and reprogramming immune effector cells to fulfill specific functions at defined body sites. In this study, we developed a "proof-of-principle" strategy to recruit and modulate host immune cells in vivo using synthetic mRNA encoding chemokines known for their ability to recruit monocytes, memory T cells, neutrophils, and NK cells (33–37). We found that circulating monocytes recruited by synthetic mRNA encoding CCL3 retained a nonprogrammed state, without adopting either a bactericidal or tissue-repairing phenotype. Most significantly, however, the addition of synthetic mRNA encoding either IFN-γ or IL-4 selectively drove the recruited monocytes to divergent polarization states. Synthetic mRNA for *Ifn-γ* activated the recruited monocytes and resident macrophages into the adoption of an antimicrobial state. In contrast, the addition of *Il-4* mRNA drove the cells toward expression of markers of alternative activation or wound-healing behavior. The data provide a new perspective into how synthetic mRNA may be used for localized delivery to both recruit and reprogram immune effector cells with the capacity to adopt polar opposite behaviors to either increase microbial killing or to enhance localized tissue repair.

## Materials and Methods

### *Mice*

C57BL/6J mice and CD45.1$^+$ mice (B6.SJL-Ptprc$^a$ Pepc$^b$/BoyJ) were obtained from the Jackson Laboratory and housed under pathogen-free

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The online version of this article contains supplemental material.

Abbreviations used in this article: BMDM, bone marrow–derived macrophage; iNOS, inducible NO synthase; IVT, in vitro–transcribed mRNA; 5meC, 5-methylcytidine; 5moC, 5-methoxycytidine; 5moU, 5-methoxyuridine; m1Y, N-1-methyl pseudouridine; NCBI, National Center for Biotechnology Information; rCCL3, recombinant CCL3; UTR, untranslated region; VR, Viromer Red.

conditions. All experiments were approved by Cornell University Institutional Animal Care and Use Committee.

### *Tissue culture cells*

HeLa cells, Vero cells and J774 cells were purchased from American Type Culture Collection. Bone marrow–derived macrophages (BMDMs) were generated from hematopoietic stem cells isolated from CD45.1$^+$ or CD45.2$^+$ mice bone marrow and maintained in DMEM (Corning Cellgro) containing 10% FBS (Thermo Fisher Scientific), 10% L929-cell conditioned media (containing M-CSF), 2 mM L-glutamine, 1 mM sodium pyruvate, and antibiotics (penicillin/streptomycin) (Corning Cellgro) at 37°C in a 5% CO2 incubator. After 6 d culture, cells were either split for further expansion or frozen with FBS containing 10% DMSO in liquid nitrogen for later use (38).

### *In vitro–transcribed mRNA synthesis and transfection*

In vitro–transcribed mRNA (IVT) mRNAs encoding for mouse CCL2 (NM_011333; National Center for Biotechnology Information [NCBI]), CCL3 (NM_011337; NCBI), IFN-γ (NM_008337.4; NCBI), and IL-4 (NM_021283.2; NCBI) were synthesized from template plasmids (Life Technologies) containing a T7 promoter, followed by a Kozak consensus sequence, the gene sequence of interest, and the mouse α-globin 3′ untranslated region (UTR). A NotI restriction site was inserted following the 3′ UTR sequence, and plasmids were linearized overnight. The digested DNA template was purified and used for in vitro mRNA transcription (Cellscript). A 2'-*O*-methyl cap-1 structure and poly(A) tail were added enzymatically using the manufacturer's instructions, which increases their stability and expression efficiency. mRNAs were synthesized with a complete substitution of uridine to N-1-methyl pseudouridine (m1Y) (TriLink BioTechnologies), which has been shown to increase protein expression and decrease innate immune activation.

(5). mRNA was treated with Antarctic phosphatase (New England Biolabs) for 30 min to remove residual triphosphates before a final RNA purification step using an RNeasy Midi kit (Qiagen). Finally, all mRNAs were quantified on a NanoDrop 2000 (Thermo Fisher Scientific) and stored frozen at –80°C.

For delivery of synthetic mRNA, 5 × 10$^5$ J774 or Vero cells seeded in 24-well plates or 2 × 10$^6$ BMDMs (freshly cultured or thawed from frozen stocks) seeded in 6-well plates were transfected with mRNA using Viromer Red (VR) transfection reagent following manufacture's procedure. Briefly, 2 μg of mRNA per 1 × 10$^6$ cells were diluted in transfection buffer and incubated with VR particle for 20 min before being added to the cells. At indicated time points, the supernatant was collected for ELISA. J774 or Vero cells were fixed for immunofluorescence. In all experiments involving i.p. injection of cells, BMDMs (1 × 10$^6$ cells per mouse) were scraped and washed before injection to animals i.p. For direct injection of naked mRNA to animals, mRNAs were diluted in transfection buffer and incubated with or without VR particles for 20 min before injection to the animals.

### *Optimization of chemical modifications of mRNA*

mRNAs with modification of 5-methylcytidine (5meC), pseudouridine (Y), m1Y, 5-methoxycytidine (5moC), 5-methoxyuridine (5moU), and combinations between these modifications were synthesized by TriLink.

A total of 1 × 10$^5$ HeLa cells were plated in 24-well plates the day prior to transfection with 200 ng of mRNA and 1 μl of Lipofectamine 2000 per well in duplicate wells per construct and time point. At 12 h posttransfection, cells were harvested and GFP expression was detected by flow cytometry and luciferase by Promega luciferase assay systems. For stress granule formation assays, the same transfection conditions were used; cells were fixed in 4% paraformaldehyde, permeabilized with Triton X-100, and stained for G3BP (61126; BD Biosciences), a known stress granule marker. Quantitative PCR was performed using quantitative PCR primers (Qiagen) for IFN-β and IL-6, with and without the addition of C16 (2 μM; Sigma-Aldrich) or 2AP (10 mM; Sigma-Aldrich), both of which are PKR inhibitors.

### *Immunofluorescence*

Cells were plated onto no. 1.5 glass coverslips the day before use. At the indicated time points, cells were fixed with 4% paraformaldehyde for 10 min before permeabilization with 0.2% Triton X-100 for 5 min at room temperature (Sigma-Aldrich). Cells were then blocked in 10% donkey serum and 5% BSA for 30 min at 37°C before incubation with primary anti-GFP Ab (8 μg/ml, a11122; Life Technologies) for 30 min at 37°C. Cells were then washed with PBS and incubated with AlexaFluor 488–conjugated secondary Ab (Jackson ImmunoResearch) for 30 min at 37°C. Nuclei were stained with DAPI (Life Technologies) for 5 min and mounted onto glass slides with Prolong gold.

### *Collection of peritoneal exudate cells and peritoneal fluid*

To collect peritoneal exudate cells and peritoneal fluid, sacrificed mice were injected with 10 ml of cold PBS + 5% FBS i.p. without pulling out the syringe. The abdomen of the animals was massaged a few times before withdrawing liquid. The withdrawn fluid was centrifuged at 1000 G to separate the peritoneal exudate cells and the peritoneal fluid.

### *ELISA*

A total of 5 × 10$^5$ J774 or Vero cells were plated into each well of a 24-well plate. After incubating overnight, cells were transfected with 1 μg of either CCL2 or CCL3 mRNA or were incubated with 10 μg/ml LPS or control media for 24 h. Cell supernatants were collected and centrifuged at 10$^4$ G for 10 min to clear the supernatant. Supernatants or peritoneal fluid from different mice was diluted and tested by ELISA kits for mouse CCL2 (MJE00; R&D), CCL3 (MMA00; R&D), IFN-γ (MIF00; R&D), and IL-4 (M4000B; R&D).

### *Isolation of monocytes from bone marrow of CD45.1$^+$ mice and adoptive transfer*

Procedure followed the instruction of monocyte isolation kit from Miltenyi Biotec (130-100-629). Briefly, bone marrow from two CD45.1$^+$ mice was isolated from femur and tibias. These bone marrow cells were labeled with magnetic beads and passed through an LS column. The monocyte-enriched fraction was injected into CD45.2$^+$ mice (1 × 10$^6$ cells per mouse) through a retro-orbital route.

### *Flow cytometry*

For surface staining, cells were washed with PBS and blocked with PBS plus 5% FBS, 2.5% mouse serum, and 0.5% anti–Fcγ III/II (anti-CD16/32) for 15 min. Cells were washed with PBS and incubated with viability dye (1:500; eBioscience) and Abs (1:200) for 15 min. Cells were washed with PBS and analyzed by LSR II (BD Biosciences). Flow cytometric data were further analyzed with FlowJo software (Tree Star).

For intracellular staining, cells were incubated with Brefeldin A (1:1000; eBioscience) in RPMI 1640 medium plus 10% FBS in 37°C for 4–5 h. Then cells were washed, blocked, and stained according to the intracellular staining protocol (eBioscience). Cells were washed with PBS and analyzed by BD LSR II.

Abs used in this paper include Ly6C-allophycocyanin Cy7, Ly6C-allophycocyanin, F4/80-PE, F4/80-e450, Ly6G 1A8-PE Cy7, Ly6G 1A8-FITC (BD), CD45.2-allophycocyanin, CD45.2-AF700, CD45.1-PE, CD45.1-AF700, CD3-e450, NK1.1-AF780, CD11b-PerCP Cy5.5 (Becton Dickinson), CD206-PE, Arginase1-PE (R&D Systems), and Anti-mouse Relm-α Ab (PeproTech). Abs were from eBioscience unless stated otherwise.

### *Detecting intracellular superoxide burst by OxyBURST assay*

Preparation of OxyBURST reporter beads is described previously (39). For the 96-well plate assay, a 10-μl suspension of beads (a bead-to-cell ratio of 2:1) was added to each well and the plate was agitated immediately to distribute the beads. The plate was transferred to a plate reader (PerkinElmer) and analyzed for 2 h. For flow cytometry, a 100-μl suspension of beads (a bead-to-cell ratio of 2:1) was added to each well of a six-well plate and the plate was agitated immediately. At indicated time points, the cells were washed, fixed by 4% PFA, and stained for analysis by flow cytometry.

### *Statistics*

Two-tailed unpaired Student *t* test with Welch correction and one-way ANOVA with Tukey multiple comparison tests were conducted in Prism (GraphPad). All experiments were repeated at least twice. Number of mice used in each experiment is indicated in figures.

## Results

### *Expression of CCL2 and CCL3 in vitro using synthetic mRNA*

Prior to initiating macrophage transfection experiments, we used permissive HeLa cells to explore different modifications of our synthetic mRNAs to optimize expression and stability and to minimize their cellular activation properties. mRNAs were synthesized with chemical modifications, including 5′ capping, alteration of

5′ and 3′ UTRs, coding region optimization, and 3′ poly(A) tail elongation (detailed procedures are included in *Materials and Methods*). Previous studies had shown that mRNA synthesized with substitution of uridine with m1Y increases protein expression and reduces innate immune activation (5, 6). We tested the impact of a variety of naturally occurred mRNA modifications on protein expression and immune activation, including 5meC, pseudouridine (Y), m1Y, 5moC, and 5moU using mRNAs encoding GFP and luciferase as readouts. We found that mRNA with modification of m1Y yielded the highest GFP and luciferase production (Fig. 1A), whereas the percentage of transfected cells was similar across all chemical modifications. Moreover, we assessed the degree of immune activation of different mRNA modifications by detecting stress granule formation. We observed significantly lower stress granule production with m1Y modification for mRNAs encoding GFP and luciferase (Fig. 1B), indicating that mRNAs with m1Y modification minimizes perturbation of the target cells. The reduced activation shown by the m1Y modification was also reflected in reduced production of IFN-β and IL-6 transcripts (Fig. 1C). We found that PKR inhibitors (2AP and C16) can further reduce the production of IFN-β and IL-6 transcripts (Fig. 1C); however, the reduction was minimal and not considered biologically significant. In all subsequent experiments we synthesized our mRNAs with the m1Y substitution.

Upon delivery of synthetic mRNA encoding GFP to HeLa cells, we observed GFP expression as early as 5 h posttransfection. The GFP signal peaked between 8 and 24 h posttransfection and lasted for 72 h (Supplemental Fig. 1A). Interestingly, we found that macrophages, traditionally thought of as resistant to transfection, were very effective at expressing foreign genes when delivered in the form of synthetic mRNA. The murine macrophage-like cell

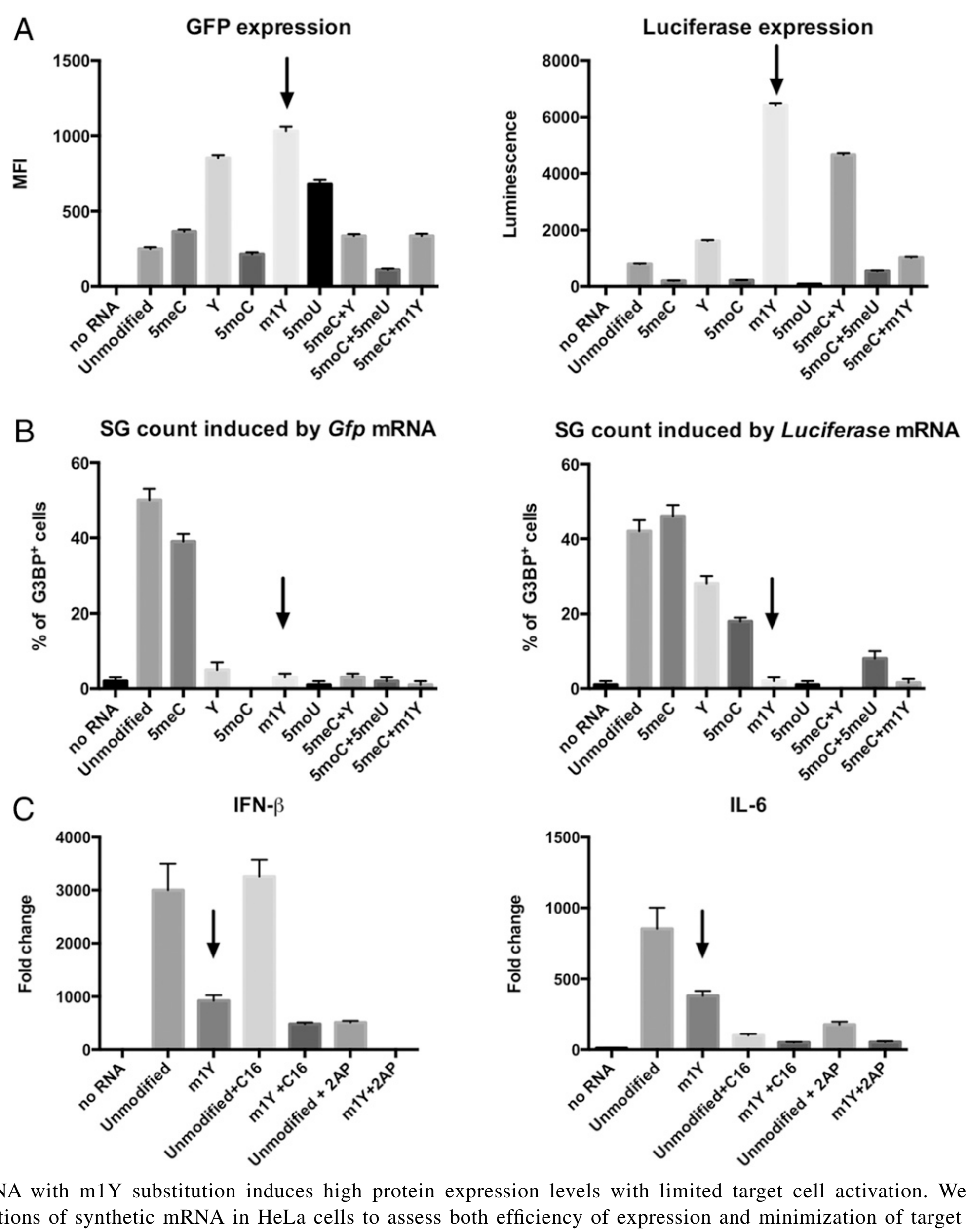


**FIGURE 1.** mRNA with m1Y substitution induces high protein expression levels with limited target cell activation. We explored different chemical modifications of synthetic mRNA in HeLa cells to assess both efficiency of expression and minimization of target cell activation. (**A**) Expression of GFP and luciferase by mRNAs with different chemical modifications (5meC, Y, 5moC, m1Y, 5moU, and combinations of these modifications) in HeLa cells. (**B**) Percentage of G3BP$^+$ HeLa cells (contain stress granules) following transfection with mRNAs with different modifications. (**C**) Expression of IFN-β and IL-6 in HeLa cells when transfected with *Gfp* mRNAs with different modifications as demonstration of immune activation.

line, J774, was transfected with *Gfp* mRNA, and ~70% of the cells expressed GFP protein 24 h posttransfection (Fig. 2A).

To explore the potential for manipulating tissue responses we decided to focus on mRNAs encoding chemokines known to stimulate recruitment of immune effector cells (33–37). CCL2 (MCP1) and CCL3 (MIP1α) recruit different immune cells including monocyte, memory T cell, neutrophil and NK cell. We transfected the J774 cells with *Ccl2* or *Ccl3* synthetic mRNAs and verified the presence of CCL2 or CCL3 in the medium by ELISA 24 h posttransfection (Fig. 2B). Although both mRNAs were expressed, we focused primarily on *Ccl3* synthetic mRNA owing to its more sustained expression (Fig. 2B).

### *Monocyte recruitment by CCL3 expressed from synthetic mRNA*

To demonstrate that CCL3 expressed from synthetic mRNAs was functionally active and could be generated in sufficient amounts to modify cell behavior, we developed an experimental cell recruitment assay. We transfected murine BMDMs with *Ccl3* mRNA and injected these cells into the peritoneal cavity of mice (Fig. 3A). We used congenic CD45.1 and CD45.2 mice pairs to discriminate between the *Ccl3* mRNA–transfected BMDMs and the recipient mouse cells present in the peritoneum (Supplemental Fig. 1B). We reasoned that the peritoneal cavity was a suitable site for ease of sampling for subsequent analysis of both cell recruitment and cellular phenotype. BMDMs from CD45.1$^+$ mice were transfected with *Ccl3* mRNA using VR particles and cultured for 8 h prior to inoculation into the peritoneum. Peritoneal exudate cells and fluid were collected 16 h postinoculation with the *Ccl3* mRNA–transfected BMDMs. The presence of CCL3 protein was confirmed in both the tissue culture medium from the transfected BMDMs prior to their inoculation, as well as in peritoneal fluid from mice injected with the transfected BMDMs (Fig. 3B).

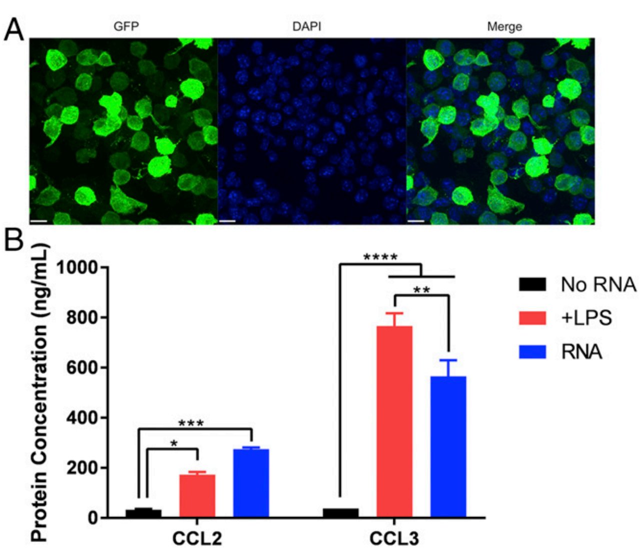


**FIGURE 2.** J774 cells transfected with synthetic mRNA encoding GFP, CCL2, or CCL3, respectively. We examined the expression of synthetic mRNAs encoding GFP, CCL2, and CCL3 in the murine macrophage-like cell line J774. (**A**) Cells were transfected with 1 μg of *Gfp* mRNA and fixed 24 h posttransfection. Cells were stained for GFP (green) and nuclei were stained with DAPI (blue). Scale bar, 12 μm. (**B**) CCL2 and CCL3 were detected by ELISA in in tissue culture supernatants following transfection of cells with 1 μg of either *Ccl2* or *Ccl3* mRNA (blue) or following incubation with 10 μg/ml LPS for 24 h. Scale bars represent SD. Data represent mean of two independent experiments. Statistical significance was measured by two-way ANOVA. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

The majority of phagocytes in an unperturbed peritoneal cavity are resident peritoneal macrophages that express high levels of F4/80 (Supplemental Fig. 2B). In contrast, in the mice receiving transfected BMDMs expressing CCL3, we observed an increase in monocytes expressing high or intermediate levels of Ly6C and low levels of F4/80 (Fig. 3C, 3D, Supplemental Fig. 2A, 2B). This suggested that these monocyte populations were distinct from F4/80$^{high}$, resident peritoneal macrophages. Interestingly, the number of F4/80$^{high}$ peritoneal macrophages and their F4/80 expression was markedly decreased in these mice (Fig. 3C, 3D, mental Fig. 2C). This apparent "disappearance" of resident peritoneal macrophages has been reported previously upon inflammation induced by i.p. inoculation of thioglycolate broth, LPS, or bacteria (40–42). The total numbers of peritoneal exudate cells, the F4/80$^{low}$Ly6C$^{low}$MHCII$^+$ peritoneal macrophages, and Ly6C$^{low}$ monocytes recovered from the peritoneal cavity were similar between the mice receiving *Ccl3* mRNA–transfected BMDMs and mice in the control groups (Supplemental Fig. 2D). Moreover, the numbers of donor cells recovered 16 h postinjection are similar between groups and were mostly dead (Supplemental Fig. 2D). To demonstrate that the recruitment was specific to CCL3-encoding mRNA, we inoculated the peritoneal cavities of mice with BMDMs transfected with uncoated VR particles, VR particles coated with *Gfp* mRNA, and VR particles coated with *Ccl3* mRNA. Although we did observe modest cellular recruitment to VR alone and *Gfp* mRNA–coated VR, consistent with minimal immune activation, there was a substantial increase in the Ly6C$^{int}$ monocyte population that was specific to the *Ccl3* mRNA (Fig. 3E). This indicates that cellular recruitment phenomenon is a function specific to the CCL3-encoding mRNA.

Previous studies reported that i.p. injection of recombinant CCL3 (rCCL3) triggers recruitment of neutrophils, detectable at 2 h and peaking at 8 h postinoculation (21). Injection of rCCL3 into an air pouch animal model also drove monocyte recruitment (22). To examine the differences between the delivery of a bolus of rCCL3 protein versus the sustained localized expression from *Ccl3* mRNA, we compared the activities of rCCL3 and BMDMs transfected with *Ccl3* mRNA in the peritoneal cavity at 4 and 16 h (Supplemental Fig. 2E, 2F). As previously reported, rCCL3 injection recruited neutrophils at 4 and 16 h. In comparison, injection of *Ccl3* mRNA–transfected BMDMs did result in neutrophil recruitment at 4 h but not at 16 h (Supplemental Fig. 2E, 2F). In contrast, rCCL3 did not recruit monocytes either at 4 or 16 h, whereas synthetic *Ccl3* mRNA–transfected BMDMs exhibited marked monocyte recruitment at both time point (Supplemental Fig. 2E, 2F). The data indicate that there are both quantitative and qualitative differences between delivery of a single dose of recombinant protein versus the sustained local expression of the chemokine CCL3.

### *Monocytes are recruited through blood stream to the peritoneal cavity in a nonactivated state*

Upon acute inflammation or pathogen infection, monocytes are recruited via the blood stream to peripheral tissues in response to various inflammatory cytokines and chemokines, such as CCL3 (36, 43, 44). To verify that the increased Ly6C$^+$ monocyte populations in the peritoneal cavity were indeed recruited from the blood stream, we isolated monocytes from CD45.1$^+$ mice and injected them i.v. to CD45.2$^+$ mice that were subsequently inoculated with BMDMs transfected with VR particles with or without *Ccl3* mRNA. We observed an increase in CD45.1$^+$ cells in the peritoneum of mice injected with *Ccl3* transfected BMDMs, demonstrating that these cells came from the peripheral blood (Fig. 4).

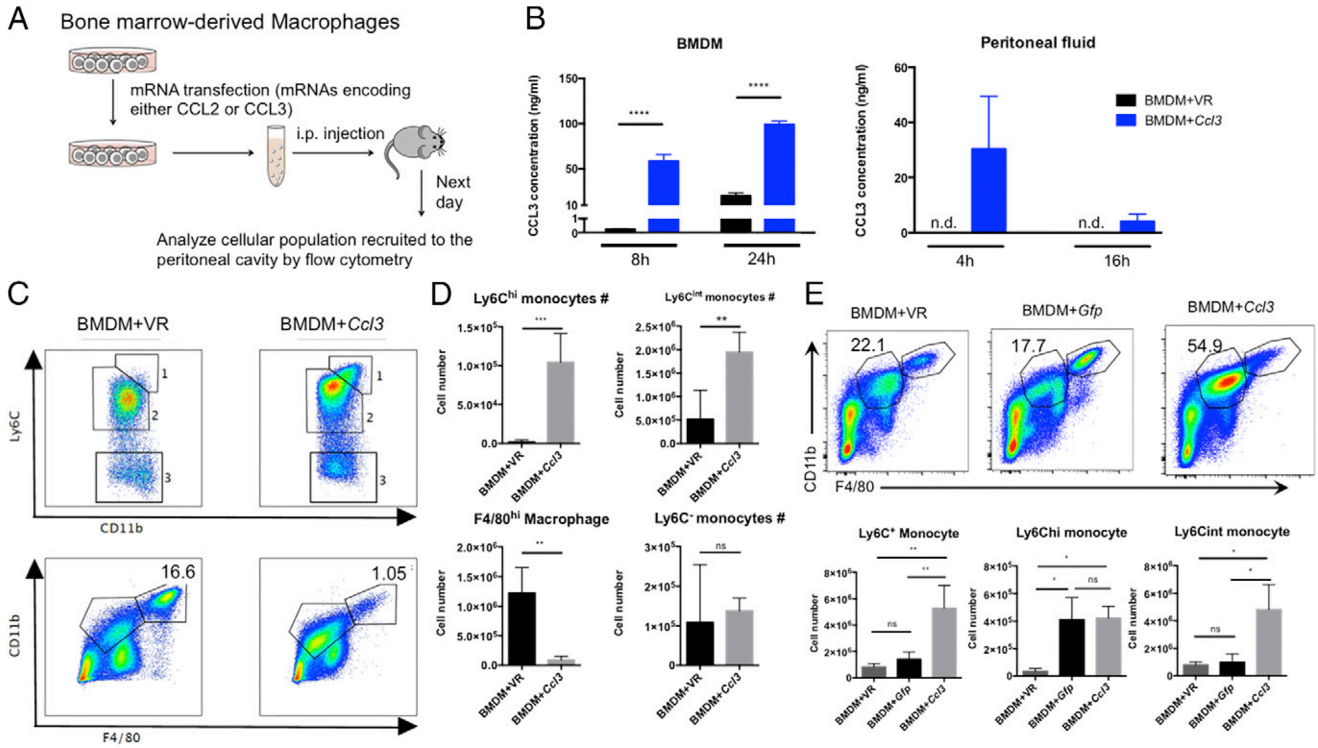


**FIGURE 3.** *Ccl3* synthetic mRNA transfected BMDM modify the cellular populations in the peritoneal cavity. We examined the biological impact of introducing BMDMs transfected with synthetic mRNA encoding CCL3 into the peritoneal cavity of mice. (**A**) Experiment setup. In brief, we transfected synthetic mRNAs encoding either CCL2 or CCL3 into BMDM generated from CD45.1$^+$ congenic mice. As control, we transfected BMDM with VR without mRNA. Eight hours after transfection, the cells were harvested, washed with PBS, and injected i.p. into CD45.2$^+$ congenic mice. Sixteen hours later, we collected the cells from the peritoneal cavity and analyzed them by flow cytometry. The use of the congenic CD45.1$^+$/CD45.2$^+$ mouse strains enabled us to discriminate between the donor BMDM transfected with the chemokine mRNAs and the resident tissue macrophages. (**B**) Demonstration of the presence of CCL3 protein in the supernatant from BMDMs transfected with *Ccl3* mRNA, as well as in the peritoneal fluid from mice inoculated with these BMDMs. (**C**) *Ccl3* synthetic mRNA–transfected BMDM recruited a population of Ly6C$^{hi}$CD11b$^+$ monocytes (Gate 1), increased the number of Ly6C$^{int}$CD11b$^+$ monocytes (Gate 2), and decreased the number of F4/80$^{hi}$ large peritoneal macrophages. Experiments were repeated three times. Data showed representative flow plot from one experiment. (**D**) The numbers of Ly6C$^{hi}$ and Ly6C$^{int}$CD11b$^+$ monocytes, F4/80$^{hi}$ large peritoneal macrophage, and Ly6G$^+$ neutrophil were quantified. (**E**) As an additional control to demonstrate the recruitment phenotype was due to CCL3-encoding mRNA, we compared the levels of cellular recruitment to VR alone, VR coated with *Gfp* mRNA, and VR coated with *Ccl3* mRNA. The recruitment of the cell population of significance, the Ly6C$^{int}$ CD11b$^+$ monocytes was clearly specific to the presence of the *Ccl3* mRNA. Data represent mean ± SD. ns: $p > 0.05$, $**p < 0.01$, $***p < 0.001$, unpaired $t$ test with Wench correction.

In acute inflammatory challenge models, such as thioglycollate broth injection, monocytes are recruited by a complex mix of inflammatory cytokines and chemokines that both recruit cells and drive them into an inflammatory, or M1, activation state (36, 45–49). We therefore wanted to determine the activation state of the monocytes recruited by BMDM transfected with synthetic *Ccl3* mRNA. We examined the expression levels of the inducible NO synthase (iNOS) and RELM-α proteins to determine whether the recruited monocytes had acquired either a classically activated (M1) or alternatively activated (M2) phenotype. Intriguingly, we found that very few monocytes recruited through CCL3 activity expressed either iNOS or RELM-α (Fig. 5A, 5B, upper panels). The data indicate that in the absence of additional stimuli, the recruited monocytes adopted neither bactericidal nor tissue-repairing phenotypes but remained "unprogrammed". This result suggests that CCL3 is responsible for immune cell recruitment, but without additional signals, it is not involved in the activation or polarization of the recruited cells. We also examined the levels of surface expression of MHC class II, CD80, and CD86 on recruited monocytes, as well as on resident macrophages and neutrophils. Again, the ectopic expression of *Ccl3* mRNA in donor BMDMs did not increase the expression of these markers on the cell types examined, with the exception of a marginal increase in CD80 expression on recruited monocytes (Supplemental Fig. 3).

*Recruited monocytes can be reprogrammed by coexpression of additional synthetic mRNAs*

We next asked if it were feasible to specifically drive these nonactivated monocytes toward M1 and M2 states of activation. We designed and synthesized two synthetic mRNAs that encoded *Ifn-γ* and *Il-4* transcripts, respectively. The expression of the mRNAs was validated through the detection of IFN-γ and IL-4 in the supernatant following their transfection into Vero cells (Supplemental Fig. 4A). The *Ifn-γ* or *Il-4* mRNAs were mixed with the *Ccl3* mRNA and transfected into CD45.1$^+$ BMDM and then injected into CD45.2$^+$ mice peritoneum. IFN-γ and IL-4 expression was detected in the peritoneal fluid (Supplemental Fig. 4B). Moreover, *Ifn-γ* mRNA–transfected CD45.1$^+$ BMDMs recovered from the peritoneum were actively expressing IFN-γ (Supplemental Fig. 4C). Phenotypic characterization of the peritoneal exudate cells revealed a marked increase in the number of iNOS-positive monocytes recruited in response to synthetic mRNAs encoding *Ccl3* and *Ifn-γ* (~30-fold above the controls) (Fig. 5A, 5B, upper panels). We also observed a similar increase in the number of RELM-α positive monocytes recruited by the *Ccl3* and *Il-4* mRNA combination (~20-fold above the controls), (Fig. 5A, 5B, upper panels). RELM-α is an accepted marker for M2 polarization of macrophages (50, 51).

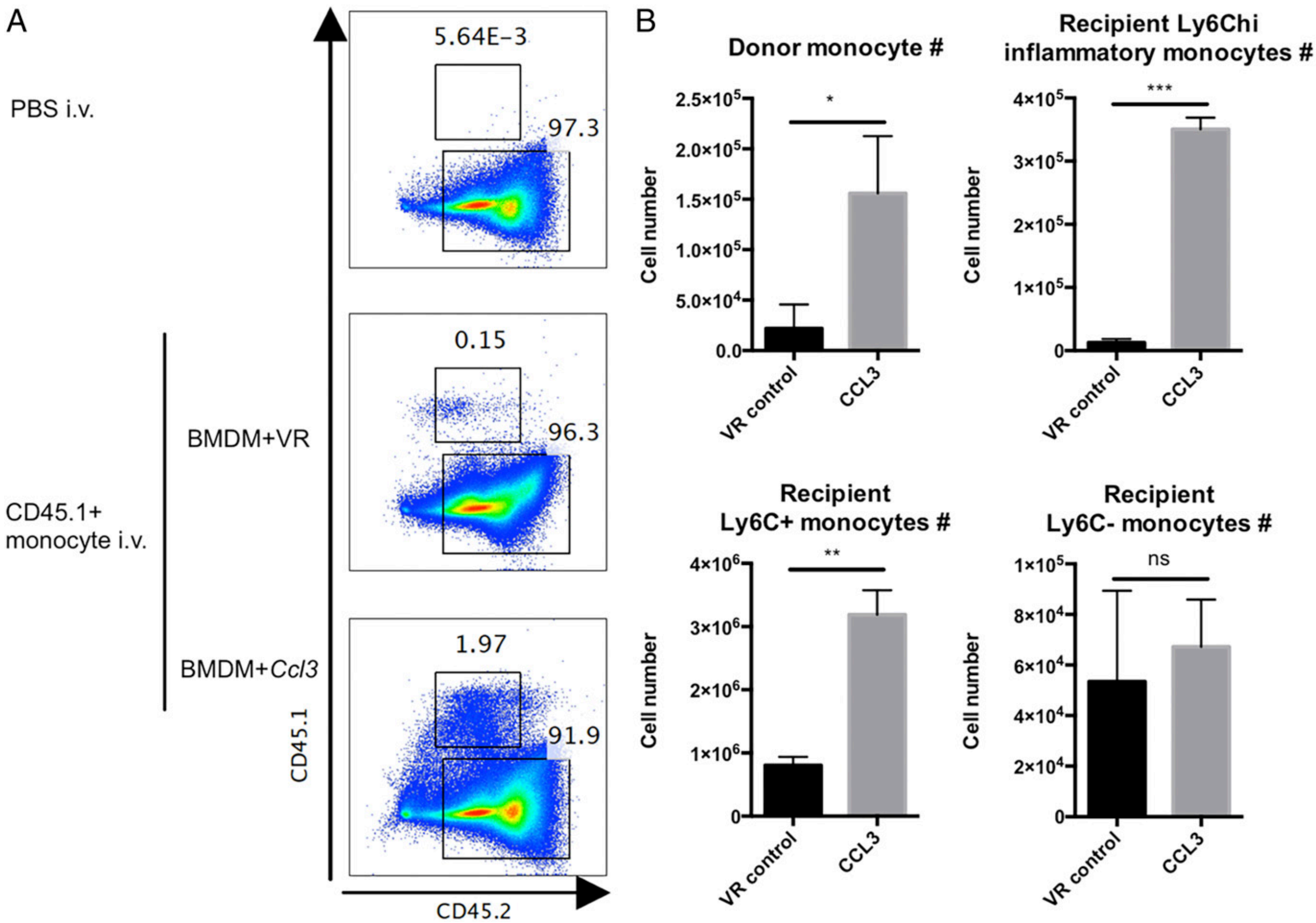


**FIGURE 4.** Peripheral blood–derived monocytes are recruited to the peritoneal cavity. We demonstrate that the cells recruited into the peritoneal cavity in response to CCL3 had their origin in the blood. (**A**) Representative flow plots showing mice in which peripheral blood monocytes were isolated from CD45.1$^+$ mice and transferred i.v. ($1 \times 10^6$ monocytes transferred per mouse) to CD45.2$^+$ mice. These CD45.2$^+$ mice were injected i.p. with BMDMs transfected with or without *Ccl3* mRNA and the presence of recruited CD45.1$^+$ monocytes in the peritoneal cavity was assessed. Experiments were repeated two times. (**B**) The number of donor CD45.1$^+$ monocytes, Ly6C$^{hi}$, Ly6C$^{int}$ and Ly6C$^{low}$ monocytes were quantified. Data represent mean ± SD. ns: $p > 0.05$, *$p < 0.05$, ***$p < 0.001$, unpaired *t* test with Wench correction.

Unsurprisingly, the expression of IFN-γ and IL-4 by the BMDMs inoculated into the peritoneum also affected the resident macrophage population (F4/80$^{high}$CD11b$^+$), which adopted M1 or M2 phenotypes, respectively. The iNOS-positive resident macrophages increased by ~50 fold in response to the BMDMs transfected with synthetic *Ccl3* and *Ifn-γ* mRNAs (Fig. 5A, 5B, lower panels), whereas the number of RELM-α positive resident macrophages increased by ~40 fold in response to *Ccl3* and *Il-4* mRNA-transfected BMDMs (Fig. 5A, 5B, lower panels).

Further characterization of the peritoneal exudate cells demonstrated that the resident macrophages and recruited monocytes from mice receiving BMDMs transfected with *Ccl3* and *Ifn-γ* mRNA also upregulated MHC class II expression (Supplemental Fig. 3). Surface expression of CD80 was increased in recruited monocytes but not in resident macrophages or neutrophils (Supplemental Fig. 3). *Ccl3* and *Il-4* mRNA had little effect on the expression of the panel of surface markers that we used for cell typing (Supplemental Fig. 3).

*Functional demonstration of phagocyte activation*

To provide a functional assessment of the polarization state of the phagocytes, we quantified the magnitude of the superoxide burst, which had been shown previously to be amplified in IFN-γ–activated macrophages (39). Carboxy-methylated silica beads were covalently modified with BSA-Oxyburst-SE and a calibration fluorochrome to quantify phagosomal NADPH oxidase activity. We confirmed the utility of the assay by demonstrating that treatment of BMDM with rIFN-γ led to increased production of reactive oxygen species, whereas exposure to rIL-4 reduced oxygen radical production in BMDM (Fig. 6A).

We harvested the peritoneal exudate cells from the mice injected with BMDM transfected with synthetic mRNA, plated the cells in 96-well plates, and added the Oxyburst reporter beads. Peritoneal exudate cells from mice injected with *Ccl3*-transfected BMDM showed levels of reactive oxygen production comparable to those from control mice. However, the peritoneal exudate cells from mice injected with *Ccl3* and *Ifn-γ* mRNA transfected BMDM exhibited increased production of oxygen radicals, whereas the peritoneal exudate cells from mice injected with *Ccl3* and *Il-4* mRNA transfected BMDM exhibited reduced reactive oxygen production (Fig. 6B).

To further validate this phenotype at the level of the individual cells, we incubated the peritoneal exudate cells with Oxyburst reporter beads in 96-well plates. At indicated time points, cells were fixed and analyzed by flow cytometry. We found that recruited monocytes from mice injected with *Ccl3* and *Ifn-γ* mRNA transfected BMDM showed elevated levels of reactive oxygen species as early as 10 min after addition of reporter beads (Fig. 6C, 6D). This elevated level of superoxide burst in the recruited monocytes from mice injected with *Ccl3* and *Ifn-γ*

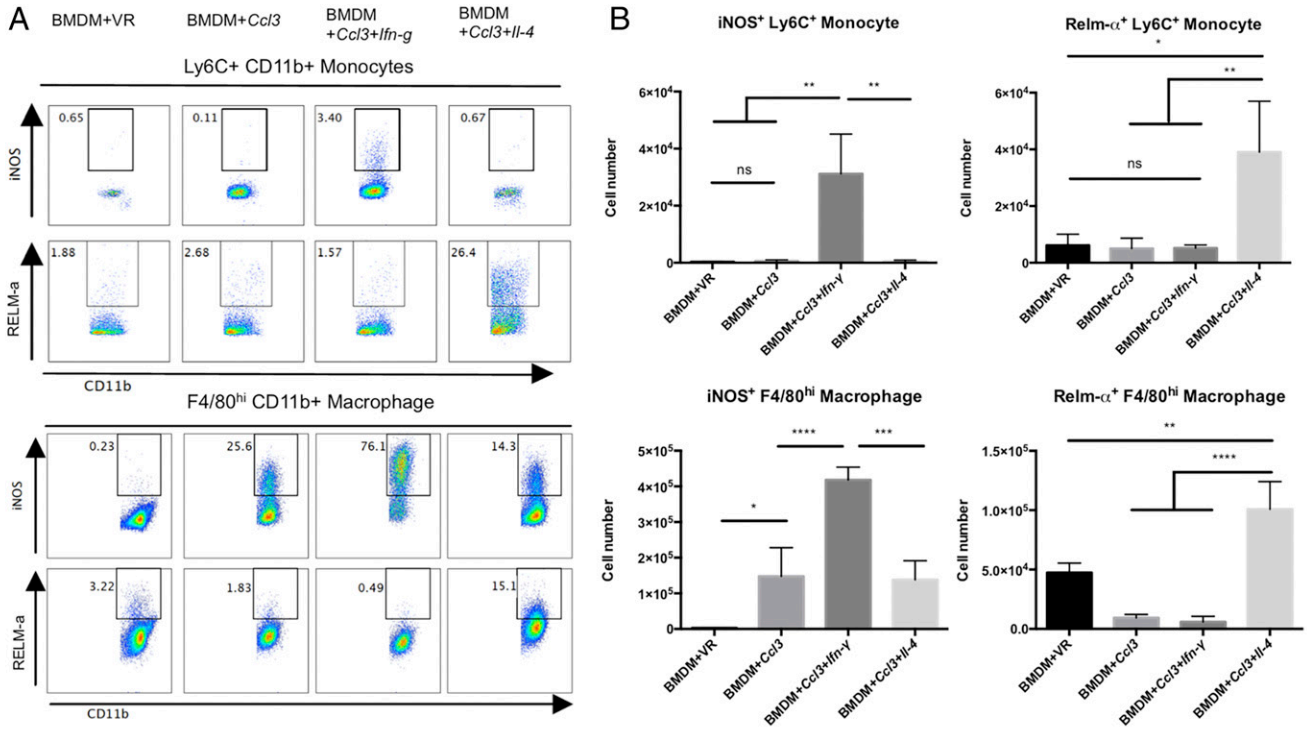


**FIGURE 5.** Cotransfection of *Ifn-γ* or *Il-4* synthetic mRNA, in combination with *Ccl3* mRNA reprograms both resident and recruited cell phenotypes. To demonstrate that the recruited cells can be polarized toward M1- and M2-like macrophage phenotypes, we cotransfected BMDMs with cytokine-encoding mRNAs. (**A**) Representative flow plots showing the expression of iNOS and RELM-α in recruited monocytes ($Ly6C^+CD11b^+$, upper panel) and in large peritoneal macrophages ($F4/80^{hi}CD11b^+$, lower panel) following their induction of *Ccl3* mRNA in combination with *Ifn-γ* or *Il-4* mRNA. (**B**) The numbers of $iNOS^+$ or RELM-$\alpha^+$ recruited monocytes and $F4/80^{hi}$ macrophages were quantified and shown to increase in the presence of appropriate cytokine-encoding mRNAs Experiments were repeated three times with comparable results. Data represent mean ± SD. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$, one-way ANOVA with Tukey multiple comparison test.

mRNA transfected BMDM was sustained throughout the duration of the experiment (Fig. 6C, 6D).

The data confirm that whereas CCL3 alone recruits monocytes in a relatively nonactivated state, coexpression of other synthetic mRNAs can be exploited to reprogram the cells to adopt specific physiologically states associated with either microbicidal activity or wound healing.

### *Naked mRNA complexed with VR particles induces comparable monocyte recruitment*

In our initial experiments we deliberately used ex vivo transfection of BMDMs so we could effectively control and track the source of chemokines and cytokines and discriminate between the effector and responder cell populations. However, to have therapeutic potential we need to demonstrate that direct delivery of synthetic mRNA to a body site induces a comparable response. In these experiments we inoculated PBS, uncoated VR particles, naked *Ccl3* mRNA, VR particles coated for *Gfp* mRNA, and VR particles coated with *Ccl3* mRNA directly into the peritoneum. Of the GFP-positive cells recovered from the peritoneal cavity at time of analysis, 62% of these cells were $F4/80^{hi}$ or resident peritoneal macrophages (Supplemental Fig. 4D). The inoculation of PBS, uncoated VR particles, and naked *Ccl3* mRNA all failed to induce significant recruitment of the $Ly6C^{hi}$ or $Ly6C^{int}$ monocytes (Fig. 7A–C). In contrast, i.p. injection of *Ccl3* mRNA–coated VR induced recruitment of $Ly6^{hi}$ and $Ly6^{int}$ monocytes to levels comparable to those observed with transfected BMDM (Fig. 7A–C). Furthermore, inoculation of VR particles coated with *Gfp*-encoding mRNA or *Ccl3* mRNA had minimal impact on phagocyte polarization, whereas the inoculation of VR particles coated with *Ccl3* plus *Ifn-γ* mRNA or with *Ccl3* and *Il-4* mRNA induced marked upregulation of expression of the polarization markers iNOS (*Ifn-γ*) and RELM-α (*Il-4*). Synthetic mRNA, delivered complexed to VR particles, induced phenotypes comparable to those observed following inoculation of ex vivo–transfected BMDMs. These data validate the general approach and demonstrate the utility of synthetic mRNA to induce localized and controlled modulation of immune environments in vivo.

## Discussion

Synthetic mRNA therapy is a promising therapeutic approach to deliver proteins of interest (1, 2, 10, 11). It has low risk of genomic insertional mutagenesis sometimes associated with DNA-based therapy and bypasses the necessity of nuclear delivery for expression (1, 2, 10, 11). Compared with recombinant protein therapy, synthetic mRNA can potentially maintain expression of a desired protein at physiological levels for an extended time period time period, which has the potential to reduce the frequency of administration (2, 10). Moreover, mRNA transfected cells can actively migrate to targeted tissues or organs to provide the localized release of desired proteins (1, 2, 13). Synthetic mRNA therapy has already been explored in several preclinical and clinical studies predominantly to express proteins with direct therapeutic effect or proteins to trigger an Ag-specific immune response (2, 12, 13).

In this study, we examined the potential for using synthetic mRNA in cell-based therapies to recruit effector cells and modify their behavior locally in vivo. Our findings demonstrated that *Ccl3* synthetic mRNA–transfected BMDMs were able to recruit

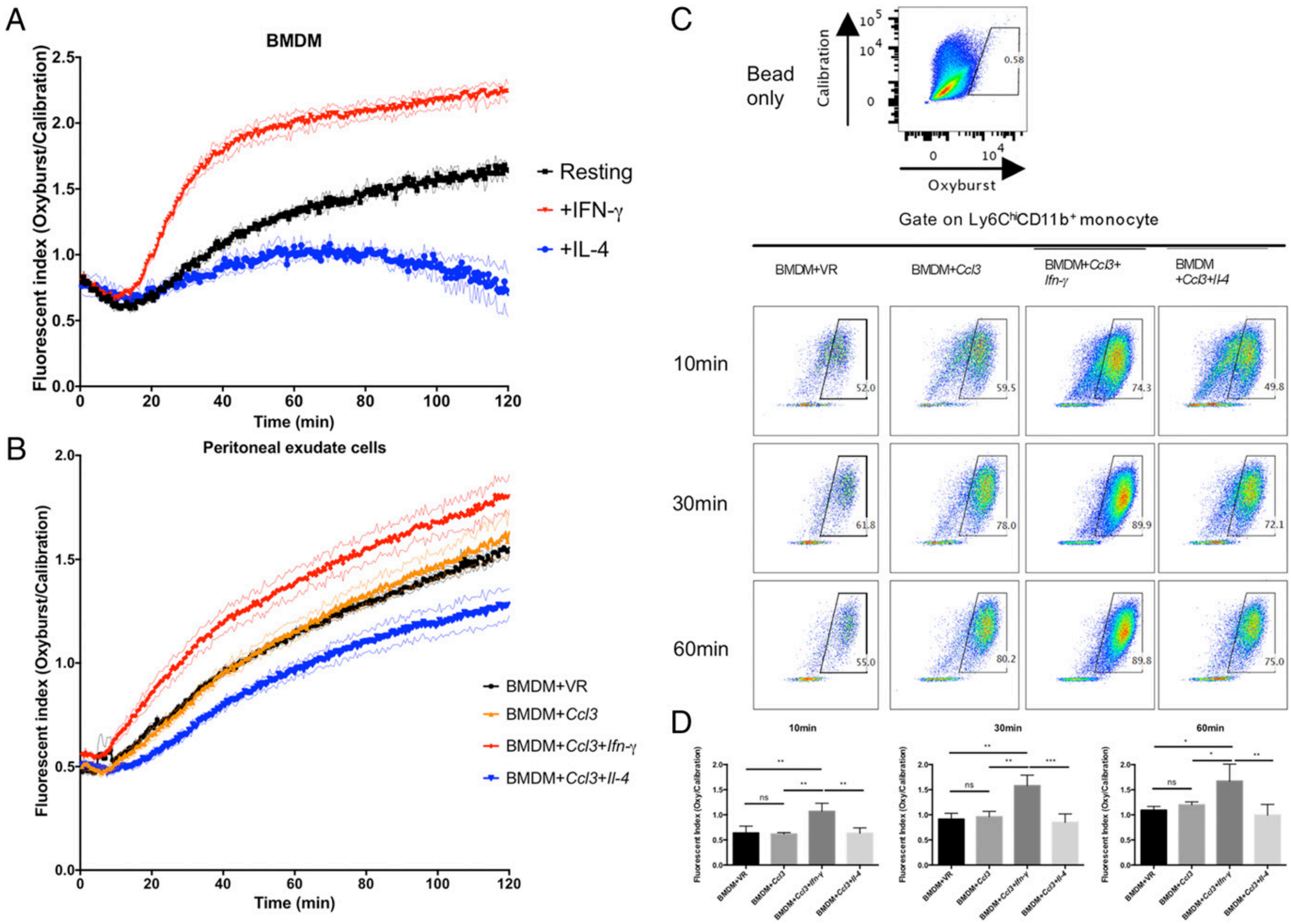


**FIGURE 6.** Quantification of NADPH Oxidase activity in the reprogrammed phagocytes correlates with the M1 or M2 activation status. To generate functional data demonstrating the polarization of the recruited macrophages we quantified the intensity of their superoxide burst. (**A**) The kinetics and magnitude of superoxide burst generated by BMDMs treated with or without recombinant IFN-γ and IL-4. Activation with IFN-γ enhanced the superoxide burst (Oxyburst beads), whereas alternative activation with IL-4 resulted in its decrease. Analysis was performed on a Perkin Elmer Envision fluorescent plate reader and the experiment was repeated three times. (**B**) The kinetics and magnitude of superoxide burst generated by peritoneal cells from mice ($n = 3$) injected with BMDMs transfected with mRNAs encoding CCL3 alone and in combination with either *Ifn-γ* and *Il-4* mRNAs. The experiment was repeated twice. (**C**) Peritoneal cells from mice ($n = 3$) injected with different mRNA transfected BMDMs were analyzed by flow cytometry. Representative flow plots showing the gating of positive Oxyburst signal. Oxyburst beads without cells served as the negative control (upper panel). The lower panels were gated on recruited monocytes (Ly6C$^+$CD11b$^+$) from four groups incubated with Oxyburst beads and harvested at different times (10, 30, and 60 min). The experiment was repeated three times. (**D**) Quantification of fluorescent index of mean Oxyburst bead fluorescent intensity. The mean Oxyburst fluorescent intensity of all the recruited monocytes that were positive for calibration Fluor were calculated. The number was divided by the mean calibration fluorescent intensity to generate a fluorescent index. The experiment was repeated three times with comparable results. Data represent mean ± SD. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, one-way ANOVA with Tukey multiple comparison test.

monocytes to the peritoneal cavity. Instead of being polarized to either a bactericidal or a tissue-repairing phenotype, the recruited monocytes remained in a relatively naive state through the migration process. Once recruited, the monocytes were responsive to both the addition of *Ifn-γ* mRNA and driven into an M1, antimicrobial state, or to *Il-4* mRNA to adopt an M2, tissue repair phenotype. These data demonstrate how synthetic mRNAs could be used in combination to expand and diversify the biological functions of both transfected and bystander cells.

Chemokines, such as CCL3, are reported to recruit neutrophils as well as monocytes (35, 52–55); however, much of these data were generated in proinflammatory models. In the current study, although we did observe transient recruitment of neutrophils, the monocytes that were recruited appeared to be predominantly in a “resting” or nonactivated state. Synthetic mRNAs have the potential to activate mammalian cells through pattern recognition receptors such as TLR 7/8, RIG-1, and NOD2, discussed by Steinle and colleagues (56). However, the modifications introduced into the synthetic mRNA used in the current study appear to minimize cellular activation. Previous work by Andries et al. (5) showed that m1Y modification increased luciferase expression and decreased production of IFN-β and CCL5 in A549 cells. We also found that m1Y, in combination with other modifications, minimized host cell activation, assessed by stress granule formation (a result of PKR activation), and reduced production of IFN-β and IL-6. Evidence that the single-stranded synthetic mRNAs themselves play a minimal role in the phenotype of the recruited and resident phagocytes is provided by the absence of activation markers in mice receiving BMDMs transfected with *Ccl3* mRNA (Supplemental Fig. 3) and in mice that received VR particles complexed with *Ccl3* mRNA (Fig. 7). The absence of an in vivo proinflammatory response to the mRNA itself is important if one envisions using this as a strategy for recruitment of immune effector cells capable of subsequent reprograming.

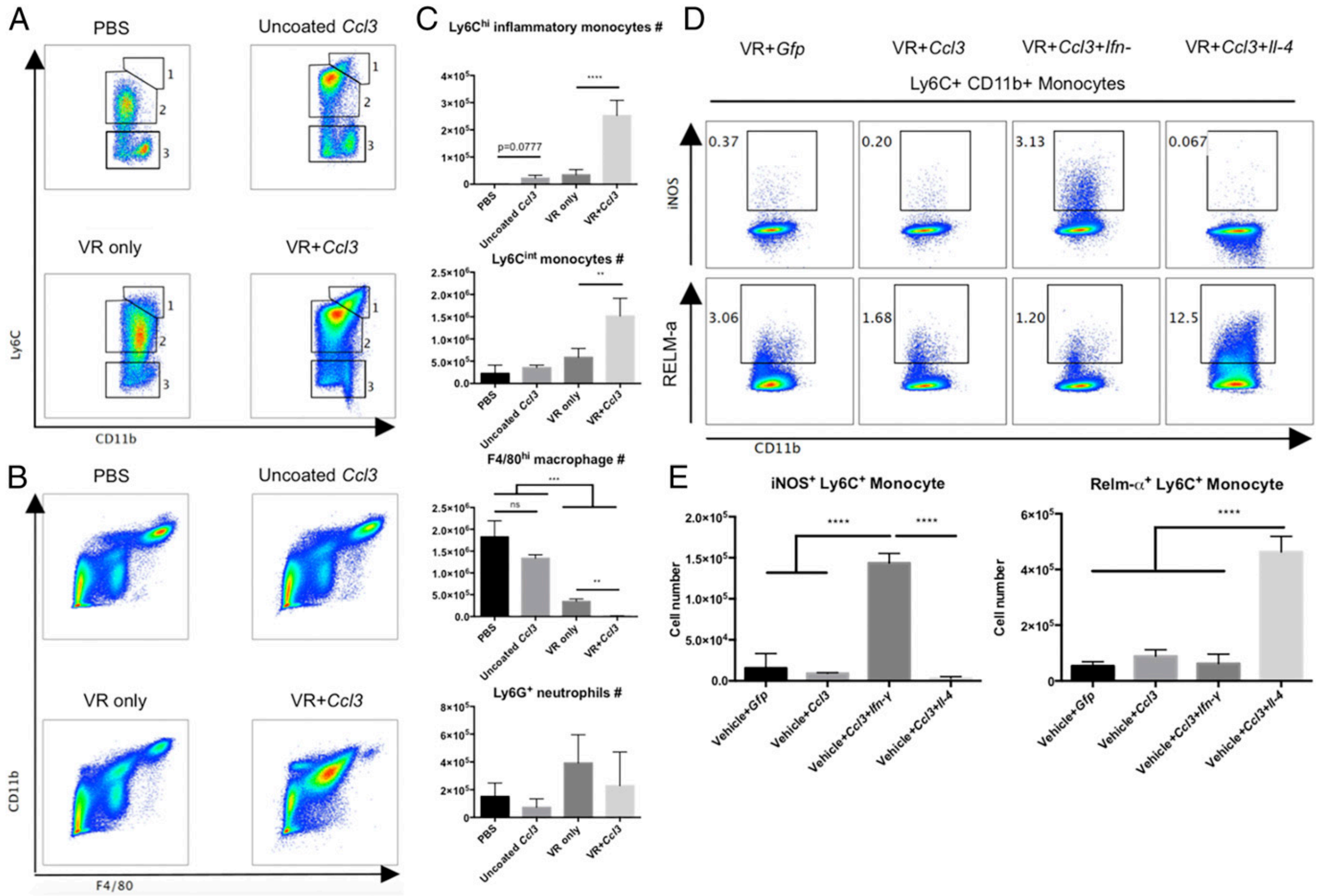


**FIGURE 7.** mRNA complexed with VR particles can recruit similar populations of monocytes and differentiate them toward different activation statues. The potential of this approach for therapeutic applications is supported by the demonstration that the mRNA-coated VR particles can mediate comparable effects following direct inoculation. (**A**) Representative flow plots of monocyte populations in the peritoneal cavity after injection of naked, *Ccl3* mRNA or *Ccl3* mRNA-coated VR particles. (**B**) Representative flow plots of F4/80$^{hi}$ large peritoneal macrophage in the peritoneal cavity after injection of naked, *Ccl3* mRNA or *Ccl3* mRNA-coated VR particles. (**C**) Quantification of the numbers of Ly6C$^{hi}$ and Ly6C$^{int}$ monocytes, F4/80$^{hi}$ large peritoneal macrophage, and Ly6G$^{+}$ neutrophil. (**D**) Representative flow plots of iNOS and RELM-α expression in these monocytes recruited by VR complexed mRNAs. (**E**) Quantification of iNOS$^{+}$ and RELM-α$^{+}$ number of monocyte recruited by VR-complexed mRNAs. Experiment was repeated two times. Data represent mean ± SD. ns: $p > 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$, one-way ANOVA with Tukey multiple comparison test.

The ability to recruit specific cell types that can subsequently be reprogrammed represents an important new concept in the field of IVT-based therapy. In vaccination strategies, one could include synthetic mRNAs encoding cytokines to recruit professional APCs, such as dendritic cells, together with the foreign Ag mRNA to enhance the efficiency of Ag presentation and the robustness of the immune response. One could envision new antimicrobials based on expression of chemokines for recruitment in combination with activating cytokines, such as IFN-γ, or specific antimicrobial proteins, such as cathelicidin. Macrophages and monocytes are ideal target cells for such strategies because they are known to respond to well-characterized recruitment signals. Moreover, they are extremely plastic and can be reprogrammed to fulfill a broad range of beneficial activities that include killing foreign microbes or tumor cells, tissue remodeling and repair, and antifibrotic functions. They are an ideal target for combinatorial IVT strategies, and we believe that this proof-of-principle study opens new avenues for future therapies that could further expand the potential applications of this rapidly evolving field.

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## Disclosures

The authors have no financial conflicts of interest.

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