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Transport of PEGylated liposomes from the splenic marginal zone to the follicle in the induction phase of the accelerated blood clearance phenomenon

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ABSTRACT

The accelerated blood clearance (ABC) phenomenon has been reported to enhance the clearance of PEGylated liposomes from the blood circulation when the liposomes are injected into the same animal repeatedly. We have shown that anti-PEG IgM production from splenic B cells is crucial in the ABC phenomenon. In this study, we describe the crucial role of marginal zone (MZ) B cells in the anti-PEG IgM production and recognition of PEGylated liposomes in the induction phase of ABC phenomenon. Suppression of the anti-PEG IgM production was correlated with the disappearance of IgM^{high} cells in the MZ, particularly MZ-B cells, following cyclophosphamide (CPA)-treatment, confirming that splenic MZ-B cells are responsible for anti-PEG IgM production. The MZ-B cells stimulated by a first dose of PEGylated liposomes internalized the second dose of PEGylated liposomes in a PEG modification-dependent manner and transported the liposomes into the follicle (FO) region. To the best of our knowledge, this is the first report showing that PEGylated liposome is recognized by MZ-B cells and transported to the FO region like blood-borne antigens or immune complexes. It is likely that PEGylated liposomes are recognized as a TI-2 antigen by the first line of defense against life-threatening infections by blood-borne organisms. Our study may have implications for immunogenicity of synthesized polymer-grafted therapeutics including nanocarriers, nucleic acids and proteins.

Introduction

It is well recognized that the presence of polyethylene glycol (PEG) on the surface of liposomes attracts a water shell, providing a steric barrier against plasma proteins or opsonins (Lasic et al. 1991; Senior et al. 1991; Torchilin et al. 1994). This, in turn, results in a decrease in both the rate and extent of uptake of liposomes by the cells of the mononuclear phagocyte system (MPS). Finally, intravenously injected PEGylated liposomes exhibit long circulating properties. Such PEGylated liposomes have been widely used as a carrier in drug delivery to improve the lifetime of encapsulated therapeutic agents (Allen et al. 1991; Klibanov et al. 1990; Papahadjopoulos et al. 1991).

Despite this defined concept, unexpected alterations in the pharmacokinetic behavior of PEGylated liposomes were observed when repeatedly injected at certain intervals into mice, rats or rhesus monkeys (referred to as the accelerated blood clearance (ABC) phenomenon) (Dams et al. 2000; Ishida et al. 2003; Laverman et al. 2001). An intravenous injection of PEGylated liposomes causes a second dose, injected a few days later, to lose its long-circulating characteristics and accumulate extensively in the liver. By further studies of our group, it was elucidated that anti-PEG IgM, produced from spleen in response to an injected first dose of PEGylated liposomes, is responsible for the alteration in the pharmacokinetics of a subsequent dose of PEGylated liposomes (Ishida et al. 2006b,c; Wang et al. 2007). Besides, the anti-PEG IgM production was detected in nude mice (Ishida et al. 2007), lacking T cells, but not in splenectomized mice (Ichihara et al. 2011) indicating that the anti-PEG IgM is secreted from splenic B cells without T-cell help. Furthermore, we observed that the level of anti-PEG IgM in serum began to increase at day 3, peaked at day 5, and then gradually decreased again (Ichihara et al. 2011; Ishida et al. 2007). It seems that two to three days are necessary for splenic B cells to mature and release anti-PEG IgM into the blood stream following injection of PEGylated liposomes.

The spleen is a crucial secondary lymphoid organ in the body's response against blood-borne antigens (Cyster 2000). The bulk of

Abbreviations: ABC, accelerated blood clearance; Chol, cholesterol; CPA, cyclophosphamide; DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; DiO, 3,3′-dioctadecyloxacarbocyanine perchlorate; FO, follicle; HEPC, hydrogenated egg phosphatidylcholine; HRP, horseradish peroxidase; $mPEG_{2000}$-DSPE, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-n-[methoxy (polyethylene glycol)-2000]; MPS, mononuclear phagocyte system; MZ, marginal zone; PE, phycoerythrin; PEG, polyethylene glycol; TI, T-cell independent; TI-1, T-cell independent type 1; TI-2, T-cell independent type 2.

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the spleen is composed of red pulp, which is the site of red blood cell disposal. The lymphocytes surround the arterioles entering the organ, forming areas of white pulp. The marginal zone (MZ) of the spleen is located at the border between the white pulp and red pulp, and outside the marginal sinus. Much of the antigen entering the spleen is retained at the marginal sinus, from where it can percolate through the marginal zone into the red pulp. The architectural structure of the MZ results in a strongly reduced blood-flow allowing intimate contact between antigens and effector cells (Harms et al. 1996). Thus, B cells in the MZ (MZ-B cells) are easily and continuously exposed to blood as opposed to B cells in the follicles (follicular B (FO-B) cells) and extensively contribute to a rapid first line of defense able to produce large amounts of specific IgM within 3–4 days after antigen stimulation (Martin and Kearney 2001; Zandvoort and Timens 2002). We therefore assumed that among the splenic B cells the MZ-B cells are responsible for the anti-PEG IgM production in the ABC phenomenon. However, so far, direct evidence showing the contribution of MZ-B cells to the anti-PEG IgM response was lacking. In addition, we had not yet elucidated how the MZ-B cells are activated by the first dose of PEGylated liposomes.

In this study, we investigated the involvement of MZ-B cells in anti-PEG IgM production by depletion of MZ-B cells by means of an intravenous injection of cyclophosphamide. In addition, we studied the behavior of the MZ-B cells in the spleen after the first dose of PEGylated liposomes with fluorescently labeled PEGylated test liposomes as an marker of activated, anti-PEG IgM over-expressing, MZ-B cells.

Materials and methods

Materials

Hydrogenated egg phosphatidylcholine (HEPC) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-n-[methoxy (polyethylene glycol)-2000] ($mPEG_{2000}$-DSPE) were generously donated by NOF (Tokyo, Japan). Cholesterol (Chol) was purchased from Wako Pure Chemical (Osaka, Japan) and cyclophosphamide monophosphate (CPA) was purchased from Sigma (MO, USA). DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate) and DiO (3,3′-dioctadecyloxacarbocyanine perchlorate) were purchased from Invitrogen (Paisley, UK). All other reagents were of analytical grade.

Animals

Male Wistar rats (250–300 g) were purchased from Japan SLC (Shizuoka, Japan). All animal experiments were evaluated and approved by the Animal and Ethics Review Committee of the University of Tokushima.

Preparation of liposomes

PEGylated liposomes composed of HEPC:$mPEG_{2000}$-DSPE:Chol (1.85:0.15:1 molar ratio) and non-PEGylated conventional liposomes composed of HEPC:Chol (2:1 molar ratio) were prepared as described previously (Ishida et al. 2003). To detect the liposome distribution in spleen and their association with spleen cells, the liposomes were labeled with the hydrophobic fluorescent dye DiI or DiO (1 mol% of liposomal phospholipids). The mean diameter of the resulting liposomes was determined by using a NICOMP 370 HPL submicron particle analyzer (Particle Sizing System, CA, USA), and was 102 ± 12 nm for PEGylated liposomes and 109 ± 15 nm for conventional liposomes, respectively. Phospholipid concentration in the liposome preparations was determined by colorimetric assay (Bartlett 1959).

ELISA for detection of anti-PEG IgM

A simple ELISA as described previously was employed to detect anti-PEG IgM (Ishida et al. 2007). Briefly, 10 nmol of PEG_{2000}-DSPE in 50 μl ethanol was added to each well of a 96-well plate. The plate was allowed to air dry completely for 2 h. The lipid-coated plates were then blocked for 1 h with Tris-buffered saline (pH 8.0) containing 1% BSA and were subsequently washed three times with Tris-buffered saline (pH 8.0) containing 0.05% Tween 20 (wash solution). Diluted serum samples (1:100) (100 μl) were then applied to the wells, incubated for 1 h and washed five times with wash solution. Horseradish peroxidase (HRP)-conjugated antibody (100 μl, 1 μg/ml, Goat anti-rat IgM-HRP conjugate; Bethyl Laboratories, TX, USA) was added to the wells. After 1 h incubation, the wells were washed five times with wash solution. The coloration was initiated by adding 100 μl of *o*-phenylenediamine (1 mg/ml) (Sigma, MO, USA). After a 15-min incubation, the reaction was stopped by adding 100 μl of 2 M H_2SO_4. The absorbance was measured at 490 nm using a microplate reader (Wallac1420 ARVOsx, PerkinElmer Life Science). All incubations were performed at room temperature.

Depletion of MZ-B cells by CPA treatment

Depletion of splenic MZ-B cells with CPA was carried out according to a previously described method (Zandvoort et al. 2001). CPA, freshly dissolved in sterile saline at 20 mg/ml, was intravenously injected at a dose of either 30 or 40 mg/kg. Instead of CPA solution, saline was intravenously injected as a control. Eight days later, MZ-B cells in the spleen were detected by flow cytometry as described below.

Distribution of PEGylated liposomes in spleen

To evaluate the distribution of PEGylated liposomes in spleen, DiI-labeled PEGylated liposomes or DiO-labeled PEGylated liposomes (5 μmol/kg rat) were intravenously injected into rats. At 24 h post-injection, spleens were harvested and snap-frozen in optimal cutting temperature compound (Sakura Fintechnical, Tokyo, Japan) by dry-iced acetone. Sections of frozen samples (7 μm thick) were immediately examined using an AxioImager A1 (Zeiss, Oberkohen, Germany) and processed with Photoshop software (Adobe Systems, CA, USA). DiI was visualized using 549 nm excitation and 565 nm emission filter sets. DiO was visualized using 470 nm excitation and 525 nm emission filter sets.

Immunohistochemical analysis

For immunohistochemical analysis, after acquiring the image of liposome distribution in the frozen spleen section, frozen sections were subsequently fixed by acetone and were stained with FITC-labeled anti-rat IgM, or mouse anti-rat MZ-B (His57, Becton Dickinson, NJ, USA) and then phycoerythrin (PE)-labeled anti-mouse IgG (H&L, Rockland Immunochemicals, PA, USA). Images were acquired by AxioImager A1 and processed with Photoshop software.

Preparation of spleen cell suspensions and flow cytometry

Spleen cell suspensions were prepared as described previously (Ishida et al. 2007). Briefly, spleen slices were squeezed through a Cell Strainer (100 μm, Becton Dickinson, NJ, USA). The cells were suspended in PBS containing 0.5 mM EDTA (EDTA-PBS). Red blood

cells in the suspension were lysed by treatment with ammonium chloride lysis buffer (0.83% NH_4Cl) for 5 min on ice. Cells were washed twice with EDTA-PBS and filtered to remove clumps using a Cell Strainer.

For flow cytometry analysis, Guava EasyCyte Mini System (GE Healthcare Bio-Sciences, Uppsala, Sweden) was used. DiI-labeled liposomes associated with spleen cells were detected at 488 nm excitation and 580–583 nm emission. In addition, in order to distinguish spleen cell populations, the cells were stained with FITC-labeled anti-rat IgM (H&L, American Qualex International, CA, USA) and analyzed at 488 nm excitation and 525 nm emission. MZ-B cells were gated on IgM^{high} population and FO-B cells were gated on IgM^{low} population. The data were analyzed with WinMDI version 2.9 (The Scripps Research Institute, CA, USA).

Statistics

All values are expressed as the mean ± SD. Statistical analysis was performed with a two-tailed unpaired *t*-test using GraphPad InStat software (GraphPad Software, La Jola, CA, USA). The level of significance was set at $p < 0.05$.

Results

Anti-PEG IgM production in CPA-treated rats

Reduction of splenic MZ-B cells was performed by intravenous injection of CPA according to Peset Llopis et al. (1996). Histochemical evaluation demonstrated that the area occupied by IgM^+ cells in the MZ decreased as compared to the area of IgM^+ cells in the follicular (FO) region in a CPA-dose dependent manner (Fig. 1A). Intravenous injection of PEGylated liposomes at low dose (0.001 μmol/kg) induced a significant anti-PEG IgM response in non-treated rats (Fig. 1B), which is consistent with our previous reports (Wang et al. 2007). The immune response against PEG was attenuated by pre-treatment with CPA in a CPA-dose dependent manner. These results show that MZ-B cells, the IgM^+-B cells in MZ (Peset Llopis et al. 1996), are responsible for the anti-PEG IgM response following intravenous injection of PEGylated liposomes.

Identification of the cell population over-expressing anti-PEG IgM

We then determined if the MZ-B cells activated by PEGylated liposomes over-express anti-PEG IgM. DiI-labeled test liposomes with or without PEGylation were used as an indicator of anti-PEG IgM over-expressing cells. Low-dose PEGylated liposomes (0.001 μmol/kg) were intravenously injected to trigger the immune response, as shown in Fig. 1B. On day 2 following the injection, DiI-labeled test liposomes were intravenously injected on the basis of our assumption that anti-PEG IgM is retained mainly on the surface of the over-expressing cells, and not released into the blood stream (Ishida et al. 2007). Twenty-four hours later, we identified the cell population to which the DiI-labeled test liposomes were associated in the spleen cell suspension. In the non-B cell population ($IgM^{negative}$), minor association of PEGylated or conventional liposomes was detected irrespective of administration of a pre-dose (Fig. 2A). In the FO-B cell population (IgM^{low}), a somewhat higher level of association was detected, but only of the PEGylated liposomes, not of the conventional liposomes, and also in this case irrespective of pre-dosing (Fig. 2B). In the MZ-B cell population (IgM^{high}), extensive association of PEGylated liposomes was detected, but not of conventional liposome (Fig. 2C). These findings indicate that MZ-B cells over-express IgM reactive to PEGylated liposomes in response to a low intravenous pre-dose of PEGylated liposomes.

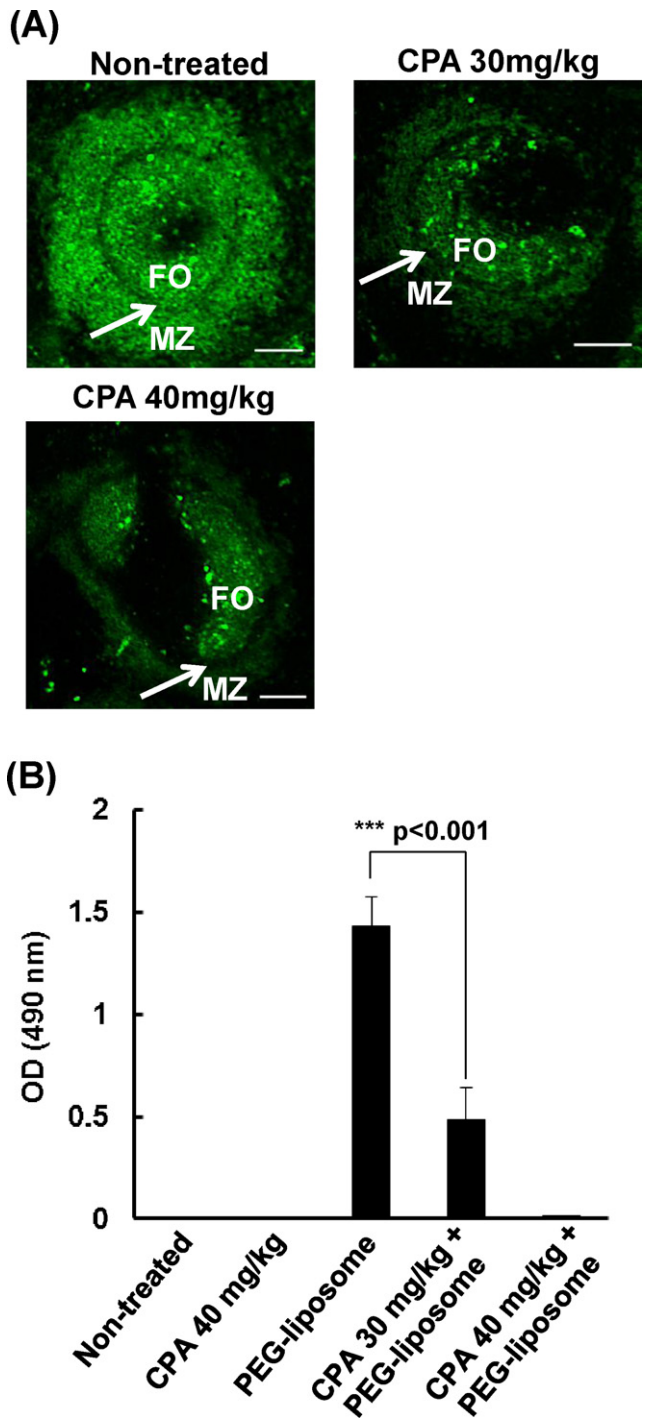

Fig. 1. Anti-PEG IgM production in CPA-treated rats. (A) Reduction of splenic MZ-B cells by CPA. At day 8 following intravenous injection of CPA (30 or 40 mg/kg), IgM^{high} cells in the spleen were visualized immunohistochemically. (B) Effect of depletion of IgM^{high} cells in the MZ on anti-PEG IgM production. On day 8 following CPA-treatment, a low dose of PEGylated liposomes (0.001 μmol/kg) was intravenously injected to induce the anti-PEG IgM response. Five days later serum was collected and anti-PEG IgM was determined. Each value represents the mean ± SD ($n = 3$). *** $p < 0.001$.

Distribution of second-dose PEGylated liposomes in the spleen

The distribution of second-dose test PEGylated liposomes in the PEGylated liposome-treated spleen was studied histologically (Fig. 3). Pre-injection of low-dose PEGylated liposomes (0.001 μmol/kg) resulted in substantially enhanced splenic

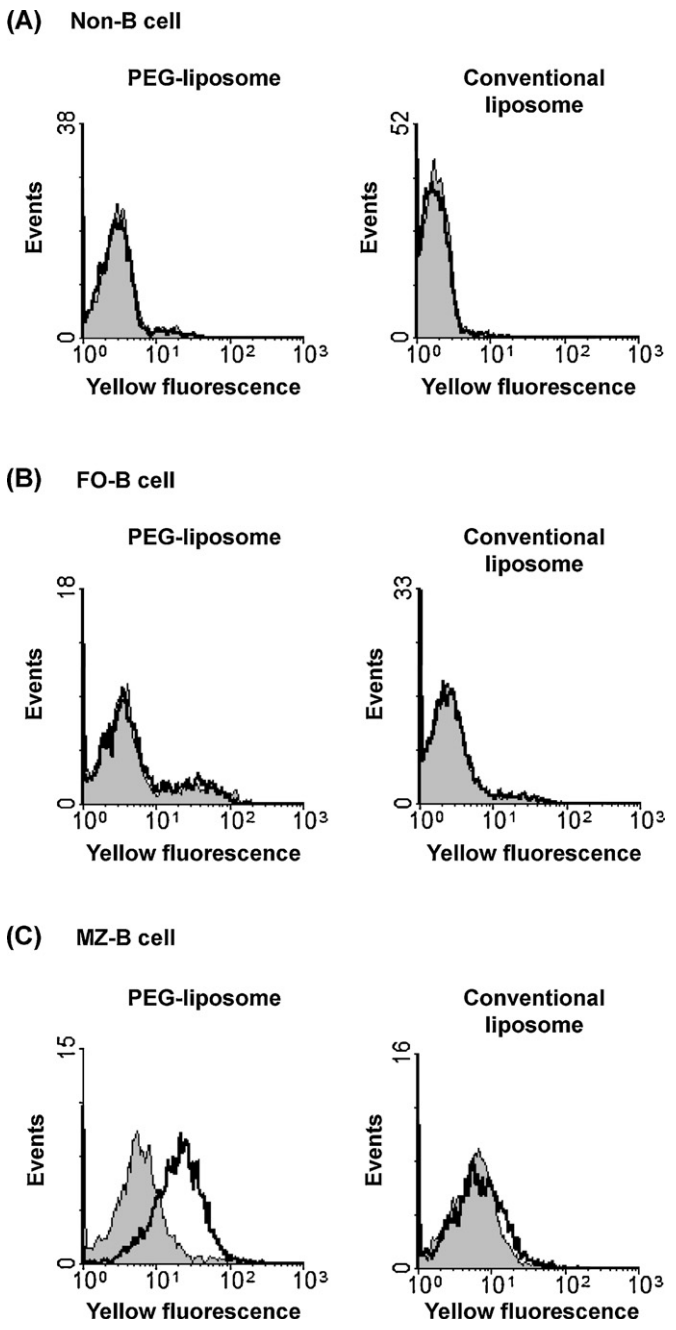

Fig. 2. Cell population associated with the second-dose PEGylated test liposomes in spleen. As a first dose, PEGylated liposomes or conventional liposomes were intravenously injected into rats at low dose (0.001 μmol/kg). Two days later, DiI-labeled PEGylated liposomes or conventional liposomes were intravenously injected. At 24 h post injection, spleen cell suspensions were analyzed by flow cytometry. (A) Non-B cell population. (B) FO-B cell population. (C) MZ-B cell population. The thin line represents control spleen cells. The data shown are representative of three independent experiments.

accumulation of the second-dose test liposomes (Fig. 3B) surprisingly mainly in the follicle (FO) region, and not in the MZ. On the other hand, pre-injection of a higher dose (5 μmol/kg), which does not promote anti-PEG IgM induction (Wang et al. 2007), did not lead to enhanced accumulation of the test dose in the spleen (Fig. 3C). In this case, a small amount of the test dose was mainly scattered in the area surrounding of FO region, similar to the observation in control (non-treated) spleen (Fig. 3A).

Transport of PEGylated liposomes from the MZ to the FO region

While from Figs. 1 and 2 it is clear that MZ-B cells over-express anti-PEG IgM, Fig. 3 shows that second-dose PEGylated test liposomes extensively accumulated in the FO region, where generally no MZ-B cells are found. To elucidate this discrepancy a time course study was performed on the intra-splenic distribution of second-dose PEGylated test liposomes in rats pretreated with low-dose PEGylated liposomes 3 days before (Fig. 4). While at the earliest time point (10 min) the test dose accumulated extensively in the MZ, but not in the FO region, it gradually moved from the MZ to the FO region, passing through the barrier between the MZ and the FO area, the so called marginal sinus, at 30 min. At 2 h, the test dose was already concentrating in the FO region, while at 24 h it was completely localized there, which is consistent with our earlier observation (Fig. 3B).

In a further approach to assess the transport of PEGylated liposomes from the MZ to the FO region, we sought to determine whether any MZ-B cells that became labeled with the test-dose liposomes might become refractory to association with a subsequently third dose of PEGylated liposomes. To initiate the immune response, a low dose of PEGylated liposomes (0.001 μmol/kg) was injected. Three days later, sequential doses of DiI-labeled PEGylated liposomes (second dose; red) and DiO-labeled PEGylated liposomes (third dose; green) were injected with a 4-h interval. We reasoned that, if the DiI-labeled MZ-B cells were rapidly migrating to the FO region, due to the architecture of the MZ, the third dose (DiO-labeled) should be not able to come in contact with the same MZ-B cells. As can be seen in Fig. 5A, the second dose (red), injected 4 h before the third, had already accumulated in FO region and concentrated there in a time-dependent manner (Fig. 5A). The third dose PEGylated liposomes gradually but massively moved into FO region. The third dose began to associate with the cells in the MZ at 10 min after injection and by 1 h post-injection it localized in MZ surrounding FO region where the second dose had already accumulated. At 4 h post-injection, the third dose had accumulated in FO region surrounding the second dose and partly co-localized with the second dose (yellow). By contrast, simultaneously injected DiI-labeled PEGylated liposomes and DiO-labeled PEGylated liposomes perfectly co-localized in the FO region at 4 h post-injection. These findings collectively indicate that the second dose and third dose, when injected with a 4-h interval, migrated separately from the MZ to the FO region by means of MZ-B cells.

Discussion

The first report concerning the ABC phenomenon came from Dams et al. (2000). They showed that upon injection of PEGylated liposomes, a second dose of PEGylated liposomes was cleared very rapidly from the blood when the interval between the first and second injection was between 5 and 21 days. Our research group expanded their research to elucidate the underlying mechanism of the phenomenon. On the basis of our results (Ishida et al., 2006b,c), we proposed the following tentative mechanism for the cause of this phenomenon: anti-PEG IgM, which is produced in the spleen in response to a first dose, selectively binds to the PEG of the second dose liposomes injected several days later and subsequently activates the complement system. As a consequence, these liposomes are avidly taken up by the Kupffer cells in the liver. In addition, we established that PEGylated liposomes elicit an anti-PEG IgM response without T-cell help (Ishida et al. 2007; Koide et al. 2010). Laverman et al. (2001) distinguished two phases in the ABC phenomenon: the induction phase, following the first injection in which the immune system is primed (reflected in the production of anti-PEG IgM) and the effectuation phase, following the second

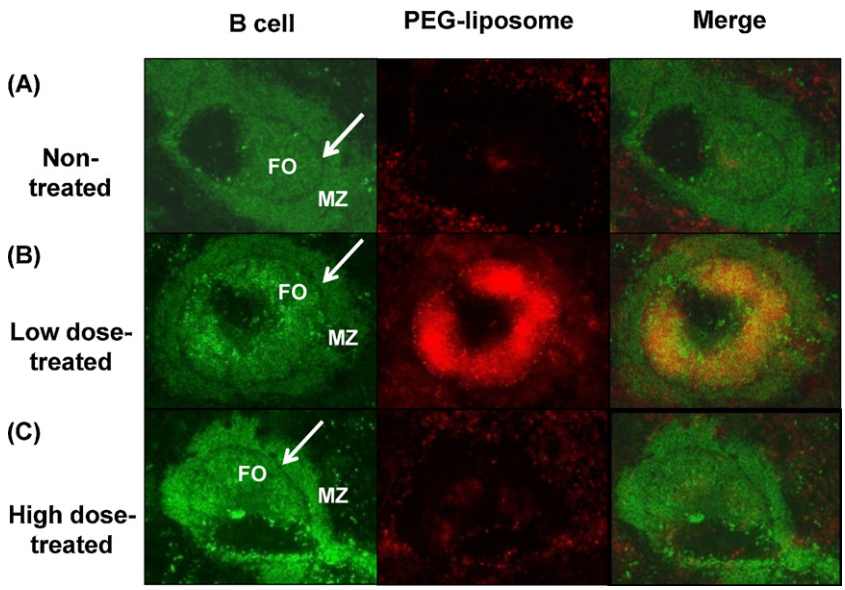

Fig. 3. Distribution of second-dose PEGylated test liposomes in spleen. PEGylated liposomes, either at low dose (0.001 μmol/kg) or high dose (5 μmol/kg) were intravenously injected into rats. Two days later, DiI-labeled PEGylated test liposomes (red) were injected. At 24 h post injection, thin sections of spleen were examined by fluorescence microscopy following staining with FITC-labeled anti-rat IgM (green). (A) Control spleen. (B) Low dose-treated spleen. (C) High dose-treated spleen. Arrows indicate the marginal sinus. The picture shown is a representative section of 12 slices from three independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

injection in which the PEGylated liposomes are rapidly cleared on the account of the produced anti-PEG IgM. In this study, we showed that, following CPA treatment, the suppression in the anti-PEG IgM production (Fig. 1). This clearly lends support to the notion that splenic MZ-B cells are responsible for the immune response against PEGylated liposomes in the induction phase of ABC phenomenon.

Because of the central position of the spleen in the blood stream, the splenic MZ is the first lymphoid compartment where B cells

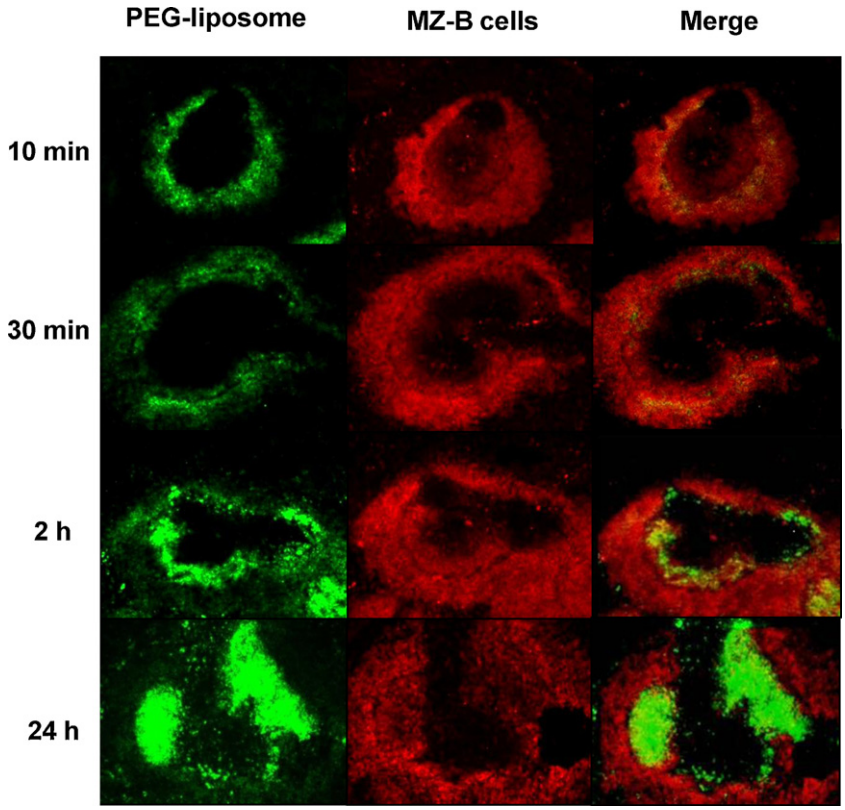

Fig. 4. Time course study on intrasplenic distribution of PEGylated test dose liposomes. To cause an immune response to PEGylated liposomes, a low dose of PEGylated liposomes (0.001 μmol/kg) was intravenously injected into rats. Three days later, DiO-labeled PEGylated liposomes (green) were intravenously injected. At the indicated time points post injection, spleen sections were examined by fluorescence microscopy following staining with anti-rat MZ-B IgG and secondary PE-labeled anti-mouse IgG (red). The picture shown is a representative section of 12 slices from three independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

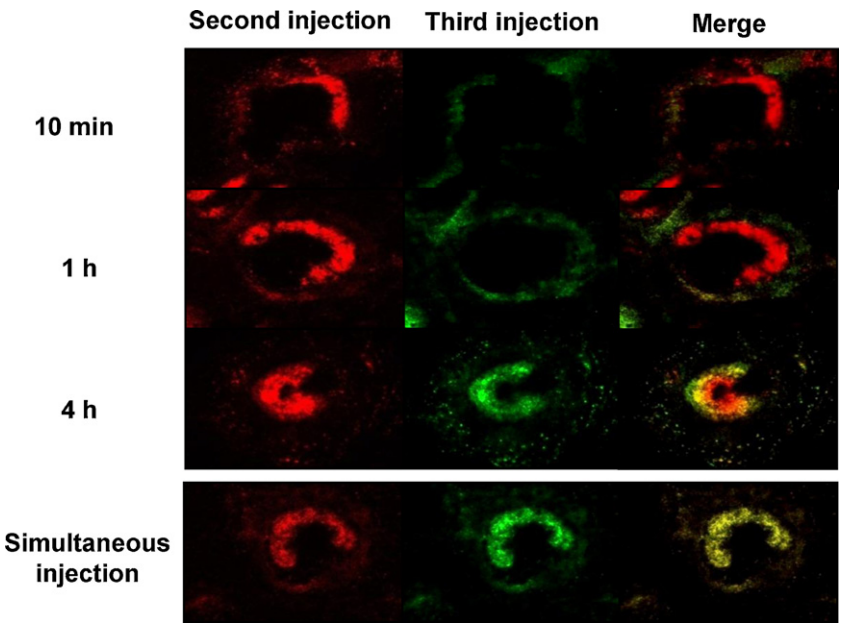

Fig. 5. Transport of test PEGylated liposomes from MZ to FO region by MZ-B cells. To initiate an immune response, a low dose of PEGylated liposomes (0.001 μmol/kg) was intravenously injected into rats. Three days later, DiI-labeled PEGylated liposomes (red) were intravenously injected as a second dose. Four hours later, DiO-labeled PEGylated liposomes (green) were injected as a third dose. At 10 min, 1 h and 4 h after the third injection, the spleens were examined. Intrasplenic distribution of the second dose (DiI-labeled) and third dose (DiO-labeled) PEGylated liposomes. The pictures shown are representative of three independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

meet and can respond to blood-borne antigens such as pathogenic microorganism (Zandvoort and Timens 2002). This implies that the MZ-B cells involve all three major classes of antigens, T-cell dependent (TD) antigen, T-cell independent type 1 (TI-1) antigen and T-cell independent type 2 (TI-2) antigen (MacLennan and Liu 1991). Among their unique futures, MZ-B cells are notably capable of responding more efficiently to TI-2 antigens without highly specific B-cell receptors and the need of specific T-cell contact (Harms et al. 1996; Kraal 1992; Zandvoort and Timens 2002). Martin et al. (2001) reported that MZ-B cells can rapidly produce large amounts of neutralizing IgM against TI-2 antigens within 3–4 days after antigenic stimulation. In our previous study, we observed a similar immune response against first-dose PEGylated liposomes; anti-PEG IgM production occurred onwards from day 3 after the first-dose injection (Ichihara et al. 2011; Ishida et al. 2007). In addition, we observed an anti-PEG IgM response to PEGylated liposomes in nude mice, which lack T-cell populations (Ichihara et al. 2011; Ishida et al. 2007), suggesting that B cells directly respond to PEGylated liposomes without T-cell help. Generally, typical characteristics of TI antigen are large molecular weights, highly repetitive structures and poor *in vivo* degradability (Mond et al. 1995). PEG has a repeating $O–CH_2–CH_2$ subunit structure, typical of TI-2 antigens, such as capsular polysaccharide. In earlier reports, repeating polymers such as polyvinylpyrrolidone and multilamellar liposomes have been put forward as examples of TI-2 antigens (Mosier 1982). Taken together, MZ-B cells appear to recognize the artificial nanocarrier, PEGylated liposomes (i.e. liposomes coated with a polymer consisting of repeating ethyleneglycol units), as TI-2 antigens and rapidly produce anti-PEG IgM to clear such particles from the blood circulation, thus executing their function in the first line of defense against invading pathogens.

In our earlier reports, we demonstrated that there was a strong inverse relationship between the quantity of the first-dose PEGylated liposomes and the extent to which the ABC phenomenon was induced as well as the level of anti-PEG IgM produced: the higher the dose the smaller the phenomenon and the anti-PEG IgM production (Ichihara et al. 2011; Ishida et al. 2005, 2006a; Wang et al. 2007). In this study, the low-dose PEGylated liposomes (0.001 μmol/kg) resulted in more extensively enhanced association of MZ-B cells with the second-dose PEGylated liposomes than the higher dose (5 μmol/kg) (Figs. 2 and 3) presumably as a result of a higher stimulation level. In line with our earlier observations, the lipid dose is apparently critically important for an optimal priming of the immune system in the induction phase of ABC phenomenon. It is known that TI-2 antigens act by simultaneously cross-linking a critical number of B-cell receptors of mature antigen-specific B cells (Janeway et al. 2005) and that optimal antigen dosing is a prerequisite to induce an optimal antibody response against TI antigens in terms of specificity and amount (Dintzis et al. 1989; Morisaki et al. 1983). The extent of B-cell receptor cross-linking by low-dose PEGylated liposomes might be sufficient to activate the cells and promote the production of specific antibody against PEG, i.e. anti-PEG IgM. On the other hand, it has been reported that if the density of TI-2 antigens is too high, the B cell becomes anergic (Janeway et al. 2005). This situation might prevail with the higher dose PEGylated liposomes (5 μmol/kg), which might cause MZ-B cells to induce immune tolerance or anergy. In addition, Gauld et al. (2005) showed that continuous binding of antigens and subsequent receptor signaling are essential for the maintenance of anergy on B cells. Due to prolonged blood circulation of PEGylated liposomes at higher dose (Laverman et al. 2000), splenic MZ-B cells are exposed to PEGylated liposomes for a long time which might also contribute to induction of immune tolerance or anergy.

In the spleen of rats treated with low dose PEGylated liposomes, the binding of the second dose of PEGylated liposomes to MZ-B cells was shown to depend on PEG modification (Fig. 2C). In addition, FO-B cells, the major population of B cells in spleen, and non-B cells did not show binding to the second dose PEGylated test liposomes. These findings also support the concept described above that MZ-B cells are over-expressing anti-PEG IgM following the first-dose injection. However, so far, there are no reports describing the presence of anti-PEG IgM-expressing MZ-B cells. Several previous studies have indicated that among the splenic B cells, at least for the naïve B cells, there may be a sub-population of selected,

multireactive B cells population constituting a B-cell compartment that is able to respond rapidly to a broad spectrum of antigens including TI antigens (Dammers et al. 2000). We very recently observed that an intravenous injection of free PEG_{2000} or PEG_{20000} only induced a substantial anti-PEG IgM response at extremely high dose as compared to liposome-associated PEG (unpublished observation by Mima et al.). Richter and Akerblom (1983) also reported that subcutaneous injection of PEG_{5900} elicits only a weak and transient immune response to PEG. Due to the multireactivity of MZ-B cells to antigens and their stronger response to membrane-associated antigens (Carrasco and Batista 2006), MZ-B cells might associate with and be activated by PEG expressed on the first dose of liposomes.

Interestingly, the second dose PEGylated liposomes rapidly accumulated in MZ and transported from the MZ to the FO region (Figs. 4 and 5). A similar observation was reported with TI-2 antigen, pneumococcal polysaccharides (PPS, Pneumovax®) (Harms et al. 1996). Although MZ-B cells do not recirculate, these cells are known to undergo rapid migration to lymphoid follicles in the FO region after exposure to bacterial infection, contributing to the transport of systemic antigens to follicular dendritic cells (Ferguson et al. 2004; Lopes-Carvalho et al. 2005; MacLennan 2008). To the best of our knowledge, this is the first observation showing that PEGylated liposome is transported by MZ-B cells to the FO region in the same manner as blood-borne antigens. Several research studies indicate that MZ-B cells can shuttle between the MZ and the FO region while transporting antigens (Cinamon et al. 2008; Kraal 2008; MacLennan 2008). Cinamon et al. showed that MZ-B cells can capture TNP-ficoll, a TI-antigen, and shuttle between the MZ and FO regions. In this study, we did not observe shuttling of MZ-B cells associated with the second dose fluorescently labeled PEGylated liposomes between the MZ and FO regions (Figs. 3–5). However, the MZ-B cells which deposit the associated liposomes in the FO region might rapidly return to the MZ and subsequently bind to and transport the following dose of liposomes to the FO region. This possibility is now under investigation in our laboratory.

The TI-2 response provides the host with a combination of "the best of two worlds": the specificity of the adaptive and the rapidity of the innate immune system. In the present study, we showed that, at low dose, PEGylated liposomes are recognized by the host immune system similarly TI-2 antigens. While synthetic polymers and their haptenated counterparts have been traditional examples of the class of TI-2 antigens (Mosier 1982), the demonstration of the TI nature of PEGylated liposomes in this study may provide an alternative of viral glycoprotein antigen as a TI-2 model (Szomolanyi-Tsuda et al. 2001). Many researchers have emphasized the need to understand B-cell activation by TI-2 antigens as an integral part of the humoral immune response to pathogenic microorganisms. We as well as other groups revealed that PEG grafted on a nanocarrier or a protein becomes immunogenic and elicits an anti-PEG IgM response (Ganson et al. 2006; Ishihara et al. 2009; Wang et al. 2007; Kaminskas et al. 2011). Such an anti-PEG IgM may limit the efficacy of any drug encapsulated in the nanocarrier (e.g. liposomes) in patients, if it occurred during treatment. PEG immunogenicity of PEGylated therapeutic agents and the underlying mechanism of the immune response deserve further investigation.

Conclusion

We showed that splenic MZ-B cells are crucial in the PEG-liposome-mediated ABC phenomenon which promotes an anti-PEG IgM response against these liposomes. In addition, we demonstrated that this stimulated MZ-B cells rapidly migrate from the MZ into the FO region while carrying the trapped PEGylated liposomes. It is likely that the PEGylated liposomes are recognized as a TI-2 antigen by the first line of defense against infections by blood-borne organisms. As a consequence the ABC phenomenon is induced.

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