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Synthesis and Biological Evaluation of Ionizable Lipid Materials for the In Vivo Delivery of Messenger RNA to B Lymphocytes

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B lymphocytes regulate several aspects of immunity including antibody production, cytokine secretion, and T-cell activation; moreover, B cell misregulation is implicated in autoimmune disorders and cancers such as multiple sclerosis and non-Hodgkin's lymphomas. The delivery of messenger RNA (mRNA) into B cells can be used to modulate and study these biological functions by means of inducing functional protein expression in a dose-dependent and time-controlled manner. However, current in vivo mRNA delivery systems fail to transfect B lymphocytes and instead primarily target hepatocytes and dendritic cells. Here, the design, synthesis, and biological evaluation of a lipid nanoparticle (LNP) system that can encapsulate mRNA, navigate to the spleen, transfect B lymphocytes, and induce more than 60 pg of protein expression per million B cells within the spleen is described. Importantly, this LNP induces more than 85% of total protein production in the spleen, despite LNPs being observed transiently in the liver and other organs. These results demonstrate that LNP composition alone can be used to modulate the site of protein induction in vivo, highlighting the critical importance of designing and synthesizing new nanomaterials for nucleic acid delivery.

B lymphocytes (commonly referred to as B cells) are a versatile subpopulation of white blood cells that originate in the bone marrow, mature in the spleen, and circulate throughout the vascular and lymphatic systems in healthy individuals.[1] While B cells perform a wide range of biological functions, they are predominantly responsible for three critical tasks in vivo: (i) producing and secreting antibodies and cytokines to fight infections as well as regulate inflammation, (ii) generating memory cells that mitigate the deleterious effects of pathogen re-exposure, and (iii) stimulating T cell production through antigen presentation via major histocompatibility complex (MHC) I (in killer T cells) or MHC II (in helper T cells).[2] In addition to their essential role in disease prevention and management, B cell dysfunction can also serve as the root of disease. For example, misregulated B lymphocyte proliferation can result in non-Hodgkin's B cell lymphoma, from which nearly 20,000 patients die each year in the US alone.[3] Moreover, a cure for lymphomas, and cancers of the circulation more broadly, remains elusive. As a result, B lymphocytes represent an attractive target for drug and gene delivery as the ability to modulate B cell function in vivo could have profound impact on the study, prevention, and treatment of disease.[4]

Recently, the delivery of messenger RNA (mRNA) cargo encapsulated in lipid nanoparticles (LNPs) has emerged as a viable strategy to modulate cell activity in vivo.[5] LNPs shield the

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mRNA from nucleases, prevent unwanted clearance, and promote cellular uptake.[6] Moreover, LNPs decomplex from mRNAs upon accessing the cytosol of target cells, and subsequent mRNA translation at the ribosomal complex produces multiple copies of the pharmacologically active protein of interest per mRNA.[7] To date, several mRNA LNPs have been developed that can induce functional protein production in specific tissues (e.g., liver, spleen) following systemic administration in mice.[8] However, none of these systems has demonstrated successful translation of the delivered mRNA within B lymphocytes in vivo.

To achieve this goal, we sought to develop degradable LNP formulations capable of delivering mRNA to the spleen, the primary residence site for a large fraction of immune cell populations including B lymphocytes (**Figure 1**a).[8g] We hypothesized that this could be facilitated through formulation of target mRNA sequences with ionizable lipid materials.[8a,9] Ionizable lipids are a class of small molecule-based materials that, in conjunction with cholesterol (aids in stability),[10] lipid-anchored polyethylene glycol (PEG) (decreases aggregation and nonspecific uptake),[11] a given phospholipid (modifies bilayer structure),[12] and an mRNA of a given sequence (codes for the pharmacologically active protein of interest),[5] can be formulated into mRNA LNPs using microfluidic mixing.[13] While each of these components serves a role for promoting in vivo

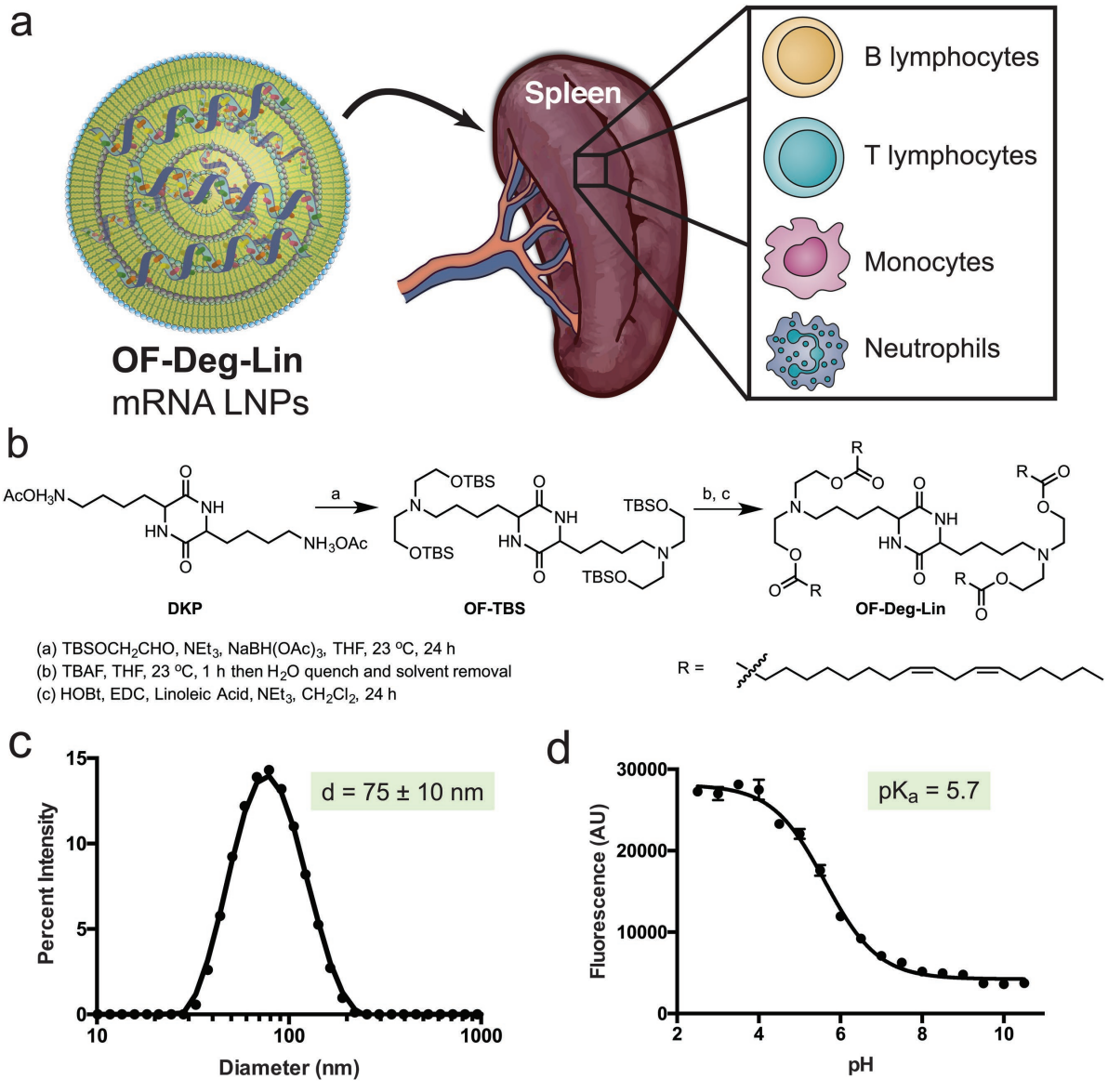

Figure 1. a) **OF-Deg-Lin** mRNA LNPs target the spleen, the primary residence site of immune cells including B lymphocytes. b) Synthesis of **OF-Deg-Lin**, a bis-lysine-derived ionizable lipid. c) Average diameter of **OF-Deg-Lin** mRNA LNPs as measured by dynamic light scattering. d) 6,P-toluidinylnaphthalene-2-sulfonate (TNS) fluorescence of **OF-Deg-Lin** mRNA LNPs as a function of pH is used to assess the surface pK_a of **OF-Deg-Lin** mRNA LNPs.

LNP efficacy, evidence within the literature has demonstrated that subtle and unique modifications to the chemical structure of the ionizable lipid can profoundly impact the overall delivery properties of the LNP.[8a,9a,d] Toward this end, we synthesized **OF-Deg-Lin**, an ionizable lipid designed to include degradable linkages, in three synthetic steps via versatile synthetic intermediate **OF-TBS** [Figure 1b; see Section S2 (Figures S1–S3) in the Supporting Information for complete synthetic procedures and molecular characterization details]. Molecular properties of **OF-Deg-Lin** include a heightened electrophilicity relative to acrylate-derived ionizable lipid materials as well as ester linkages that, upon hydrolytic cleavage, generate linoleic acid, a nontoxic fatty acid.[14]

OF-Deg-Lin was then formulated into LNPs using microfluidic mixing with 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), C14-PEG-2000, cholesterol, and unmodified mRNA coding for firefly luciferase (FLuc).[8a,b,13] Several characterization methods were performed to verify the integrity of **OF-Deg-Lin** mRNA LNPs. Dynamic light scattering confirmed the formation of LNPs, revealing an average particle diameter of 75 ± 10 nm with a polydispersity index of 0.197 [Figure 1c, representative raw data is available in Section S4 (Table S1) in the Supporting Information]. Cryogenic transmission electron microscopy highlighted the spherical morphology of **OF-Deg-Lin** LNPs (Figure S4, Supporting Information). A modified Quant-iT RiboGreen RNA assay (a fluorescence-based assay which quantifies RNA concentration between LNP samples in the presence and absence of a surfactant) demonstrated that **OF-Deg-Lin** LNPs entrapped ≈60% of the formulated FLuc mRNA [representative raw data is available in Section S4 (Table S2) in the Supporting Information].[9b] Each of these parameters were largely consistent with values observed for other mRNA LNPs. By contrast, the surface pK_a of **OF-Deg-Lin** FLuc mRNA LNPs was lower (≈5.7) than those observed for liver-targeting mRNA LNPs (≈7.0) [Figure 1d; see Section S4 (Table S3) in the Supporting Information for raw data].[8b] These data indicated that **OF-Deg-Lin** LNPs demonstrate pH responsive behavior which may be important for endosomal escape and/or other cellular uptake pathways. An in vitro dose–response study on HeLa cells was then used to validate **OF-Deg-Lin** LNPs as a viable delivery vector for mRNA (Figure S5, Supporting Information). Negligible toxicity was observed across all doses studied (Figure S6, Supporting Information) and the dose-dependent protein production over the entire dosing regime suggested that translation of the desired protein with our system in vitro was limited only by the dose of mRNA applied.

Having established **OF-Deg-Lin** LNPs as a potential delivery vehicle for mRNA transfection in vitro, our attention then shifted to investigating **OF-Deg-Lin** LNP mRNA delivery in vivo. For some applications, clinical potential of mRNA LNPs is strengthened by selective protein expression in specific tissues, whether healthy or diseased.[5] FLuc mRNA was therefore selected as a model therapeutic cargo for our study for three reasons: (i) the associated protein can be directly imaged in whole organs, allowing us to verify whether or not **OF-Deg-Lin** LNPs demonstrate protein production with requisite levels of tissue specificity; (ii) the luminescent signal can be directly correlated to the total number of individual protein molecules, allowing for robust protein quantification (Figure S7, Supporting Information); and (iii) luciferase is not secreted, and as such its presence can be used to identify successfully transfected cell populations independent of protein production in other tissues or cell types.[15]

OF-Deg-Lin FLuc-mRNA LNPs were injected intravenously via the tail vein at a 0.75 mg kg^{-1} dose in C57BL/6 mice alongside mice injected with a phosphate-buffered saline (PBS) negative control. At 6 h post-injection, each mouse was treated intraperitoneally with D-luciferin and then sacrificed. The organs (pancreas, spleen, liver, kidneys, lungs, heart, and uterus/ovaries) were isolated by standard necropsy and imaged with an IVIS Lumina LT in vivo imaging system (**Figure 2**a). Quantification further indicated that more than 85% of total protein production occurred in the spleen as measured by average radiance (Figure 2b). Notably, no significant protein expression was observed in any organ when naked FLuc mRNA was administered systemically at the same dose and analyzed at the same time point; this finding confirmed that delivery vectors such as LNPs are critical for systemic in vivo mRNA delivery (Figure S8, Supporting Information). Interestingly, however, **OF-Deg-Lin** mRNA LNPs entrapping nontranslating, fluorescently labeled mRNA demonstrated transient transport to the liver (Figure 2c). Indeed, quantification of these data demonstrated that more than 100-fold more fluorescent signal was observed in the liver than for the spleen and other organs when **OF-Deg-Lin** Cy5 mRNA LNPs were administered systemically (Figure 2d). This finding was also confirmed in a separate experiment wherein perfusate was flowed through the mice after systemic administration of **OF-Deg-Lin** Cy5 mRNA LNPs using identical doses, administration route, and time points (Figure S9a, Supporting Information). Perfusion illustrated that the fluorescent signals in the organs were due to the fluorescently labeled LNPs/mRNA rather than the volume of blood in a given organ at the time of resection. Control perfusion experiments administering the naked Cy5 mRNA as well as a PBS negative control further highlighted that the **OF-Deg-Lin** LNPs transport predominantly to the liver (Figure S9b,c; Supporting Information).

These results suggest that **OF-Deg-Lin** mRNA LNPs fail to induce protein production in liver cells, even though they still transport to the tissue of the liver. On the other hand, the same mRNA LNPs transport to and induce protein expression in the spleen, the target organ of interest. While the mechanism by which **OF-Deg-Lin** mRNA LNPs express predominantly in the spleen is still being explored, one hypothesis is that the electrophilic ester bonds in **OF-Deg-Lin** may degrade more readily in the liver than in other organs.[16] In this model, the LNPs are still transported to the liver, but may degrade before actively inducing protein expression in the cells of this organ (e.g., during cell internalization; during endosomal localization, internalization, and trafficking; or may be unable to escape the endosome due to acidic degradation). By contrast, these materials appear to survive the enzymatic conditions in the spleen, such that the same particles are not only transported to the spleen but also retain the ability to induce functional protein expression following uptake in the resident cells of the spleen.

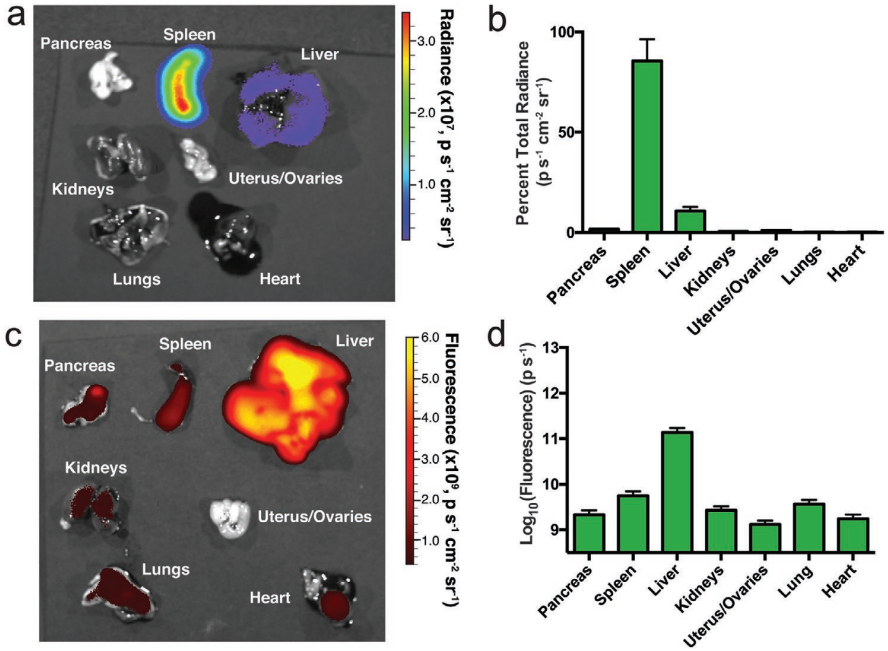

Figure 2. a) Representative biodistribution of systemically administered **OF-Deg-Lin** FLuc mRNA LNPs as measured by functional luciferase expression. b) More than 85% of the expressed luciferase is present in the spleen as measured by average radiance. c) Representative fluorescence localization of systemically administered **OF-Deg-Lin** nontranslating Cy5 mRNA LNPs. d) **OF-Deg-Lin** nontranslating Cy5 mRNA LNPs are transported to the liver roughly 100-fold over any other organs. Where applicable, data presented as mean + standard deviation ($n = 3$).

To assess this hypothesis, a different FLuc mRNA LNP comprised of nondegradable ionizable lipid OF-02 was also screened (Figure S10, Supporting Information). OF-02 was selected as a control because it is a nondegradable structural analog of ionizable lipid **OF-Deg-Lin** (Figure S10a, Supporting Information) that has been established in the literature as one of the most potent vectors for systemic administration of mRNA.[8a] Whereas **OF-Deg-Lin** contains degradable ester linkages to append the diketopiperazine core and the doubly unsaturated tails, OF-02 contains nondegradable 1,2-amino-alcohol linkages to append the same diketopiperazine core and the doubly unsaturated tails. OF-02 was then formulated into LNPs containing the nontranslating Cy5 mRNA. The resultant OF-02 Cy5 mRNA LNPs were then systemically administered at an identical dose as that used for **OF-Deg-Lin** Cy5 mRNA LNPs. After 1 h the mice were euthanized and perfused with PBS (50 mL) via the left ventricle. The resected organs were then imaged in an identical fashion as those that were resected for the **OF-Deg-Lin** Cy5 mRNA LNPs (Figure S10b, Supporting Information). Much like degradable **OF-Deg-Lin** Cy5 mRNA LNPs (Figure 2c,d and Figure S9a; Supporting Information), nondegradable OF-02 Cy5 mRNA LNPs also transported predominantly to the liver following systemic administration. The similarities end, however, when functional FLuc mRNA was used instead. Whereas degradable **OF-Deg-Lin** FLuc mRNA LNPs induced protein expression predominantly in the spleen (Figure 2a,b), nondegradable OF-02 FLuc mRNA LNPs induced protein expression predominantly in the liver (Figure S10c, Supporting Information). Quantification of these data and comparison to the corresponding **OF-Deg-Lin** mRNA LNP data indicated the following; even though both degradable **OF-Deg-Lin** mRNA LNPs and nondegradable OF-02 Cy5 mRNA LNPs transport predominantly to the liver (Figure S10d, Supporting Information), degradable **OF-Deg-Lin** FLuc mRNA LNPs induce the majority of protein expression in the spleen while nondegradable OF-02 FLuc mRNA LNPs induce the majority of protein expression in the liver (Figure S10e, Supporting Information). Ongoing mechanistic work in our laboratory will continue to explore this phenomenon, but we believe this comparison to a nondegradable, structurally analogous ionizable lipid highlights the importance of degradable ester bonds to the unique biodistribution profile of **OF-Deg-Lin**.

Having established that **OF-Deg-Lin** LNPs can deliver to cells in the spleen, we hypothesized that **OF-Deg-Lin** LNPs may be employed to target B cells in vivo, largely because the spleen is the primary residence site of a large fraction of immune cells.[8g] To test this hypothesis, fluorescence-activated cell sorting (FACS) was performed on cells isolated from the spleen. Briefly, **OF-Deg-Lin** Cy5 mRNA LNPs were formulated as before. These LNPs were then injected at a 0.75 mg kg^{-1} intravenous dose, alongside a PBS control group. After 1 h, the mice were sacrificed, the spleens were isolated, red blood cells were lysed, and the remaining cells were analyzed via FACS. FACS analysis was performed on four discrete $CD45^+$ cell populations: B cells ($CD19^+$), T cells ($TCR\text{-}\beta^+$), neutrophils ($Ly\text{-}6G^+$), and monocytes/macrophages ($CD11b^+/Ly\text{-}6G^-$) (**Figure 3**a). **OF-Deg-Lin** Cy5 mRNA LNPs label the B cell and monocyte/macrophage populations as well as a small percentage of the neutrophil population and very few of the T cells. These data indicated that ≈7% of the total B-cell population was labeled

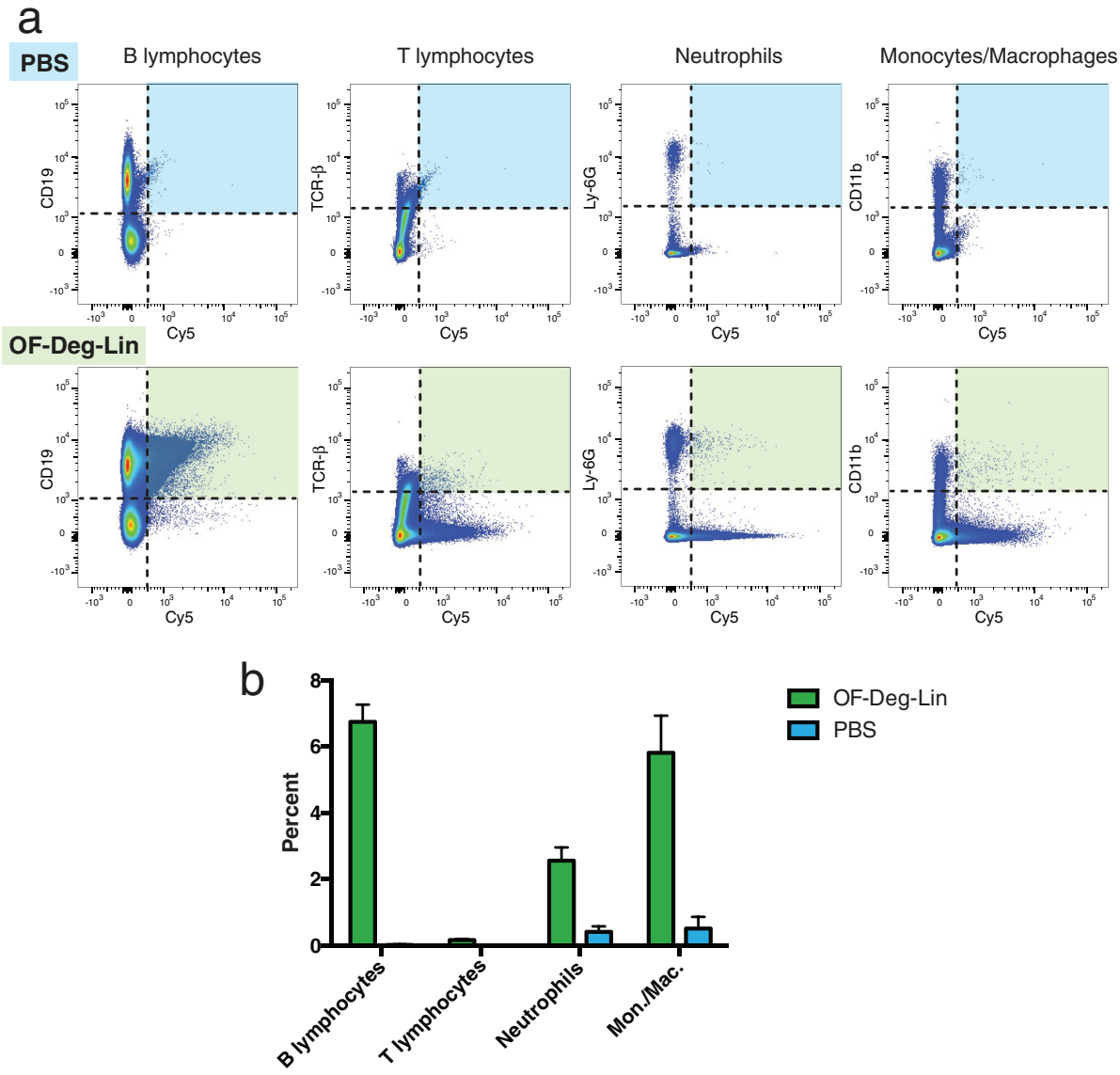

Figure 3. a) Representative flow cytometry dot-plots demonstrating association of fluorescently labeled mRNA in B cells ($CD19^+$ expressing), T cells ($TCR\text{-}\beta^+$ expressing), Neutrophils ($Ly\text{-}6G^+$ expressing), and Monocytes/Macrophages ($CD11b^+$ expressing and $Ly\text{-}6G^-$) for mice treated with PBS and **OF-Deg-Lin** Cy5 mRNA LNPs. b) Average percent labeling of respective cell populations with **OF-Deg-Lin** Cy5 mRNA LNPs. Data presented as mean + standard deviation ($n = 3$).

with the Cy5 mRNA following a single intravenous injection, which occurs alongside other immune cell population labeling in the spleen (Figure 3b).

To directly measure levels of functional protein production within our target B cells induced by **OF-Deg-Lin** mRNA LNPs, we performed an in vivo dose–response study specifically on the B cell population of mice treated with **OF-Deg-Lin** FLuc mRNA LNPs (**Figure 4**a,b). C57BL/6 mice were injected intravenously with **OF-Deg-Lin** FLuc mRNA LNPs at doses ranging from 0.75 mg kg^{-1} to 2.25 mg kg^{-1}. After 6 h, the mice were sacrificed and the spleens were isolated and processed. The B cells were then isolated and verified to be greater than 98% pure by flow cytometry (Figure S11, Supporting Information). The isolated cells were plated at a seeding density of ≈2 million B lymphocytes per well. Luciferase expression was quantified directly on the cultured B cells and the total luminescent signal was converted into total mass of protein produced per million cells (Figure S7, Supporting Information). Protein production across all doses studied was linear, suggesting that protein production was limited only by the administered dose of mRNA. Approximately 60 pg of luciferase protein was produced per million B cells at the highest **OF-Deg-Lin** LNP dose. Notably,

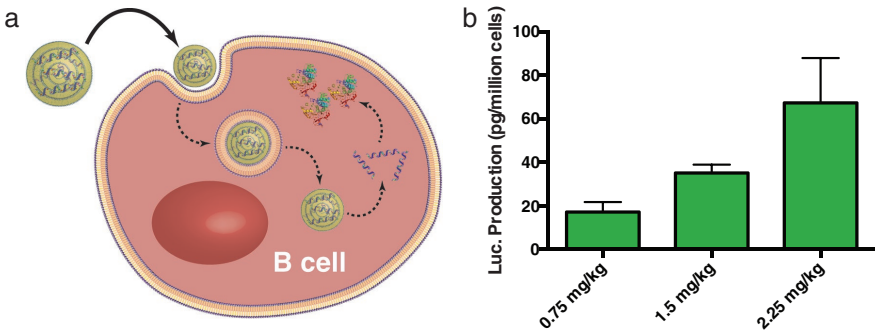

Figure 4. a) Cartoon representation of **OF-Deg-Lin** mRNA LNPs promoting functional protein production in a B lymphocyte population. b) Dose–response curve detailing total luciferase production in the B-lymphocyte population after systemic administration of **OF-Deg-Lin** mRNA LNPs. Data presented as mean + standard deviation ($n = 3$).

negligible toxicity of the **OF-Deg-Lin** LNP components has been observed in vivo at doses up to 4 mg kg^{-1} (Figure S12, Supporting Information).

To the best of our knowledge, **OF-Deg-Lin** LNPs are the first description of mRNA LNPs that can induce protein expression within B lymphocytes in vivo. Whereas published formulations such as **cKK-E12**,[8a] **OF-02**,[8a] and **C12-200**[8b] produce the majority of protein production in cells of the liver, intravenous dosing of **OF-Deg-Lin** mRNA LNPs resulted primarily in expression in the spleen, with minimal translation in the liver, and negligible production in the cells of other organs. Although other mRNA formulations have accessed the spleen, they failed to induce protein expression within B lymphocytes and instead targeted dendritic cells.[8g] Uniquely, **OF-Deg-Lin** mRNA LNPs both target the spleen (with >85% specificity) and induce protein expression within the B cell population following routine intravenous dosing. We hope that **OF-Deg-Lin** mRNA LNPs may provide a tool to modulate and study pathways associated with cytokine production from B lymphocytes in vivo and ongoing work in our lab will aim to further analyze these biological responses.

In summary, we report on the design, synthesis, and characterization of a spleen-targeting, mRNA-based LNP system designated **OF-Deg-Lin**. **OF-Deg-Lin** LNPs induce the majority of protein expression (>85% by percent of total protein) in the spleen and induce functional protein production within B lymphocytes. Future work will focus on the introduction of alternative proteins to study and modulate B-cell activity in vivo. Moreover, efforts toward elucidating structure–function relationships within this class of LNP materials will also be explored. In total, this study not only highlights **OF-Deg-Lin** mRNA LNPs as a delivery vector to induce protein expression within B lymphocytes, but also demonstrates the importance of designing and synthesizing new nanomaterials to better understand and improve nucleic acid delivery in vivo.

Experimental Section

Animal Experiments: All animal studies were approved by the MIT Institutional Animal Care and Use Committee and were consistent with local, state, and federal regulations as applicable. LNPs were intravenously injected in female C57BL/6 mice (Charles River Labs, 18–22 g) via the tail vein. Six hours after injection of FLuc mRNA LNPs, mice were injected intraperitoneally with 130 µL of D-luciferin (30 mg mL^{-1} in PBS). After 15 min, mice were sacrificed and the organs were isolated (pancreas, spleen, liver, kidneys, lungs, heart, uterus, and ovaries) and imaged with an IVIS imaging system (Perkin Elmer, Waltham, MA). Luminescence was quantified using Living Image software (Perkin Elmer).

Supporting Information

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

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

biomaterials, drug delivery, nanoparticles, proteins, RNA

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