Chinese Chemical Letters 36 (2025) 110285

Contents lists available at ScienceDirect

Chinese Chemical Letters

journal homepage: www.elsevier.com/locate/cclet

Folic acid ameliorates the immunogenicity of PEGylated liposomes

Xiying Wu[a], Anze Liu[b], Yuzhong Yan[c,*], Ying Lu[b,*], Huan Wang[b,*]

[a] Shanghai Skin Disease Hospital, Tongji University School of Medicine, Shanghai 200433, China
[b] Department of Pharmaceutical Sciences, School of Pharmacy, Naval Medical University, Shanghai 200433, China
[c] Department of Research and Discipline Development, Zhoupu Hospital, Shanghai University of Medicine and Health Sciences, Shanghai 201318, China

ARTICLE INFO

Article history:
Received 29 May 2024
Revised 12 July 2024
Accepted 18 July 2024
Available online 19 July 2024

Keywords:
Folic acid
Pegylated liposomes
Immunogenicity
Marginal zone B cells
Accelerated blood clearance

ABSTRACT

As PEGylated liposomes have witnessed remarkable advancements in drug delivery, their immunogenicity has emerged as a notable challenge. In this study, we discovered that a simple pre-injection of folic acid (FA) effectively mitigated the immunogenicity of PEGylated liposomes and enhanced their *in vivo* performance by tolerating splenic marginal zone B cells. FA specifically inhibited the internalization of PEGylated liposomes by splenic marginal zone B cells, thereby reducing splenic lymphocyte proliferation and specific IgM secretion. This modulation alleviated IgM-mediated accelerated blood clearance and adverse accumulation of the PEGylated liposomes in the skin. These findings provide new insights into the immunomodulatory effects of FA and promising avenues to enhance the efficacy and safety of PEGylated liposomal nanomedicines.

In recent years, PEGylated liposomes have witnessed significant progress as vehicles for drug delivery [1-3]. Their application in nanomedicines (*e.g.*, Doxil, Onpattro and BNT162b) have greatly improved the druggability of therapeutics by optimizing the pharmacodynamics or pharmacokinetics, thus offering renewed hope for the treatment of numerous diseases [4,5]. However, the previously overlooked immunogenicity of PEGylated liposomal nanomedicines has gradually emerged as a critical concern as the field advances [6,7]. In the early days, PEGylated liposomes were primarily encapsulated with cytotoxic drugs such as doxorubicin and irinotecan [8]. The cytotoxic drugs can suppress the immune function and mask the intrinsic immunogenicity of PEGylated liposomes. However, as the focus shifted to encapsulating therapeutics like nucleic acid drugs, especially when administered to a large population, the immunogenicity issues of PEGylated liposomal nanomedicines became increasingly prominent [9-11]. Notable examples include the accelerated blood clearance (ABC), complement activation-related *pseudo*-allergy (CARPA), hand-foot syndrome (HFS) [12,13]. The immunogenicity may not only compromise the therapeutic effects but also induce adverse reactions in patients. This underscores the urgent need to address this immunogenicity issues to ensure the safety and efficacy of PEGylated liposomal nanomedicines.

The splenic marginal zone B (MZB) cells possess dual functions of antigen presentation and antibody secretion, playing critical roles in the immune effects against foreign substances [14-16]. Blockade or deletion of splenic MZB cells effectively alleviate the immunogenicity of PEGylated liposomal nanomedicines [17,18]. For instance, infusion of free polyethylene glycol (PEG) can safely and efficiently mitigate the production of anti-PEG antibodies, alleviate allergic reactions, and restore the prolonged circulation of various PEGylated therapeutics [17,19]. It appears most likely that free PEG temporarily saturates the B cell receptors (BCRs) on anti-PEG B-cells, thereby preventing subsequently injected PEGylated liposomes from activating BCRs. Recently, we have first discovered that folic acid (FA) can induce splenic MZB cells anergy by disrupting the stability of the B cell receptors complex and interfering the antigen signals transmission. FA can serve as a safe and effective immunomodulator to induce immune evasion and mitigate the production of anti-drug antibodies against therapeutic biologics [20]. The induced MZB cells anergy by FA provides an immunological escaping window for the subsequently injected therapeutics. Based on these findings, we hypothesize that FA might also ameliorate the immunogenicity of PEGylated liposomes and improve their *in vivo* performance *via* tolerating splenic MZB cells. To test this hypothesis, we administered FA prior to intravenous injection of PEGylated liposomes. The effect of FA on performance of PEGylated liposomes (*e.g.*, cellular uptake, pharmacokinetic profiles and the ABC phenomenon) were investigated.

A classic plain PEGylated liposome (sLip) with an average diameter of 86 nm was prepared according to the prescription

* Corresponding authors.
E-mail addresses: zp_yanyz@sumhs.edu.cn (Y. Yan), acuace@163.com (Y. Lu), wang568855641@163.com (H. Wang).

https://doi.org/10.1016/j.cclet.2024.110285
1001-8417/

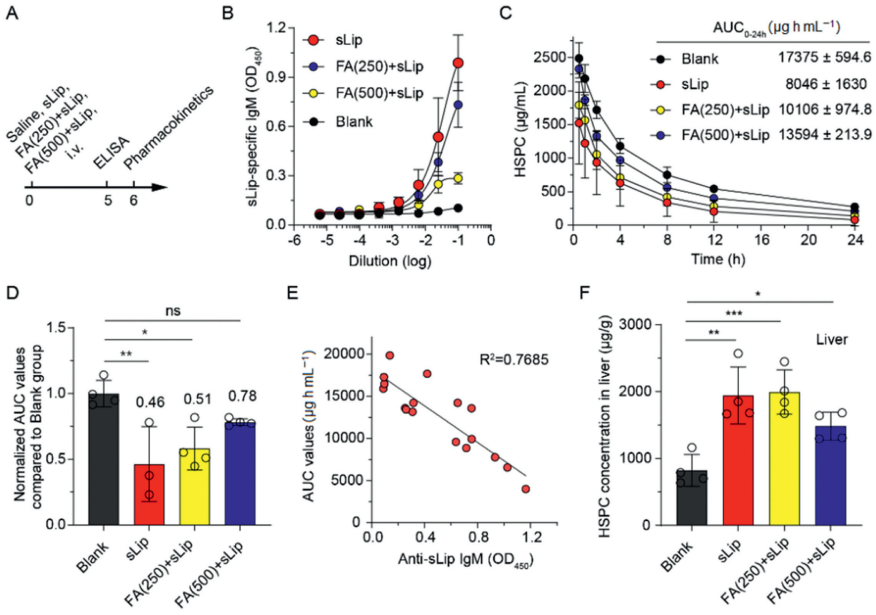

Fig. 1. Effects of FA on the pharmacokinetics of sLip in mice. (A) ICR mice were injected i.v. with FA 30 min before sLip administration on day 0. Blood of each mouse was sampled for ELSIA assay on Day 5. sLip/DiD were injected i.v. on Day 6 to investigate the effects of FA on the pharmacokinetics of sLip. (B) Anti-sLip IgM levels were detected on the sLip precoated ELISA plates. (C) Lipid concentrations of sLip/DiD in plasma of the pretreated mice. (D) Normalized AUC values compared to blank group. (E) Correlation analysis between AUC values and anti-sLip IgM levels. (F) Lipid concentrations of sLip/DiD in the liver 24 h post-administration. Data are means ± SDs ($n = 4$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ by one-way ANOVA test.

of Doxil (Fig. S1 in Supporting information). To investigate the ABC phenomenon, mice were initially injected with a low dose of sLip to induce sLip-specific antibodies and study the pharmacokinetics of the sLip administered subsequently (Fig. 1A). All animal experiments were approved by the Animal Experimental Ethics Committee of Naval Medical University. As shown in Fig. 1B, notable elevated levels of sLip-specific IgM were induced by administration of sLip. Conversely, pre-treatment with FA significant mitigated the production of sLip-specific IgM as well as PEG-specific IgM, detected using PEG-coated ELISA plates (Fig. S2 in Supporting information). In addition, the reduction was observed to be dose-dependent with FA. In our previous study, we found that FA-functionalized caused enhanced immunogenicity of sLip [18,21,22]. Interestingly, pre-treatment with FA attenuated the production of FA-functionalized sLip (FA-sLip)-specific IgM to some extent, indicating the potential of FA in ameliorating the immunogenicity of targeted nanoparticles (Fig. S3 in Supporting information). The subsequent pharmacokinetic profiles of sLip were evaluated in the pre-treated mice. As shown in Figs. 1C and D, a prominent ABC phenomenon was witnessed of the sLip group, resulting in a nearly halved area under the curve (AUC) value. In contrast, FA pre-treatment efficiently mitigated the ABC phenomenon of the post-administered sLip. It has been well established that IgM is the main culprit of ABC phenomenon [23,24]. Consistently, as depicted in Fig. 1E, the AUC values exhibited a negative correlation with anti-sLip IgM levels. The accumulation of sLip in liver was also reduced by FA pre-treatment (Fig. 1F). The above results indicated that FA effectively alleviated the ABC phenomenon of sLip by reducing the production of sLip-specific IgM.

Splenic MZB cells play a pivotal role in the immunogenicity observed with PEGylated liposomal nanomedicines [14,15,20]. To investigate the inhibitory effect of FA on the uptake of sLip by splenic MZB cells, mice were injected with a high dose of FA prior to the administration of sLip/DiD. As shown in Fig. 2A, a substantial decrease in the uptake of sLip by splenic MZB cells ($CD19^{+}CD21^{high}CD23^{low}$) was found. Since splenic MZB cells possess the ability to secrete IgM in a T cell-independent manner,

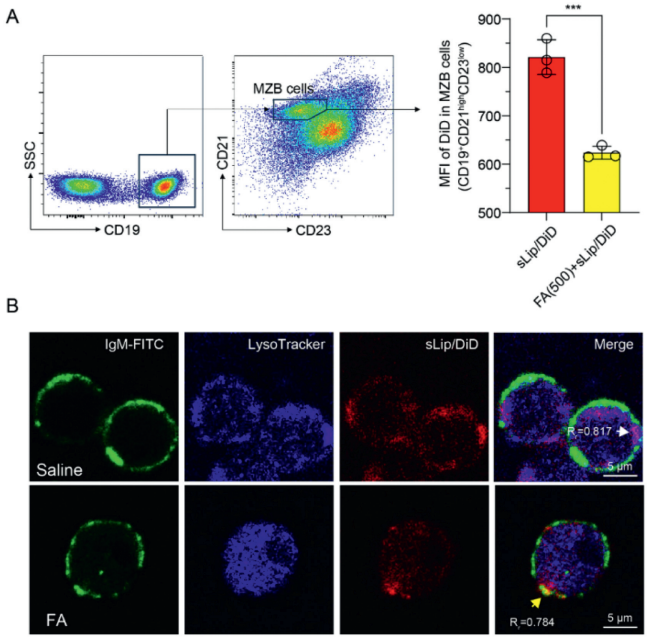

Fig. 2. Effect of FA on the splenic MZB cellular endocytosis of sLip. (A) MFI of DiD in $CD19^{+}CD21^{high}CD23^{low}$ splenic MZB cells 2 h after i.v. injection of sLip/DiD using flow cytometry assay ($n = 3$). (B) Representative confocal microscopic images of MZB cellular uptake of sLip/DiD. MZB cells were labeled with anti-IgM antibodies (green) and lysosomes were stained with LysoTracker dyer (blue). The white arrow indicated the colocalization of sLip with lysosomes and the yellow arrow indicated the colocalization of sLip with mIgM. Scale bar: 5 μm. Data are means ± SDs ($n = 3$). $^{***}P < 0.001$ by Student's *t*-test.

the lysosomal processing of sLip *in vitro* was further explored. As shown in Fig. 2B, in the absence of FA pretreatment, the internalized sLip merged well with lysosomes with a Pearson's correlation $R_r = 0.817$. However, the presence of FA not only decreased the uptake of sLip but also inhibited the lysosomal processing of

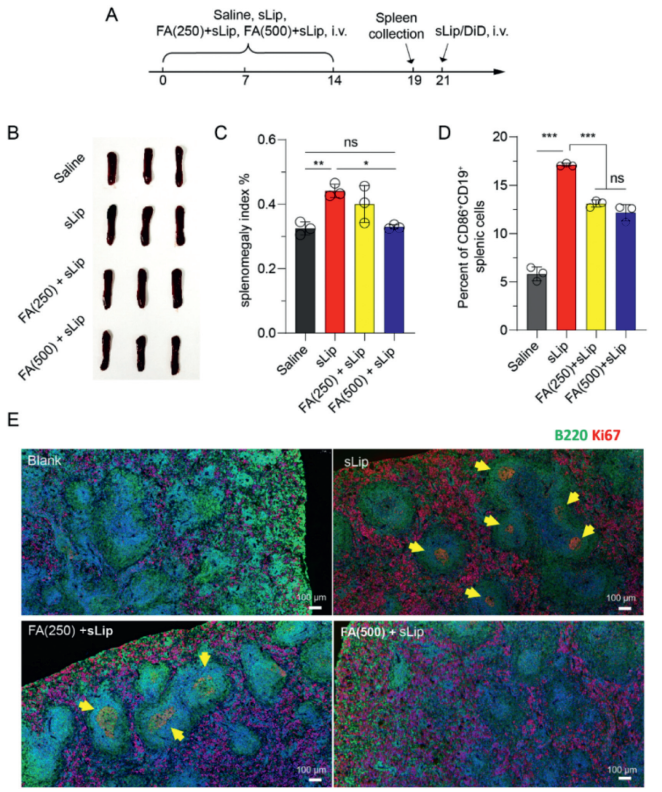

Fig. 3. Effect of FA on sLip-induced proliferation of splenic lymphocytes. (A) ICR mice received 3 consecutive injections of FA plus sLip administration on Days 0, 7 and 14. Spleens were collected for proliferation analysis on Day 19 and the remaining mice were injected i.v. with sLip/DiD on day 21 for intrasplenic distribution. (B) Pictures of spleens from mice on Day 19. (C) Splenomegaly index according to the ratios of spleens weight to body weight. (D) Analysis of frequency of $CD86^+$ splenic B cells using flow cytometry. (E) Representative microscopic immunofluorescence images of spleens. Nuclei were stained with DAPI (blue), proliferative splenic B cells were labeled with anti-B220 antibodies (green) and Ki67 (red). The yellow allows indicate germinal centers. Scale bar: 100 μm. Data are means ± SDs ($n = 3$). ns, non-significant. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ by one-way ANOVA test.

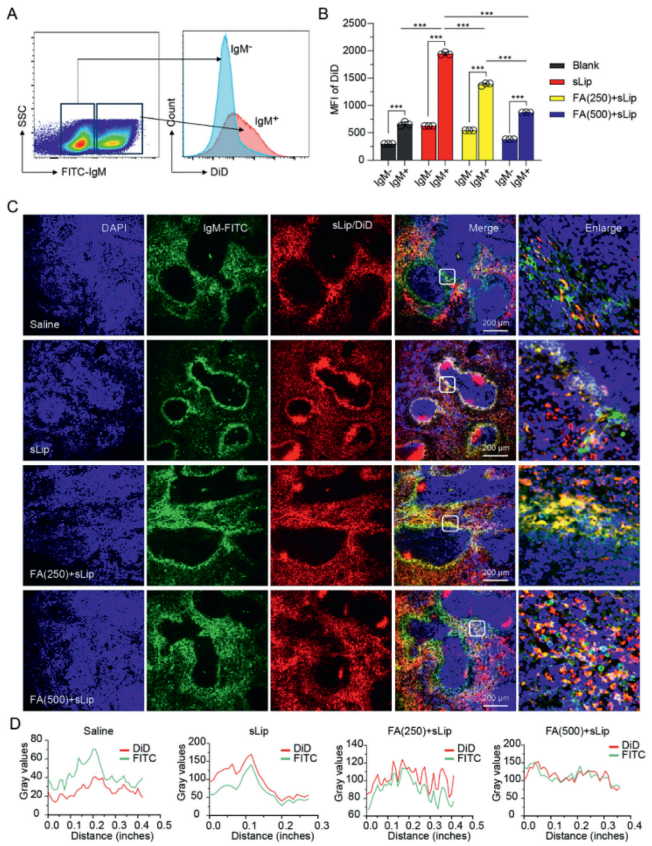

Fig. 4. Effect of FA on the intrasplenic distribution of sLip. (A) Flow sorting of IgM^+ MZB cells and (B) analysis of cellular uptake of sLip/DiD. (C) Representative confocal microscopic images of frozen sections of spleens 24 h after injections of sLip/DiD on Day 21. Nuclei were stained with DAPI (blue) and MZB cells were labeled with anti-IgM antibodies (green). Scale bar: 200 μm. (D) Plot profiles according to the rectangular areas in C. Data are means ± SDs ($n = 3$). $^{***}P < 0.001$ by Student's *t*-test.

sLip. The sLip merged well with mIgM with a Pearson's correlation $R_r = 0.784$.

To examine the effect of FA on the multiply dosing of sLip, mice underwent three consecutive weekly injections of sLip (Fig. 3A). Repeated administration of sLip triggered the spleens to secret a robust production of antibodies, initially represented by IgM and subsequently switched to IgG as shown in Fig. S4 (Supporting information). Splenomegaly, a visual indicator of splenic lymphocyte proliferation, was evident in the sLip-stimulated group as shown in Figs. 3B and C. However, FA pre-treatment effectively decreased the splenomegaly index. The percent of $CD86^+$ splenic B cells in FA groups was also reduced compared to that of sLip group (Fig. 3D). Spleen slices were prepared to directly observe the lymphocyte proliferation as shown in Fig. 3E. Notable formation of germinal centers were found in the spleen of the sLip-repeated mice. In contrast, FA pre-treatment efficiently suppressed the formation of germinal centers in a dose-dependent manner. Collectively, the above results suggest that FA pre-treatment effectively inhibited sLip-induced splenic lymphocyte proliferation.

An additional injection of sLip/DiD was administered to the sLip-stimulated mice to examine the intrasplenic distribution. As shown in Figs. 4A and B, the splenic MZB cells (IgM^+) proficiently internalized the injected sLip/DiD, yet this uptake was significantly attenuated upon pretreatment with FA. Furthermore, Figs. 4C and D revealed that repeated stimulation with sLip triggered a significant proliferation of splenic MZB cells, identified by IgM labeling around the white pulp. The sLip/DiD tended to aggregate in the marginal zone and colocalized well with the splenic MZB cells. However, FA pretreatment, particularly at higher dose, efficiently inhibited the proliferation of splenic MZB cells, resulting in a more homogeneous distribution of sLip/DiD throughout the spleen.

PEGylated liposomal nanomedicines encounter dose-limiting skin toxicity due to the accumulation of liposomes in the dermis and the subsequent local release of the anticancer drug [25,26]. Mice were initially injected with three consecutive weekly injections of sLip, followed by one injection of sLip/DiD. As shown in Figs. S5A and B (Supporting information), a notable accumulation of sLip/DiD was observed in the ears. Nevertheless, pretreatment with high doses of FA effectively mitigated this accumulation. Furthermore, FA pre-treatment also demonstrated a suppressive effect of sLip skin accumulation in single injection as shown in Figs. S5C and D (Supporting information).

In summary, the present study delved into the role of FA in reducing the immunogenicity of PEGylated liposomes and enhancing their *in vivo* performance. Our experiments found that FA significantly inhibited the internalization of sLip by splenic MZB cells, thereby reducing the immune response triggered by the sLip. Additionally, FA reduced splenic lymphocyte proliferation, decreased sLip-specific IgM secretion, effectively alleviated IgM-mediated ABC phenomenon, and significantly mitigated the adverse accumulation of sLip in the skin. Although similar studies have been reported to used free PEG to inhibit the induction of anti-PEG antibodies, prolong the circulation time, and attenuate allergic reactions of PE-

Gylated liposomes [17,19]. In contrast, as FA has long been widely used in clinics and in life, there is a higher degree of public acceptance for FA. In the future, it is possible to develop lyophilized formulations of free FA with liposomal drugs for clinical applications. Overall, these findings provide new insights into the immunomodulatory effects of FA and offer new strategies for optimizing the efficacy and safety of PEGylated liposomal nanomedicines.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Xiying Wu: Writing – original draft, Methodology, Investigation, Funding acquisition. **Anze Liu:** Methodology, Investigation, Data curation. **Yuzhong Yan:** Validation, Supervision, Methodology, Formal analysis, Data curation. **Ying Lu:** Writing – review & editing, Supervision, Resources, Investigation. **Huan Wang:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (Nos. 82373817 and 82003659), Shanghai Natural Science Foundation (No. 23ZR1477500) and Pudong Health Bureau of Shanghai (No. YC-2023-0401).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cclet.2024.110285.

References

[1] X. Li, J. Gu, Q. Xiao, et al., Chin. Chem. Lett. 34 (2023) 107483.
[2] M.J. Mitchell, M.M. Billingsley, R.M. Haley, et al., Nat. Rev. Drug Discov. 20 (2021) 101–124.
[3] M. Fu, X. Han, B. Chen, et al., Acta Mater. Med. 1 (2022) 486–506.
[4] K. Elumalai, S. Srinivasan, A. Shanmugam, Biomed. Tech. 5 (2024) 109–122.
[5] L.J. Kubiatowicz, A. Mohapatra, N. Krishnan, R.H. Fang, L. Zhang, Exploration 2 (2022) 20210217.
[6] Y. Li, S. Jacques, H. Gaikwad, et al., Nat. Nanotechnol. 19 (2024) 246–254.
[7] M. Ibrahim, E. Ramadan, N.E. Elsadek, et al., J. Control. Release 351 (2022) 215–230.
[8] Y.C. Barenholz, J. Control. Release 160 (2012) 117–134.
[9] M. Hu, X. Li, Z. You, R. Cai, C. Chen, Adv. Mater. 36 (2023) e2303266.
[10] X. Wang, H. Wang, K. Jiang, et al., J. Control. Release 293 (2019) 201–214.
[11] Y. Bavli, B. Chen, G. Gross, et al., J. Control. Release 354 (2023) 316–322.
[12] G. Miao, Y. He, K. Lai, et al., J. Control. Release 363 (2023) 12–26.
[13] G.T. Kozma, T. Shimizu, T. Ishida, J. Szebeni, Adv. Drug Deliv. Rev. 154-155 (2020) 163–175.
[14] F. Martin, J.F. Kearney, Nat. Rev. Immunol. 2 (2002) 323–335.
[15] A. Cerutti, M. Cols, I. Puga, Nat. Rev. Immunol. 13 (2013) 118–132.
[16] P. Schriek, A.C. Ching, N.S. Moily, et al., Science 375 (2022) eabf7470.
[17] M.D. Mcsweeney, L. Shen, A.C. Dewalle, et al., J. Control. Release 329 (2021) 774–781.
[18] H. Wang, S. Lin, X. Wu, et al., Adv. Sci. 10 (2023) e2301777.
[19] L. Shen, Z. Li, A. Ma, et al., ACS Nano 18 (2024) 8733–8744.
[20] H. Wang, Z. Jiang, Z. Guo, et al., Acta Pharm. Sin. B 14 (2024) 808–820.
[21] H. Wang, T. Ding, J. Guan, et al., ACS Nano 14 (2020) 14779–14789.
[22] H. Wang, S. Lin, S. Wang, et al., Nano Lett. 22 (2022) 6516–6522.
[23] T. Ding, J. Guan, M. Wang, et al., J. Control. Release 319 (2020) 371–381.
[24] J. Fu, E. Wu, G. Li, B. Wang, C. Zhan, Nano Today 55 (2024) 102163.
[25] Y. Li, L. Lofchy, G. Wang, et al., ACS Nano 16 (2022) 6349–6358.
[26] J.I. Griffin, G. Wang, W.J. Smith, et al., ACS Nano 11 (2017) 11584–11593.