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Vaccine Adjuvant MF59 Promotes Retention of Unprocessed Antigen in Lymph Node Macrophage Compartments and Follicular Dendritic Cells

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Ag retention within lymph nodes (LNs) upon vaccination is critical for the development of adaptive immune responses, because it facilitates the encounter of the Ag with cognate lymphocytes. During a secondary exposure of the immune system to an Ag, immune complexes (ICs) that contain the unprocessed Ag are captured by subcapsular sinus macrophages and are transferred onto follicular dendritic cells, where they persist for weeks, facilitating Ag presentation to cognate memory B cells. The impact of adjuvants on Ag retention within the draining LNs is unknown. In this article, we provide the first evidence, to our knowledge, that the oil-in-water emulsion adjuvant MF59 localizes in subcapsular sinus and medullary macrophage compartments of mouse draining LNs, where it persists for at least 2 wk. In addition, we demonstrate that MF59 promotes accumulation of the unprocessed Ag within these LN compartments and facilitates the consequent deposition of the IC-trapped Ag onto activated follicular dendritic cells. These findings correlate with the ability of MF59 to boost germinal center generation and Ag-specific Ab titers. Our data suggest that the adjuvant effect of MF59 is, at least in part, due to an enhancement of IC-bound Ag retention within the LN and offer insights to improve the efficacy of new vaccine adjuvants. The Journal of Immunology, 2015, 194: 1717–1725.

Ag retention within lymphoid organs is a crucial step for the development of an immune response (1–3). Follicular dendritic cells (FDCs) have been described to play a key role in the induction of B cell responses, particularly during re-exposure of the immune system to a protein Ag (3–6). FDCs capture circulating immune complexes (ICs) through the CR2 (CD21/35) and retain them for a long period (up to 3 wk) (3, 5, 7–9). A prolonged exposure of the ICs on the FDC surface facilitates the encounter of the unprocessed Ag with cognate memory B cells (9, 10). Moreover, FDCs secrete chemokines, such as CXCL13, to attract and interact with cognate B cells (5, 6, 9). Through this interaction, FDCs provide Ag-specific B cells with signals from the cognate Ag and the costimulatory surface or soluble molecules, which have to be integrated to achieve the selective activation of the memory B cells (4–6, 8–11). FDCs maintain intact and unprocessed ICs, internalizing and recirculating them on the surface through a nondegradative vesicle trafficking pathway (12). In addition, it has been demonstrated that cognate B cells acquire a portion of the FDC membrane together with the Ag (9). B cell activation determines both the expansion of the Ag-specific memory B cell compartment and the formation of plasma cells (13–15). Thus, FDCs exert a primary role in the induction of a humoral immune response. In addition, it has been discovered that lymphoid organ dendritic cells (DCs) sample the Ag-loaded FDCs to acquire Ag for T cell activation, suggesting that FDCs have a broader function in the immune response and highlighting their importance (16). Also, the lymphoid organ macrophages have been described to play a critical role in the induction of the humoral immune response (3, 17–20). In particular, it has been observed that the subcapsular sinus macrophages (SCSMs) transport ICs onto noncognate B cells that shuttle them onto FDCs (3, 19, 20). At the same time, IC-loaded SCSMs can relay intact Ag directly to cognate memory B cells, providing the signal for B cell activation (3, 17–20). These findings unveiled an unexpected and peculiar feature of the SCSMs that, differently from the “conventional” macrophages, display a low degradation rate of the engulfed Ag (20).

Adjuvants can be added to vaccine formulations, to significantly enhance the humoral and cellular immune response to a vaccine Ag, and this may be particularly relevant for subunit vaccines, which tend to be poorly immunogenic (21–24). The oil-in-water emulsion MF59 is a very potent and safe adjuvant licensed for human use in the European Union (21–24). Previous studies have shown that, upon i.m. immunization, MF59 facilitates Ag uptake by immune cells (25), stimulates the expression of innate immune genes at the injection site, resulting in the recruitment of immune cells within the muscle, and increases the number of Ag-positive leukocytes in the draining lymph nodes (LNs) (26, 27). It has also been demonstrated that MF59 promotes the differentiation of human monocytes to DCs in vitro (25). However, no information exists on the effect of MF59 or other adjuvants on the Ag distribution within the intact lymphoid organs during vaccination. Further, most of the studies analyzing Ag deposition in LNs were performed in passively immunized animals (9, 10, 19, 20).

In this study, we evaluated Ag capture within draining LNs of mice, during an endogenous immune response to an Ag formulated with MF59. As a model Ag, we used PE, taking advantage of the strong intrinsic fluorescence of this protein (28). Because the characteristic fluorescence of PE is sensitive to protein processing and
requires the integrity of PE phycobilisome (28, 29), the use of this protein allows the detection of intact and unprocessed Ag, which retains all B cell epitopes and their immunogenic potential for B cell activation. Although previous studies on Ag deposition have been conducted upon s.c. or footpad injection of the Ag (10, 15, 19, 20, 30, 31), we decided to use i.m. immunization because this is the preferred route for vaccine administration in humans.

Materials and Methods

Mice and immunizations

C57BL/6 mice aged 4–6 wk were purchased from Charles River Laboratories. Animal experiments were performed in compliance with the European guidelines and approved by the Novartis internal Animal Welfare Body. Mice were immunized i.m. (quadriiceps) in one leg (unless stated otherwise) with a volume of 50 μl, using PBS as dilution buffer. PE (Molecular Probes, Invitrogen Life Technologies) Ag was used at 6 μg/mouse. MF59 was diluted 50% v/v per dose. Immunization schedules are described in the Results. MF59 labeled with DiD lipophilic tracer (Molecular Probes, Invitrogen Life Technologies) was used to track the adjuvant localization within LNs.

Confocal microscopy

LNs of mice were collected under dry conditions at the appropriate time points, were immediately frozen using liquid nitrogen, and were stored at −80°C until processing. Cryosections of LNs obtained with the cryostat CM1950 (Leica) were stained using the following Abs: anti–CD21/35-FITC (Serotec) and anti–IgD-biotin (eBioscience) plus streptavidin-Alexa Fluor 405 (Invitrogen Life Technologies) to identify germinal center (GC) B cells; and anti-CD16/35-Pacific Blue (Biolegend) and anti–IgD-biotin (eBioscience) plus streptavidin-Alexa Fluor 405 (Invitrogen Life Technologies) to identify cortical (C) B cells; and anti-CD16/35-Pacific Blue or -PE to identify FDCs. The cryosections (8 μm thick) were cut along the entire organ to analyze all the planes of the organs. The cryosections were fixed using PBS/3% formaldehyde for 10 min at room temperature, washed twice with PBS, and permeabilized with PBS/3% saponin (permeabilization buffer) for 30 min at room temperature. Tissue sections were then incubated with the appropriate Abs diluted in permeabilization buffer for 1 h at room temperature in the dark. An additional incubation step with streptavidin-Alexa Fluor 405 was performed for IgD staining. After washing three times with permeabilization buffer and once with PBS, stained tissue sections were sealed using Gold Anti-fade reagent (Invitrogen Life Technologies) and a coverslip. Images were acquired with the Axio Observer LSM700 confocal microscope (Zeiss) at 20°C, using FLULAR 5X or Plan-Apochromat 40X objective lenses with 0.25 or 1.3 of numerical aperture, respectively. The 40X objective lens was used with the Zeiss Immersed 518F imaging medium. Images were processed with Zen 2008 software (Zeiss).

Flow cytometry

LNs of mice were collected and immediately subjected to enzymatic digestion. In brief, LNs of each group of mice specifically treated were pooled in RPMI 1640 medium (Life Technologies, Invitrogen Life Technologies) containing Liberase Research Grade (Roche) at the working concentration of 500 μg/ml and DNase I (Roche) at the working concentration of 250 μg/ml (digestion buffer). LNs were incubated for 30 min at 37°C and then agitated by pipetting every 15 min. Supernatants were collected and fresh digestion buffer was added for 30 min at room temperature in the dark. An additional incubation step with streptavidin-Alexa Fluor 405 was performed for IgD staining. After washing three times with permeabilization buffer and once with PBS, washed tissue sections were sealed using Gold Anti-fade reagent (Invitrogen Life Technologies) and a coverslip. Images were acquired with the Axio Observer LSM700 confocal microscope (Zeiss) at 20°C, using FLULAR 5X or Plan-Apochromat 40X objective lenses with 0.25 or 1.3 of numerical aperture, respectively. The 40X objective lens was used with the Zeiss Immersed 518F imaging medium. Images were processed with Zen 2008 software (Zeiss).

Detection of anti-PE Abs

Serum PE-specific total IgG were measured by ELISA. In brief, Maxisorb 96-well plates (Nunc) were coated with a PE solution (2.5 μg/ml) in carbonate buffer (100 μl/well) overnight at 4°C. Plates were then blocked by addition of PBS 3%/polyvinylpyrrolidone (SERVA) (200 μl/well), incubated for 2 h at 37°C, and then washed once with PBS, 0.05%/Tween 20 (washing buffer). Serial dilutions (3-fold step) of standard and serum samples in PBS/0.05% Tween 20/1% BSA were added to the wells and incubated for 2 h at 37°C. Plates were then washed three times with washing buffer and incubated for 1 h at 37°C with anti-mouse IgG alkaline phosphatase (Sigma-Aldrich) solution (100 μl/well). After three washes, the substrate p-nitrophenylphosphate (100 μl/well; Sigma-Aldrich) was added for 30 min at room temperature. Absorbance to 405 nm was then measured by a plate spectrophotometer (BioTek-ASHI).

Results

Adjuvant MF59 induces Ag retention within draining LNs

We sought to determine whether the adjuvant MF59 affects Ag uptake and distribution within LNs. To analyze the distribution of Ag deposition, we immunized mice twice (14 d apart) with PE in the presence or absence of MF59 and collected draining LNs and sera of mice 1 h, 6 h, and 7 d after the second immunization (Fig. 1). After just 1 h, the Ag was already detectable within the subcapsular sinus (SCS) and medullary compartments of the LN in MF59-treated mice, as shown by colocalization with CD169 (SCSM) and F4/80 (MM), whereas no Ag was detected in mice immunized with PE alone (Fig. 1A, 1F, 1G). The PE Ag was undetectable after the primary immunization either at early or at late time points, such as 14 d upon priming, which is the booster time point (data not shown).

As previously established in passively immunized mice, 6 h after the second immunization we found some of the Ag colocalized with FDCs in mice immunized with MF59-adjuvanted PE, whereas no Ag was detectable in the absence of adjuvant (Fig. 1B, 1F, 1G). Surprisingly, at this time point, most of the Ag was still present in the area of the LN corresponding to SCS and medullary compartments (Fig. 1B, 1F, 1G), indicating a persistence of the Ag in the macrophage compartments, in the presence of MF59. Finally, as expected, 7 d after the second immunization with MF59, the Ag was totally retained by FDCs, as indicated by colocalization with the CD21/35 marker, whereas no PE was detectable onto FDCs in absence of MF59 (Fig. 1C, 1F, 1G). In addition, MF59 induced a marked generation of GCs, as detected by GL7 staining (Fig. 1C), which is consistent with a greater production of anti-PE Abs compared with immunization in the absence of adjuvant (Fig. 1D). Complement activation and binding of ICs to CD21/35 through the C3d complement fragment are both required for Ag deposition onto FDCs in passively immunized mice and for the generation of an optimal humoral immune response (8). To verify whether complement activation was necessary for PE deposition onto FDCs also in our experimental setting, we used C3−/− mice, in which the formation of ICs containing C3 fragments is abolished. When these mice were immunized with PE in the presence or absence of MF59, we observed an impaired Ag deposition onto FDCs and, more importantly, an impaired GC formation, even in the presence of the adjuvant (Fig. 1E, 1F). This finding, which is in agreement with published data, demonstrates that the increased IC-bound Ag deposition onto FDCs observed in the presence of MF59 is dependent on complement activation and is required for an optimal B cell response.

To quantify the Ag retention within draining LNs in our experimental setting, we analyzed the LNs by flow cytometry (Fig. 2). Because 6 h after the second immunization is the key time point for Ag retention in our experimental setting, we focused our attention on this time point. Quantitation of PE-loaded cells by flow...
cytometry recapitulates and extends what was observed by confocal microscopy (Fig. 2). We found a notable increase in the number and percentage of PE$^+$ total LN cells in mice immunized in the presence of MF59 (Fig. 2A), and this result was observed...
for all APC subsets, including not only SCSMs and MMs, but also DCs and B cells (Fig. 2B). Unlike confocal microscopy, by using flow cytometry, we were able to detect PE within the draining LNs even in the absence of MF59 coadministration (Fig. 2). This difference was presumably due to the greater sensitivity of flow cytometry compared with confocal microscopy. Interestingly, focusing on PE+ cells, we observed that, on immunization with MF59, most PE-loaded cells are B cells (Fig. 2C). However, the Ag loading by macrophages, revealed as PE fluorescence intensity, is much higher than the Ag loading by B cells (Fig. 2D). Similar results were obtained also at 1 h after the boost, albeit to a lesser extent (data not shown). Taken together, these findings are con-

FIGURE 2. Quantitation of Ag retention induced by MF59 within draining LNs. Similar experiments of Fig. 1. Inguinal draining LNs were collected 6 h after the second immunization and analyzed by flow cytometry. (A) Number (left panel) or percentage (right panel) of PE+ cells within total draining LN cells from mice immunized with PE alone (black bars) or PE adjuvanted with MF59 (white bars). (B) Number (left panel) or percentage (right panel) of PE+ SCSMs (phenotypically distinguished as CD169+ F4/80− CD11c−), MMs (phenotypically distinguished as CD169− F4/80− CD11c+), DCs (phenotypically distinguished as CD169− to F4/80− to CD11c+), and B cells (phenotypically distinguished as CD19+) as indicated, within total draining LN cells from mice immunized with PE alone (black bars) or PE adjuvanted with MF59 (white bars). (C) Percentage of PE+ B cells among the PE+ total LN cells from mice immunized with PE alone (black bar) or adjuvanted with MF59 (white bar). (D) Comparison of PE loading by B cells (green bars), MMs (blue bars), and SCSMs (red bars) in immunization with MF59. The mean fluorescence intensity (MFI) of PE for each cell population is depicted as the difference (∆MFI; left panel) or the ratio (MFI fold increase; right panel) with the background (PBS-injected mice). The ∆MFI reveals that the net PE fluorescence of macrophages is roughly one logarithmic higher than the net PE fluorescence of B cells, whereas the MFI fold increase confirms that the PE uptake by macrophages is higher than the PE uptake by B cells. Inguinal draining LNs from three different mice per treatment were pooled. Results from one representative experiment of two are depicted.
sistent with the confocal microscopy observation and confirm the IC-bound Ag translocation model from macrophages to non-cognate B cells toward FDCs. Thus, we demonstrated that MF59 adjuvant enhances Ag accumulation by macrophages and consequently by non-cognate B cells, over the first 6 h, ultimately leading to Ag retention onto FDCs. In contrast with the confocal microscopy data, at 7 d after the boost, we were able to detect very low amounts of PE+ cells within the total LN cells (data not shown). This expected result is likely due to the fact that the enzymatic digestion of LNs induces the loss of stromal cells, including FDCs, where all the IC-trapped Ag is located 7 d after the boost as shown in Fig. 1 and as reported in the literature (1–3, 5, 6, 9).

**FIGURE 3.** MF59 directly affects Ag retention within SCS and medullary compartments. (A–C) Mice were immunized i.m. in both legs with PE alone (B) or PE + MF59 (C), and serum anti-PE IgG titers were measured by ELISA after 2 wk (A). (A) Anti-PE IgG titers. Each symbol represents the serum anti-PE IgG titer of a single mouse. Unpaired Student t test does not reveal any statistical significant difference between the two groups. (B and C) Each group of mice was successively reimmunized in the left leg with PE alone (upper panels) and in the right leg with MF59-adjuvanted PE (PE + MF59, lower panels). Then 6 h after the second immunization, inguinal draining LNs were collected and analyzed by confocal microscopy to detect FDCs (CD21/35-Pacific Blue), SCSMs (CD169-FITC), and PE (B and C). Signals that localize CD21/35 or CD169 expression and PE are shown separately or merged as indicated. The image of one section is shown, in each panel, as example of consecutive sections of a whole LN, which is representative of three (B) or two (C) organs from three different mice. Original magnification ×5. Scale bar, 100 μm. (C) Bottom panels 1 and 2 are enlargements of the areas indicated as 1 and 2 in the Merge panel above. One representative experiment of two is shown.
Adjuvant MF59 directly affects Ag retention within draining LNs

Detection of the Ag in the macrophage area after a booster immunization in the presence of MF59 could theoretically be a consequence of a superior primary Ab response and an increased amount of circulating ICs, because of the MF59 adjuvant effect during priming immunization. Indeed, the anti–PE Ab titer 6 h after the second immunization is higher in the presence of MF59, although this difference is not statistically significant (Fig. 1D). We therefore designed an experiment to better evaluate the Ag retention within LN macrophage compartments after secondary immunization with or without MF59, but in the presence of an identical amount of circulating ICs. We first immunized mice in both legs with PE alone or adjuvanted with MF59; then for the secondary immunization (2 wk later), the animals of both groups were injected with PE alone in the left leg and with MF59-adjuvanted PE in the right leg, and the draining LNs of both legs were analyzed. In animals treated with PE alone as the first immunization and thus in the presence of very low amounts of Ag-specific Abs (and consequently ICs) (Fig. 3A), we did not detect Ag in SCS or medullary compartment 6 h after the secondary injection with either plain or adjuvanted PE (Fig. 3B). Thus, the secondary immunization with MF59 is not sufficient to accumulate within the draining LN enough Ag to be detected after priming with PE alone (in the presence of low amounts of Ag-specific Abs). On the contrary, in animals that received MF59-adjuvanted PE as the primary immunization and that displayed higher (but not statistically significant) anti–PE Ab titers (Fig. 3A), MF59 was required at the secondary immunization to promote an optimal retention of PE within the SCS and medullary compartments in the draining LNs (Fig. 3C). Indeed, the second immunization with PE resulted in an efficient Ag retention within the draining LN of the leg treated with two immunizations in the presence of MF59 (Fig. 3C). On the contrary, in the same mouse, within the draining LN of the leg that received the primary immunization with MF59-adjuvanted PE and the boost with PE alone, we did not observe an efficient Ag retention (Fig. 3C), and in only one mouse of three could we detect some PE signal within the macrophage compartments (Supplemental Fig. 1). Thus, adjuvant MF59 play a significant role for an optimal LN retention of the IC-bound Ag during the secondary immunization in the presence of determined amounts of Ag-specific Abs. These results also confirm that the primary immunization with MF59 does not promote enough Ag accumulation to be detectable at the booster time point. The IC-bound Ag was clearly detectable in the macrophage area and, as expected, in the process of translocation toward FDCs (enlarged images 1 and 2 at bottom of Fig. 3C). Ag retention by macrophages precedes its translocation inside the B cell follicles, and this step allows the subsequent deposition of the Ag onto FDCs. Thus, MF59 directly stimulates IC-bound Ag accumulation within LNs, even if the contribution of circulating ICs could be critical over a threshold amount.

To further assess the direct contribution of MF59 in the retention of the Ag within macrophage compartments, we evaluated whether MF59 reaches draining LNs upon injection. Indeed, labeled MF59 was present in the SCS and medullary compartments 1 and 6 h after injection (Fig. 4A, 4B) and remained in these compartments for a long time, being detectable after 7 (Fig. 4C) and 14 d (Fig. 4D). In addition, at day 7, some MF59 was also detected within the paracortex of the LN, which is the T cell area where DCs reside (Fig. 4C and its magnification). Therefore, MF59 never reaches the FDC area or the B cell follicles, but remains mainly within the macrophage area.

Upon immunizations with MF59, FDC activation is associated with Ag trapping and GC generation

Given the importance of FDCs for the development of a humoral immune response, we asked whether the MF59 adjuvant could affect FDC activation and whether this activation correlates with generation of GCs and Ag uptake by FDCs. We first analyzed activation of FDCs in the LNs of mice immunized with PE in the presence or absence of MF59, 7 d after a secondary immunization, checking the expression of CD16/32, a described marker for FDC activation (7, 32). The activation of FDCs was generally more evident after immunization with MF59, although it was also observed in the absence of adjuvant (Fig. 5A). However, in mice immunized with MF59-adjuvanted PE, Ag deposition onto FDCs was observed only in follicles displaying activated FDCs and

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**FIGURE 4.** MF59 is retained within SCS and medullary compartments. Mice were injected i.m. with DiD-labeled MF59, and inguinal draining LNs were collected after 1 h (**A**), 6 h (**B**), 7 d (**C**), and 14 d (**D**) and analyzed by confocal microscopy to detect SCSMs (CD169-FITC), MMs (F4/80-Alexa Fluor 450), FDCs (CD21/35-Pacific Blue), and labeled MF59. Signals that localize CD169, F4/80, or CD21/35 expression and MF59 are shown separately or merged as indicated. (**C, bottom panel**) Magnification of the indicated area in the Merge image. The image of one section is shown, in each panel, as an example of consecutive sections of a whole LN, which is representative of three organs from different mice. Original magnification $\times$5. Scale bar, 100 µm. One representative experiment of two is shown.
matched with CD16/32-expressing cells (Fig. 5B, upper and central panels), whereas CD16/32 expression could be detected also in the absence of Ag uptake by FDCs (Fig. 5B, lower panel). Using a nonfluorescent Ag (RrgB protein from the *S. pneumoniae* pilus), we confirmed that FDC activation within draining LNs, measured detecting the CD16/32 expression, also occurred in the absence of adjuvant (data not shown). More importantly, in this experimental system, we found that GC formation correlates with activation of FDCs. In fact, GCs could be observed only in the presence of FDC activation (Supplemental Fig. 2), even though B cell follicles without detectable GC reaction, but with CD16/32 expression in the absence of PE, were observed (data not shown). These results suggest that after immunizations with adjuvant MF59, FDC activation is necessary, but not sufficient, for Ag trapping and GC generation.

**Discussion**

Ag trapping onto FDCs or by SCSMs was previously assessed in passively immunized mice, to artificially induce IC formation, using s.c. (flank, tail) or footpad as route of Ag administration (9, 10, 19, 20). In these models, it has been demonstrated that the Ag reaches the SCS in minutes and within a few hours (6–24 h) is almost completely deposited in the FDC area, where it can be trapped for days and can be readily detectable at 1 wk, when GCs can be observed (9, 10, 15, 19, 20, 30, 31, 33). All these previous studies have been performed administrating a soluble Ag in mice artificially reconstituted with circulating Ag-specific Abs, whereas no study to date has evaluated the effect of vaccine adjuvants on Ag deposition on the macrophage and FDC area of draining LNs, in the context of an endogenous immune response.

We decided to investigate whether the vaccine adjuvant MF59 is able to modulate Ag distribution within LNs upon a booster immunization, by affecting trafficking of an IC-trapped Ag. The analysis of the kinetics of Ag translocation within the draining LNs during an endogenous response to an MF59-adjuvanted vaccine enables a better understanding of the mechanism of action of this adjuvant. Clarifying this mechanism is highly important because MF59 is currently used to formulate seasonal and pandemic influenza vaccines used in humans and has the potential to greatly improve the efficacy of poorly immunogenic vaccine Ags. All experiments in our study were performed using i.m. immunization, because this is the preferred route of administration for human vaccines. We chose the PE as model Ag because of its strong intrinsic fluorescent property and its rather poor immunogenicity,
which allowed us to better evaluate the effect of an adjuvant on the enhancement of a specific immune response.

Consistently with the described model for the trafficking of IC-trapped Ags within the LN of passively immunized mice (1, 2, 10, 17–20), we observed that PE formulated with MF59, drained to the LN from the injection site, first encounters SCSMs and is then translocated onto FDCs. Interestingly, however, we discovered that MF59 promotes accumulation within the SCSM and MM compartments, of the unprocessed Ag trapped in ICs, enhancing its subsequent deposition onto noncognate B cells and then FDCs. Indeed, no Ag translocation onto FDCs was detected in the absence of complement C3 factor and, therefore, in the absence of IC formation. In addition, the Ag accumulation observed after immunization with MF59 correlates with GC formation and the increase in the magnitude of the humoral immune response, which is consistent with the ability of SCSMs and FDCs to deliver Ag to cognate memory B cells, resulting in their activation. Thus, our results confirm a correlation between retention of the intact IC-bound Ag within LNs and development of an optimal humoral immune response. The novel finding that MF59 adjuvant exerts a direct effect on this process remaining mainly localized in the macrophage compartments highlights the importance of this cellular population in adjuvant activity and identifies macrophages as a critical target of MF59 function. Taken together, our data represent the first demonstration that, during a secondary immune response after re-exposure to an Ag upon i.m. immunization, the presence of the vaccine adjuvant MF59 directly induces retention for several hours of the intact, unprocessed Ag, trapped in ICs, by the compartment of SCSMs and MMs. Interestingly, noncognate B cells play a central role in Ag accumulation within the SCS and medullary compartments, either confirming the translocation model of IC-bound Ag from macrophages to B cells or demonstrating that MF59 enhances the accumulation of the Ag by all the cells involved in the process of Ag translocation onto FDCs. In addition, it is conceivable that MF59 induces the recruitment of noncognate B cells within the LN macrophage compartments to potentiate the mechanism of translocation onto FDCs. Moreover, the accumulation of unprocessed IC-bound Ag in the SCS and medullary compartments is followed by its strong deposition onto FDCs, which correlates with a robust humoral response.

The exact mechanism by which MF59 exerts this effect on Ag accumulation is still poorly understood. MF59 might affect the degradation of IC-bound Ag by APCs once engulfed, but this hypothesis has not been addressed. However, it was previously proposed that peripheral macrophages, loaded with Ag and MF59, promote Ag translocation into LNs (27); further experiments are needed to confirm this hypothesis. Certainly, it has been demonstrated that MF59 induces inflammation at injection site and increases the Ag-positive leukocytes within the draining LNs (26, 27). Thus, the migration of immune cells from injection site into the draining LN could play a critical role in the enhancement of Ag and MF59 accumulation within the node. In conclusion, although the details of these phenomena are still to be elucidated, our study has provided the first demonstration, to our knowledge, that MF59 promotes IC-bound Ag retention within draining LNs during the booster response.

FDCs are critical for the induction of a complete humoral immune response, because these cells are required to capture IC-bound Ags and stimulate cognate B cells (9, 10), leading to an increase of the Ag-specific memory B cell population and expansion of the Ab-secreting plasma cells (13–15). FDCs have also been shown to function as a reservoir of Ag for uptake by lymphonodal DCs (16). However, no study has investigated FDC activation, in vivo, after vaccination. We discovered that FDCs undergo activation upon immunization with a poorly immunogenic Ag, such as PE, even in the absence of an adjuvant, and that FDC activation is increased by addition of MF59. Therefore, we propose that, although a weak immune response can induce FDC activation, the presence of adjuvant MF59 in the immunization can modulate the function of FDCs. Moreover, our study provides the first demonstration that, upon vaccination, the activation of FDCs is necessary for both GC generation and Ag deposition. In fact, consistent with the ability of FDCs to be activated after immunization with a poorly immunogenic Ag, we observed LN follicles displaying activated FDCs without detectable GCs or Ag deposition. However, in the same organ, we also observed GC formation and Ag deposition only in follicles containing activated FDCs. In addition, the deposited Ag is clearly colocalized with the activated cells of the FDC network, indicated by the expression of CD16/32. In a study of passively immunized mice, it has previously been demonstrated that expression of CD21/35 is required for Ag deposition onto FDCs, whereas CD16/32 is dispensable (8). Therefore, based on this finding and on our data, we suggest that CD16/32 represents an alternative marker for FDCs, and that, upon vaccination, the activation of FDCs is required, but not sufficient, for Ag retention by the cells and GC formation within a single B cell follicle. Interestingly, MF59 may partially modulate this process.

IC-trapped Ag retention by LN macrophages facilitates Ag translocation to FDCs and Ag uptake by cognate B cells or DCs, which are key steps in the initiation of an immune response. It is therefore important to evaluate how this process is modulated by vaccine adjuvants (9, 10, 16–20). Our study demonstrates that vaccine adjuvant MF59 promotes retention of the intact, unprocessed Ag trapped in ICs within the LN macrophage compartments, affecting the cascade of events leading to a marked deposition of this IC-bound Ag onto activated FDCs. This increase of the overall accumulation of unprocessed Ag within the LNs is associated with the ability of MF59 to enhance the humoral immune response. In conclusion, our work identifies a new mechanism by which MF59 may increase vaccine immunogenicity and will help to improve the efficacy of new vaccine adjuvants.

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Disclosures

All authors are employees or managers of Novartis Vaccines. MF59 is a proprietary adjuvant of Novartis.

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