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Immunostimulatory Effects Triggered by Self-Assembled Microspheres with Tandem Repeats of Polymerized RNA Strands

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Self-assembled RNA particles have been exploited widely to maximize the therapeutic potential of RNA. However, the immune response via RNA particles is not fully understood. In addition, the investigation of the immunogenicity from RNA-based particles is required owing to inherent immunostimulatory effects of RNA for clinical translation. To examine the immune stimulating potency, rationally designed microsized RNA particles, called RNA microspheres (RMSs), are generated with single or double strands via rolling circle transcription. The RMSs show an exceptional stability in the presence of serum, while they are selectively degraded under endolysosomal conditions. With precisely controlled size, both RMSs are successfully taken up by macrophages. Unlike the nature of RNA fragments, RMSs induce only basal-level expression of inflammatory cytokines as well as type I interferon from macrophages, suggesting that RMSs are immunocompatible in the therapeutic dose range. Taken together, this study could help accelerate clinical translation and broaden the applicability of the self-assembled RNA-based particles without being limited by their potential immunotoxicity, while a systematic controllability study observing the release of RNA fragments from RMSs would provide self-assembled RNA-based structures with a great potential for immunomodulation.

Nucleic acids have shown great potential as a fabric for synthesis of multiscale biomaterials due to their programmability, biocompatibility, and biodegradability. With these advantages, they have been engineered to generate sophisticated functional structures[1] employed to a wide range of biological applications, such as gene therapy,[2] targeted drug delivery,[3] and biomolecular imaging.[4]

Researchers have focused largely on engineering RNA because of their numerous biological functions including storage of genetic information, regulation of target gene and transfer of molecules. Various technologies have been applied to produce RNA-based structures to utilize the biological features of RNA.[5] In particular, rolling circle transcription (RCT) has been used extensively to generate RNA structures with increased loading capacity and efficacy of therapeutic RNA.[6] In a recent study, gene regulation using an RCT-based RNA nanoball showed therapeutic potential for treatment of age-related macular degeneration.[7]

On the other hand, specific sequences or structures of RNA are known to stimulate immune responses.[8] While the aforementioned RNAs can be utilized for boosting immune responses,[9] there are concerns that the RNA-based structures may cause undesired immune activation, which could greatly limit their therapeutic efficacy.[10] To address the issue, there have been efforts to circumvent the immune systems through chemically modified RNA.[11] On the other hand, the immunogenicity of self-assemblies composed of DNA, RNA,[12] or DNA-RNA hybrid[13] has been investigated widely for the better understanding of immunogenic properties of nucleic acid-based structures and their clinical translation. However, the study of immune stimulating properties of self-assembled RNA particles with potentially immunostimulatory RNA fabricated from RCT is not well established, whereas DNA particles from rolling circle amplification have been widely exploited as adjuvants for boosting immune response.[14] In order to clinically translate the fast-evolving RNA-based therapeutics, evaluation of immune response to self-assembled RNA particles is indispensable.

In this study, possible immunostimulatory effect of RNA microspheres (RMSs) depending on the sequences or structures of RNA was fully investigated. The RMSs are made up of tandem copies of RNA strands self-assembled by RCT.[6a] The RMSs exhibited an enhanced resistance to serum nucleases, which could enhance the cellular uptake efficiency, while effectively releasing RNA strands under lysosome-mimetic

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condition. The size of RMSs enabled an effective internalization by macrophages without further complexation as reported previously.[14a] However, the RMSs showed minimal or negligible immunostimulatory effect on the cells regardless of the presence of CpG content and degree of double-stranded structure without systemic release of RNA fragments. To the best of our knowledge, this is the first report suggesting the application of self-assembled RNA-based particles should not be limited by potential immunotoxicity.

To evaluate the immunogenicity of RMSs depending on the CpG contents and degree of double-stranded structure, we prepared two types of RMSs composed of single-stranded RNA (ssRNA) or double-stranded RNA (dsRNA) according to previous reports.[6a,15] As illustrated in **Figure 1**, ssRNA-based RMSs (ssRMSs) and dsRNA-based RMSs (dsRMSs) were self-assembled via RCT or complementary RCT using template circular DNA encoded with the sequences indicated in Table S1 in the Supporting Information.

To test the immune response by precisely controlling the amount of CpG oligoribonucleotide (ORN) embedded in the ssRMSs, we encoded repeated CpG ORN sequence into generated RNA strands. Similar to CpG oligonucleotide (ODN), a well-known toll-like receptor 9 (TLR9) agonist, CpG ORN made of unmethylated CpG RNA is known as an immunostimulant.[16] For testing the immunostimulatory effect caused by double-stranded regions in RMSs, we constructed dsRMSs consisting of 4, 23, or 70 bp-long double-stranded regions by manipulating the sequences of two partially complementary circular DNAs for RCT (Figure 1), given that immunostimulatory effect of dsRNA is dependent on the length of double-stranded region.[17] For convenience, ssRMSs made up of 1, 3, or 4 CpG ORNs per repeating unit were termed as ssRMS-CG-1, ssRMS-CG-3, or ssRMS-CG-4, respectively. Likewise, the dsRMSs consisting of 4, 23, or 70 bp-long dsRNA per repeating unit were named as dsRMS-4, dsRMS-23, or dsRMS-70, respectively.

Both ssRMS and dsRMS were characterized by scanning electron microscopy (SEM), transmission electron microscopy (TEM) and gel electrophoresis (**Figure 2**). The RMS composed of single strands of multiple CpG ORN was successfully fabricated with an average diameter of 1.3 μm (Figure 2a) with a polydispersity index (PDI) of 0.466 (Figure S1a, Supporting Information). This indicates that ssRMSs have a favorable size to be phagocytosed by macrophages (Figure 2b).[14a,18] According to SEM and TEM images, the porous structures of

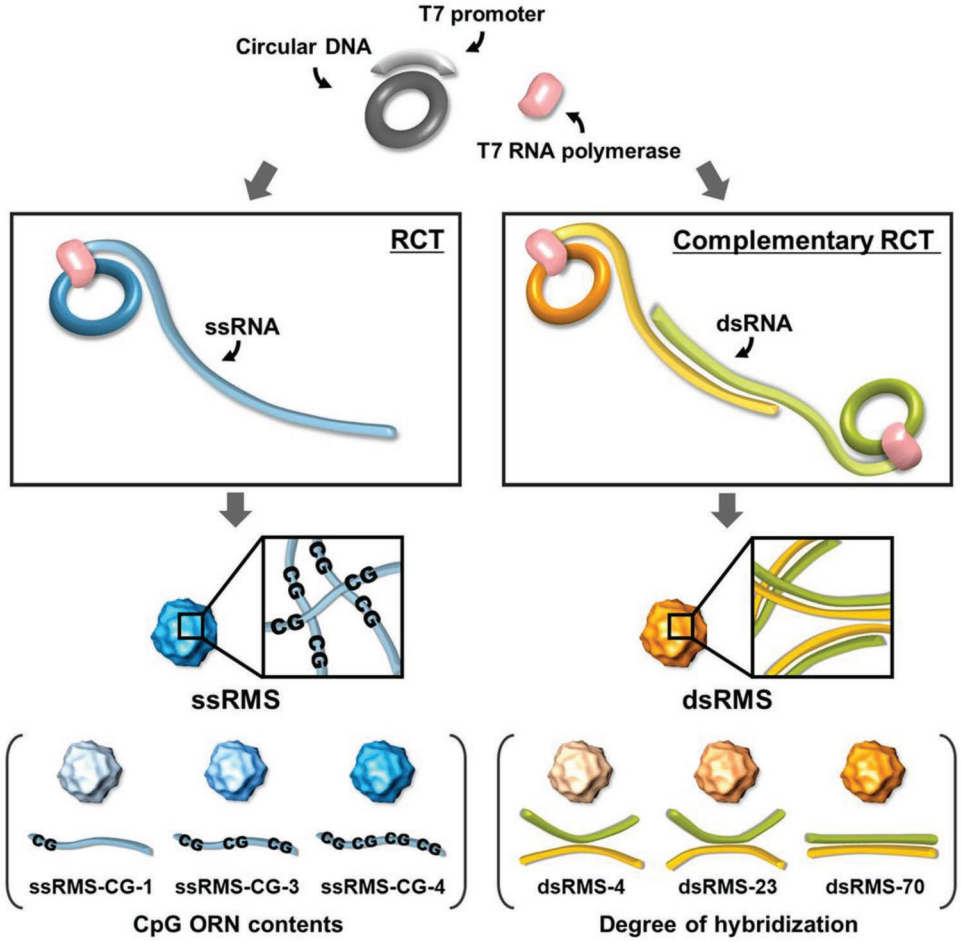

Figure 1. Schematic illustration of fabrication of RMSs composed of ssRNA-containing CpG ORN or dsRNA. The ssRMSs were synthesized via RCT with three different CpG ORN contents. Similarly, the dsRMSs were generated via complementary RCT to bear dsRNA regions with different degrees of hybridization.

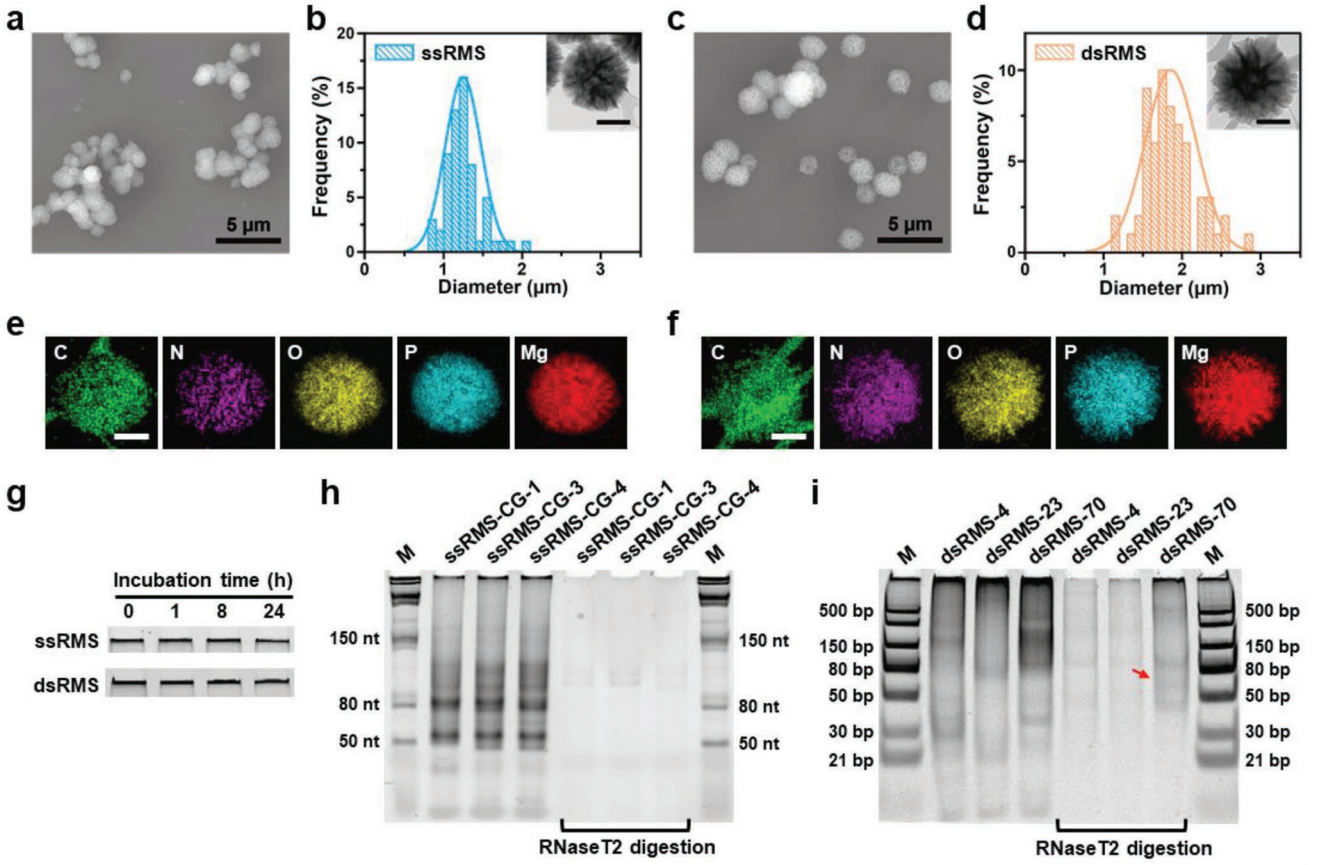

Figure 2. SEM images and SEM image-based size distribution analysis of (a,b) ssRMS or (c,d) dsRMS. Insets indicate TEM image of the RMSs, revealing their compact internal structure (scale bars: 500 nm). TEM-based energy dispersive X-ray spectroscopy (EDX) mapping analysis of (e) ssRMS or (f) dsRMS composed of carbon (C), nitrogen (N), oxygen (O), phosphorus (P), and magnesium (Mg) (inset scale bars: 500 nm). g) Gel electrophoresis results of RMSs after treatment with 10% serum, showing stability of RMSs toward serum nucleases. Gel electrophoresis result for (h) ssRMSs or (i) dsRMSs after RNase T2-mediated degradation. Red arrow indicates generated RNA strands. M: ssRNA or dsRNA marker.

ssRMS were observed with a highly packed internal structure (Figure 2b, inset). The dsRMSs also showed spongelike spherical structure with an average diameter of 1.8 μm with the PDI value of 0.243 which could also be engulfed by macrophages (Figure 2c,d and Figure S1b, Supporting Information). Similar to ssRMS, dsRMS also showed a highly packed internal structure (Figure 2d, inset). Furthermore, energy dispersive X-ray spectroscopy (EDX)-based elemental mapping revealed that both types of RMSs are composed of RNA and magnesium pyrophosphate which serves as the structural skeleton of RMSs (Figure 2e,f).[19] Moreover, the endotoxin levels of both types of RMSs (3.2 ng μL^{-1}) were found to be less than 0.06 EU ml^{-1}, which were below the US Food and Drug Administration limit for biomaterials (0.5 EU ml^{-1}) by an order of magnitude (Table S2, Supporting Information).[20]

The serum stability of RMSs was evaluated with 10% serum-containing media. As a result, 54.0% of ssRMSs and 72.8% of dsRMSs remained in the well in gel electrophoretic analysis due to their high molecular weight even at 24 h after the treatment with serum, while naked ssRNA was degraded completely after 1 h incubation (Figure 2g and Figure S2, Supporting Information). This result suggests that the RNA in RMSs were protected from serum nucleases for an extended period of time owing to compact structure compared to free RNA strands.[21] In addition, a possibility of releasing RNA strands from RMSs after the internalization of RMS in phagolysosome was investigated by incubating RMSs with RNase T2 enriched in the lysosomal compartment.[22] Interestingly, gel electrophoresis result revealed that ssRMSs were degraded to an undetectable level by the gel electrophoresis at 3 h after the treatment with RNase T2, indicating that ssRMSs were digested by RNase T2 (Figure 2h). On the other hand, when dsRMS-70 was treated with RNase T2 for 3 h, dsRNA fragments with the length ranging from 50 to 80 bp was observed among the various bands generated by the degradation of the RMSs gradually from the outside by RNase T2 due to their bulky structure and multimeric RNA (Figure 2i). This result indicates that RNase T2 digested unhybridized RNA between double-stranded regions due to the ssRNA-specific cleaving activity of RNase T2.[22,23] It was further confirmed that dsRMSs with a lower degree of hybridization (dsRMS-4 and dsRMS-23) were degraded almost completely by RNase T2. Taken together, free RNAs were not generated significantly from RMSs regardless of CpG content or degree of hybridization in lysosome-mimetic conditions unless RMSs were designed to bear over 70 bp-long dsRNA. In addition, only rationally designed dsRMS-70 could potentially boost immune

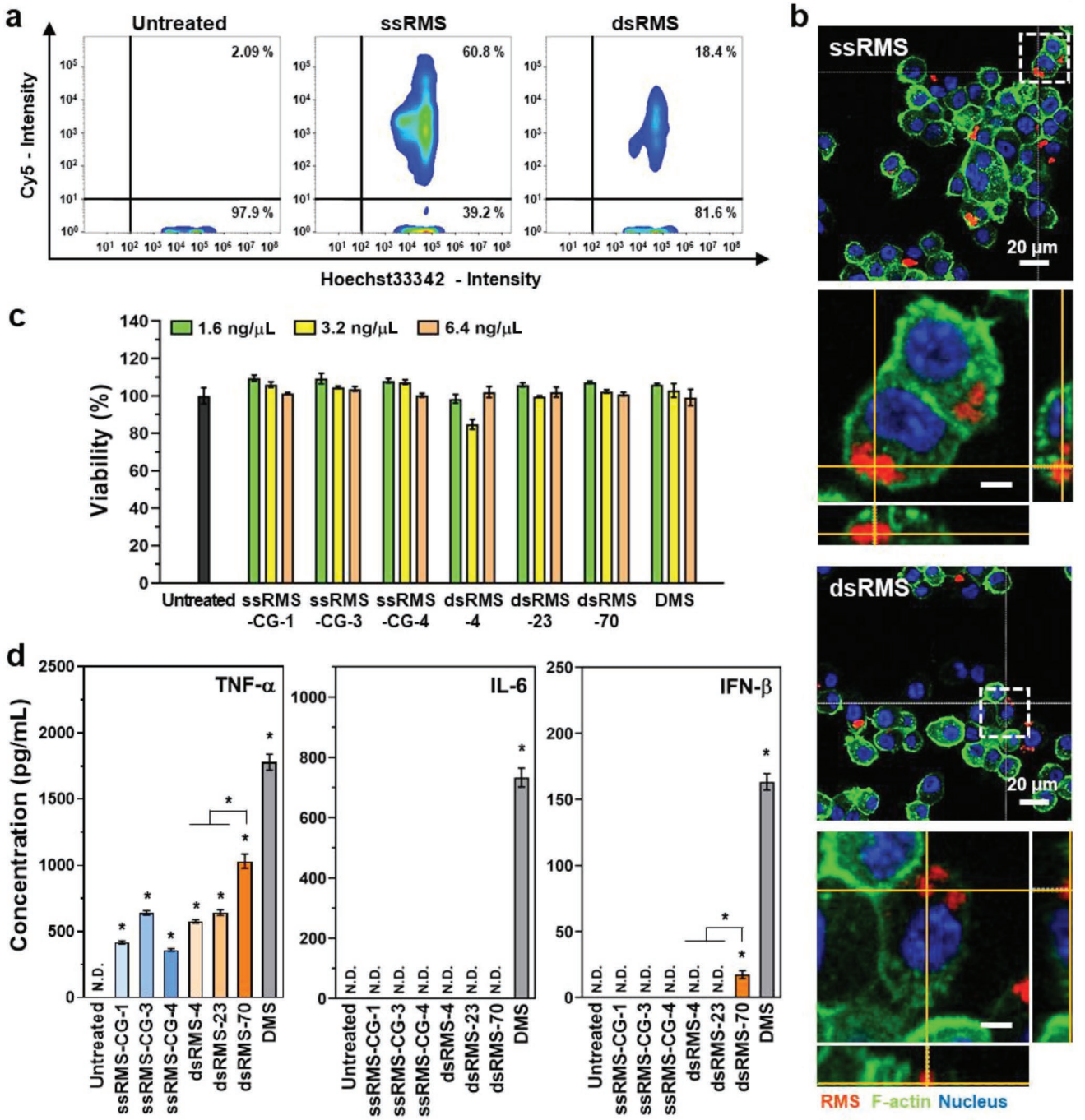

Figure 3. a) Cytometric analysis of RAW264.7 cells treated with 3.2 ng μL^{-1} cy5-labeled RMSs. b) Confocal microscopy images of RAW264.7 cells treated with 3.2 ng μL^{-1} cy5-labeled ssRMS (top) and dsRMS (bottom). The region indicated by the white box is shown at higher magnification below (inset scale bars: 5 μm). c) Viability of RAW264.7 cells after treatment with 1.6, 3.2, or 6.4 ng μL^{-1} ssRMS, dsRMS, or DMS ($n = 3$). d) Quantitative analysis of TNF-α, IL-6, and IFN-β levels expressed from RAW264.7 cells treated with 3.2 ng μL^{-1} ssRMS, dsRMS, DMS or left untreated ($n = 4$). The data are represented as mean ± standard error of the mean (S.E.M.). *$p < 0.05$ (one-way ANOVA followed by Tukey test), compared to indicated group or untreated control. N.D.: not detected.

responses due to the immune stimulating property of dsRNAs longer than 30 bp.[24]

Given the sizes of RMSs, we hypothesized that they could be delivered efficiently into macrophages. To evaluate the uptake efficiency of RMSs, macrophage-like RAW264.7 cells were treated with cy5-labeled RMSs. Both groups of RAW264.7 cells treated with cy5-labeled ssRMSs and dsRMSs showed a high level of red fluorescence intensity, which suggests a successful uptake of RMSs to the cells (**Figure 3**a). Confocal microscopy images further prove that both types of RMSs were internalized

efficiently by RAW264.7 cells (Figure 3b). Moreover, RMSs had a negligible cytotoxicity even at three times higher concentrations compared to previously reported working concentrations for immunostimulatory nucleic acids, such as CpG ODN (Figure 3c).[25]

To assess the immunostimulatory effect of RMSs, we examined the levels of proinflammatory cytokines, tumor necrosis factor alpha (TNF-α), and interleukin 6 (IL-6), released by RMS-treated RAW264.7 cells. In addition, the expression level of interferon beta (IFN-β) was analyzed to understand the immune response induced possibly by dsRNA fragments stimulating endosomal and cytosolic pathogen recognition receptors (PRRs).[8a,26] When treated with ssRMSs, they elicited mild or negligible cytokine levels regardless of the CpG ORN contents (Figure 3d). On the contrary, TNF-α and IFN-β expression levels induced by dsRMSs were dependent on the extent of hybridization of dsRNA. This result is consistent with previous reports that the greater immune response were induced as the degree of hybridization increased.[17] Meanwhile, the same amount of self-assembled CpG DNA microsphere (DMS) made up of CpG ODN was also used as a control group, which was introduced in our previous study.[14a] As expected, dsRMS-70 treated cells showed the highest expression of IFN-β among all types of RMSs with the release of dsRNA fragments by systemic degradation after cellular uptake. Taken together, it was confirmed that the RMS with 70 bp long-dsRNA region would be able to induce moderate immune response from RAW264.7 cells. Since the macrophage-like RAW264.7 cell line is known to elicit different immune responses from primary macrophages, further analysis was carried out with a primary cell type, bone marrow-derived macrophages (BMDMs).[27]

As shown in **Figure 4**a, cy5-labeled RMSs treated BMDMs showed a significant increase of cy5 fluorescence intensity compared to untreated BMDMs. This indicates that both types of RMSs were engulfed efficiently by BMDMs. Notably, cellular uptake efficiencies of both types of RMSs were not significantly different for BMDMs unlike for RAW264.7, which could be due to higher phagocytic activity of primary macrophages compared to RAW264.7.[28] We further confirmed that they were internalized by macrophages and localized at the intracellular compartment (Figure 4b).We then evaluated the immune responses of BMDMs to RMSs by measuring expression levels of proinflammatory cytokines. Unlike the result from RAW264.7, the expression level of TNF-α as well as IL-6 and IFN-β from RMSs treated BMDMs was not significantly different compared to the untreated control (Figure 4c). It should be noted that the level of proinflammatory cytokines was increased significantly for the positive control group (lipopolysaccharide (LPS) treated BMDMs; Figure S3, Supporting Information). Taken together, both types of RMSs were insufficient to activate BMDMs within the therapeutic dose range reported in previous studies,[6d] suggesting immunocompatibility of RMSs. Furthermore, the immune responses of the RMSs were further assessed on dendritic cells (DC2.4 cells), an antigen presenting cells that play an important role in initiating immune responses.[29] The RMSs were internalized to DC2.4 cells efficiently, and the RMSs induced a negligible or mild expression of cytokines at various concentrations (**Figure 5**), which

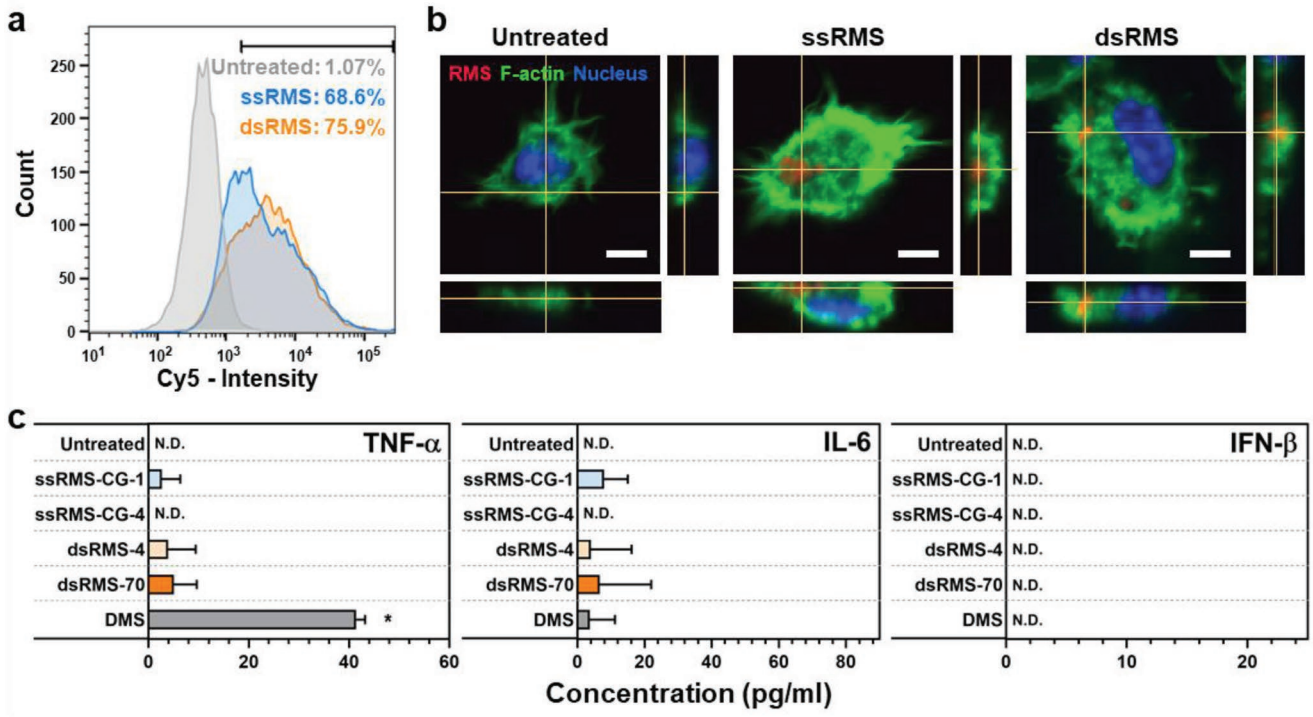

Figure 4. a) Flow cytometric analysis of BMDMs treated with 3.2 ng μL^{-1} cy5-labeled RMSs. b) Confocal microscopy images of BMDM treated with 3.2 ng μL^{-1} cy5-labeled RMSs, indicating internalization of RMSs (inset scale bars: 5 μm). c) Analysis of TNF-α, IL-6, and IFN-β levels released from BMDMs treated with 3.2 ng μL^{-1} RMSs or DMS (mean ± S.E.M., $n = 3$). *$p < 0.05$ (one-way ANOVA followed by Tukey test), compared to untreated control.

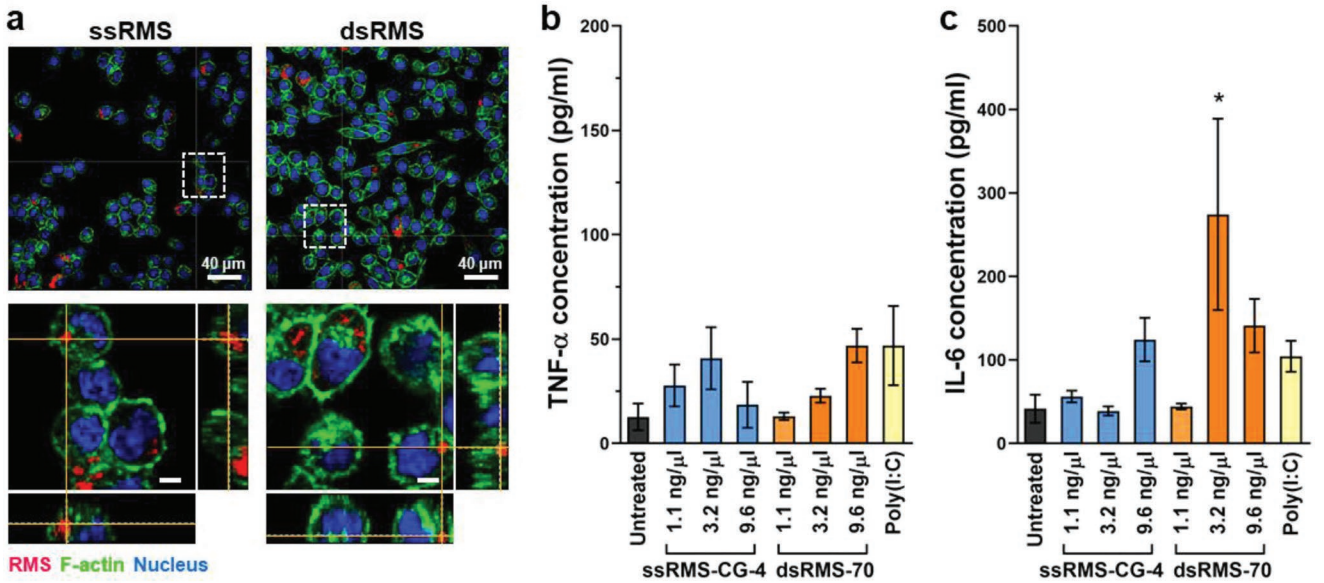

Figure 5. a) Low- and high-magnification confocal microscopy images of DC2.4 cells treated with 9.6 ng μL^{-1} cy5-labeled ssRMS (left) and dsRMS (right). The region indicated by the white box is shown at higher magnification below (inset scale bars: 10 μm). b) TNF-α and c) IL-6 concentration expressed from DC2.4 cells treated with ssRMS-CG-4, dsRMS-70, poly(I:C) (9.6 ng μL^{-1}), or left untreated (mean ± S.E.M., $n = 3$). *$p < 0.05$ (one-way ANOVA followed by Tukey test), compared to untreated control.

aligns well with the findings from macrophages. Meanwhile, the highest concentration of the RMSs (9.6 ng μL^{-1}) stimulated DC2.4 cells to produce a significant level of cytokines compared to the immunostimulatory RNA, poly(I:C) (Figure 5b,c).[30] The findings could broaden the clinical application of self-assembled RNA structures which showed a great therapeutic potential[7] without concerns on the immunogenicity mediated by macrophages or dendritic cells.

In conclusion, we have developed different types of the RMSs to determine the immunostimulatory potency depending on CpG contents and degree of hybridization. The RMSs exhibited considerable uptake efficiency to macrophages without further complexation due to the favorable size of the RMSs for phagocytosis. In addition, the RMSs were degraded efficiently by lysosomal RNase, while exhibiting resistance to degradation under serum condition. In the immunological assay on macrophage-like RAW264.7 cells, the RMSs triggered moderate levels of cytokines with dependence on the degree of hybridization. In further validation with primary cells, all RMSs were proved to be immunoquiescent on BMDMs when used within therapeutic dose range. Moreover, the RMSs showed negligible or mild immune responses from DC2.4 cells at various doses. The results from individual cell types imply that the RMSs have little possibility of causing severe immunotoxicity. Meanwhile, the dsRMS-70 induced the expression of IFN-β caused possibly by the released dsRNA motifs which stimulate PRRs at RAW264.7 cells. These findings allow us to facilitate the control on immunomodulation through engineering the structure of self-assembled RMSs with the previously reported approaches to manipulate the size, shape, content or sequence of RNA-based nanoparticles.[12,13] Taken together, this study will help accelerate clinical translation and broaden the applicability of the self-assembled RNA-based particles without being limited by immunotoxicity, while a systematic controllability to release RNA fragments from the RMSs would provide the RMSs with a great potential for immunomodulation. Furthermore, the implementation of more in vivo-like environment[31] will provide us with more accurate understanding on immunopotency of the self-assembled RNA-based therapeutics.

Supporting Information

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

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

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