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Oil-in-Water Emulsion MF59 Increases Germinal Center B Cell Differentiation and Persistence in Response to Vaccination

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Induction of persistent protective immune responses is a key attribute of a successful vaccine formulation. MF59 adjuvant, an oil-in-water emulsion used in human vaccines, is known to induce persistent high-affinity functional Ab titers and memory B cells, but how it really shapes the Ag-specific B cell compartment is poorly documented. In this study, we characterized the Ab- and Ag-specific B cell compartment in wild-type mice immunized with HlaH35L, a Staphylococcus aureus Ag known to induce measurable functional Ab responses, formulated with MF59 or aluminum salts, focusing on germinal centers (GC) in secondary lymphoid organs. Taking advantage of single-cell flow cytometry analyses, HlaH35L-specific B cells were characterized for the expression of CD38 and GL-7, markers of memory and GC, respectively, and for CD80 and CD73 activation markers. We demonstrated that immunization with MF59-, but not aluminum salt–adjuvanted HlaH35L, induced expanded Ag-specific CD73+CD80+ GC B cells in proximal- and distal-draining lymph nodes, and promoted the persistence of GC B cells, detected up to 4 mo after immunization. In addition to increasing GC B cells, MF59-adjuvanted HlaH35L also increased the frequency of T follicular helper cells. This work extends previous knowledge regarding adaptive immune responses to MF59-adjuvanted vaccines, and, to our knowledge, for the first time an adjuvant used in human licensed products is shown to promote strong and persistent Ag-specific GC responses that might benefit the rational design of new vaccination strategies.

Effective vaccines should generate specific and strong immune responses against target Ags with acceptable levels of inflammation induced by the immunization. In the past, live-attenuated or killed whole organisms were used, often causing side effects not acceptable any longer by a modern society. The next generation of vaccines mostly relies on purified subunit Ags that may be weakly immunogenic on their own and require adjuvants to enhance the strength and duration of the immune responses they induce. MF59 oil-in-water emulsion and aluminum salts (alum), including aluminum hydroxide and aluminum phosphate generally described as alum, are the two major adjuvants licensed in human vaccines, and have a long track record of safety and efficacy in humans.

Alum is empirically used in human vaccines since 1920 with the general goal to enhance Ab production against weak immunogens (1, 2). MF59 is a nanoeulsion originally licensed in 1997 to adjuvant human influenza vaccines; it consists of the naturally occurring squalene oil and of nonionic surfactants Tween 80 and Span 85 emulsified in uniform particles of ~160 nm in size. In the past years, >150 million doses of MF59-adjuvanted vaccines have been administered worldwide, and a vast body of knowledge on MF59 immunogenicity has been collected from human clinical studies, in particular with influenza vaccines. MF59 adjuvant enhances both the quantity and the quality of Ag-specific immune responses, allowing for Ag dose sparing, improving the breadth of the Ab responses, and enhancing protective immunity, in particular target populations such as children and the elderly (3–5).

MF59 and alum were initially thought to exert their adjuvant effect by the formation of an Ag depot, but subsequent studies refuted this hypothesis for both adjuvants (6–8), revealing two separate mechanisms of action. Ag adsorption on alum directly delivers the Ag to resident dendritic cells (DC) and seems critical for alum adjuvanticity (9). The mechanisms by which the Ag uptake is facilitated are not yet clear, but a recent study suggested that this may occur in the absence of uptake by alum by APCs. In particular, alum was shown to bind lipids on the surface of APCs and trigger cellular activation cascades leading to initiation of an immune response, but without itself being internalized by the cells (9), suggesting an indirect role in delivering Ag into the Ag-processing pathway; however, other studies have shown that alum is internalized by APCs, resulting in endosomal destabilization (10). In contrast, MF59 primary mechanism of action
resides in the generation of an immunocompetent environment at the injection site (muscle), characterized by the release of local extracellular ATP, expression of activation makers on resident DC, and secretion of chemokines that strongly increase phagocytic cells trafficking toward the injection site and the draining lymph nodes (LNs) (11–15). Unlike alum, MF59 does not directly activate DC, but rather promotes cell migration, a more efficient Ag uptake, and more Ag-loaded APCs in draining LNs compared with alum or nonadjuvanted vaccines (12, 13, 15). Furthermore, the adjuvant effects of MF59 were maintained even when MF59 and the Ag were administered 24 h apart at the same site (16).

Although MF59 also induces strong and persistent T cell responses (17, 18), it is better known for the powerful adjuvant effect it exerts on vaccine-specific Ab and B cell responses. In particular, MF59 promotes high-affinity Abs, increased diversity of the epitope repertoire, and enhanced frequencies of Ag-specific memory B cells, resulting in higher frequencies of responding memory B cells and higher titers of protective neutralizing Abs after Ag re-exposure (19–21). Remarkably, higher immune responses were observed with MF59 in younger (or Ag-naive) individuals compared with nonadjuvanted vaccine, suggesting that MF59 plays a major role in the activation and differentiation of Ag-specific naive B cells and priming of immune responses (5, 20).

Both of these findings suggest that MF59 strongly affects T-dependent B cell responses, in particular, shaping germinal centers (GC) where expansion, selection, and affinity maturation of Ag-specific B cells take place during primary responses. GC develop within secondary lymphoid organs from one or few B cell clones, called Ag-specific GC B cells that are defined by a switched IgM IgG3 phenotype, downregulation of CD38, and upregulation of GL-7 expression. Under the pressure of T follicular helper (Tfh) cells and follicular DC, GC B cells extensively proliferate and increase their affinity for the Ag. Subsequently, GC B cells differentiate in the following: 1) long-lived bone marrow plasma cells that constantly feed the blood circulation with high-affinity Abs, and 2) high-affinity memory B cells that quickly respond to Ag recall in case of re-exposure.

Detoxified Staphylococcus aureus α-hemolysin (HlaH35L) was used as a model Ag to study the impact of MF59 on functional B cell responses. HlaH35L is a promising vaccine candidate against S. aureus infections (22, 23), and anti-Hla Abs are linked to reduced risk of severe diseases in humans (24). Moreover, the importance of protective neutralizing Abs against Hla has been demonstrated in several animal models of S. aureus infection (22, 23, 25).

In this study, the magnitude and quality of Ab responses were evaluated in three different vaccination schedules, as follows: 2 wk, 1 mo, and 4 mo between first and second dose. The 1-mo schedule was chosen to further characterize Hla-specific B cells by ELISPOT and flow cytometry. Frequencies of Ag-specific B cells were determined in PBMC, spleen, and LNs of immunized mice up to 4 mo after the last immunization. HlaH35L plus MF59 induced higher Hla-specific functional Ab titers compared with HlaH35L plus alum, regardless of the immunization schedule. Although both adjuvant formulations induced Hla-specific GC B cells in proximal-draining LNs, only HlaH35L plus MF59 induced GC B cells in axillary distal-draining LNs. Furthermore, HlaH35L plus MF59 increased Ag-specific GC persistence and B cell memory responses up to 4 mo after immunization. Finally, increased frequencies of Tfh cells were also measured in HlaH35L plus MF59–immunized mice compared with mice in the unadjuvanted HlaH35L and HlaH35L plus alum groups.

Materials and Methods

Mice

Pathogen-free BALB/c female mice (Charles River) aged 6 wk were used in this study. All animal studies were carried out in compliance with current Italian legislation on the care and use of animals in experimentation (Legislative Decree 116/92) and with the Novartis Animal Welfare Policy and Standards. Protocols were approved by the Italian Ministry of Health (authorization 249/2011-B) and by the local Novartis Animal Welfare Body (authorization AWB 201106).

Vaccine Ag, adjuvants, and immunizations

HlaH35L was amplified by PCR from S. aureus NCTC8325 strain and cloned as tagless constructs. MF59, a Novartis Vaccines proprietary oil-in-water emulsion consisting of 4.3% (v/v) squalene, 0.5% Tween 80, and 0.5% Span 85 in citrate buffer (10 mM), was prepared as described earlier (15). Alum [Al(OH)3] was from Novartis Vaccines.

Formulations were prepared with 10 µg HlaH35L, with 2 mg/ml alum or 50% MF59. All of the formulations, including the groups saline and the nonadjuvanted HlaH35L, were adjusted with PBS and NaCl to reach final pH 6.5–7 and osmolality 0.308 ± 0.060 Osm/Kg. Ag adsorption to alum and protein integrity were analyzed by SDS-PAGE (data not shown). BALB/c mice were immunized i.m. with 50 µl vaccine injected in each hind leg quadriceps (100 µl/mouse). The immunization schemes are reported in Figs. 1A and 6A. Briefly, BALB/c mice (at least five to six mice per group per experiment) were immunized twice, 2 wk, 1 mo, or 4 mo apart, and analyses of the immune response were performed 10 or 4 mo after the second immunization.

HlaH35L labeling

Amine labeling of HlaH35L with Alexa Fluor 488 (A488) succinimidyl ester was performed according to the manufacturer’s protocol (Molecular Probes, Life Technologies). Briefly, 1 mg protein was incubated with A488 (HlaH35L/amine =10, where n is number of moles) for 1 h at room temperature in the presence of 100 mM NaHCO3. Unlabeled fluorochrome was removed using 10-kDa Amicon ultra-0.5 ml centrifugal filters (Millipore). The degree of labeling was 2.97 and calculated as follows: Amax × MW/ [protein] × edye, where Amax is the absorbance of the protein–fluoro-
cone conjugate (at 495 nm for A488), MW is the m.w. of the protein (Da), edye is the extinction coefficient of the dye at its maximum absorbance (εA488 = 71,000 cm−1 M−1), and the protein concentration is expressed in mg/ml. Protein concentration after labeling was determined by NanoDrop (Thermo Scientific), and its integrity was assessed by SDS-PAGE and size exclusion ultra pressure liquid chromatography (data not shown).

Flow cytometry staining

For the characterization of Ag-specific B cell responses, cell suspensions prepared from spleen, or pools of axillary and inguinal LNs from each mouse, were stained with Yellow live/dead cell stain (Invitrogen) for 20 min at room temperature and then incubated with Fc block (BD Biosciences) in PBS plus 1% FBS (HyClone, Thermo Scientific) for 10 min at 4°C. Approximately 107 splenocytes or 2–3 × 106 LN cells were stained for 30 min at 4°C with the following mAbs: anti-CD19 PE (clone 1D3),-anti-CD20 PE-Cy5 (clone 90) and anti-CD73 PE-Cy7 (clone TY/11.8) (eBioscience).

To identify Ag-specific B cells, samples were stained with 0.1 µg A488-labeled HlaH35L per 2–3 × 106 cells. The optimal amount of fluorescently labeled protein to be used was previously determined in titration experiments on cells from naive and immunized mice. Cells were analyzed on a special-order FACS LSRII or sorted using a FACS Aria II (BD Biosciences).

For the characterization of Tfh cell responses, inguinal LN cell suspensions were stained with Near-Infra Red live/dead cell stain (Invitrogen) for 20 min at room temperature and then incubated with Fc block (BD Biosciences) in PBS plus 1% FBS (HyClone, Thermo Scientific) for 10 min at 4°C. Approximately 2 × 106 LN cells were stained for 30 min at 4°C with the following mAbs: anti-ICOS PE (clone 7E.17G9), anti–PD-1 PE (clone 11-26c.2a), anti–GL-7 Alexa Fluor 647 (clone GL-7), and anti-CD80 PE-HESP94 (clone 16-10A1) (all from BD Biosciences); anti-CD21 BV755 (clone 17A2; BioLegend); and anti-CD38 PE-Cy5 (clone 90) and anti-CD73 PE-Cy7 (clone TY/11.8) (eBioscience).

Cells were analyzed on a special-order FACS LSRII or sorted using a FACS Aria II (BD Biosciences). Data analyses were performed using

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were immunized twice, 2 wk, 1 mo, or 4 mo apart with HlaH35L, HlaH35L expressed as the ratio between the LogEC50 values obtained by ELISA with specific IgG titers measured by ELISA. (c) Specific high-avidity Abs. (and without thiocyanate treatments; high ratio indicates sera with Hla-specific B cells. The cryosections (8 µm thick) were cut along the entire organ to analyze all the planes of the organs. The cryosections were fixed immediately frozen using liquid nitrogen, and stored at ~80°C until processing. Cryosections of LNs were obtained with the Leica CM1950 cryostat and stained using the following Abs: anti-CD21/35-Pacific Blue (BioLegend) to identify follicular DC; anti-GL-7–FITC (eBioscience) to identify GC B cells; and anti-IgD-PE (BD Pharmingen) to identify follicular B cells. The cryosections were collected, washed with medium plus 2.5 mM EDTA, and plated for ELISPOT analyses, as described above. The 40× objective lenses with 0.25 or 1.3 of numerical aperture, respectively. The 40× objective lens was used with the Zeiss Immersol 518F imaging medium. Images were processed with Zen 2008 software (Zeiss).

ELISPOT analyses of HlaH35L-specific plasma cells and memory B cells

ELISPOT analyses of HlaH35L-specific plasma cells were performed on bone marrow cells collected by washing the pair of femoral bone marrows. Cells were plated into ELISPOT plates (MultiScreen HTS-HA MSHAN45; Millipore) previously coated with 100 µL PBS containing 10 µg/mL HlaH35L or BSA, or 5 µg/mL goat anti-mouse Ig Ab (Southern Biotech), and blocked for 2 h at room temperature with PBS containing 10% FBS. Plates were washed after 4 h of incubation with cells, than incubated with biotin-conjugated goat anti-mouse IgG (1:2000; Sigma-Aldrich) followed by HRP-conjugated streptavidin (1:1000; Endogen), and developed with the 3-amino-9-ethylcarbazole substrate kit (Sigma-Aldrich). Spots were counted using the UV Spot ELISPOT plate Analyzer and the Immunospot software v5.1 Professional DC (Cellular Technology Limited). To determine the frequencies of HlaH35L-specific memory B cell, freshly collected splenocytes were harvested and cultured in vitro in complete medium with 5 µg/mL CpG (Primm) plus 1000 U/mL IL-2 (Novartis); after 5 d at 37°C 5% CO2, cells were collected, washed with medium plus 2.5 mM EDTA, and plated for ELISPOT analyses, as described above.

ELISPOT assays were also used to confirm the specificity of A488-HlaH35L B cells sorted by flow cytometry (Supplemental Fig. 2B). In particular, splenocytes from immune mice were stained with A488-HlaH35L, anti-CD3, anti-CD19, anti-IgM, and anti-IgD. HlaH35L-specific CD19+IgM+ IgD+ as well as CD19+IgM- were sorted from immune mice, mixed at a ratio of 0.5:100, and cultured in vitro at 3 × 10^6 cells in 200 µL complete medium with 5 mg/ml CpG (Primm) and 1000 U/mL IL-2. After 5 d, cells were harvested and plated for ELISPOT, as described above.

Ab ELISA and avidity

Titrations of HlaH35L-specific serum IgG was performed on individual serum samples with ELISA plates (Nunc Maxisorp, Thermo Scientific) coated overnight with HlaH35L (10 µg/ml). Plates were blocked with 2% BSA in PBS/Tween 20 0.05% for 1 h at 37°C, and then washed three times with 0.05% Tween 20 in PBS and incubated for 2 h at 37°C with individual mouse sera in 3-fold serial dilutions. Plates were washed, incubated for 2 h at 37°C with secondary anti-mouse total IgG (1:2000; Sigma-Aldrich) and washed, and p-nitrophenyl phosphate disodium (Sigma-Aldrich) was hydrolyzed in 0.05 M NaOH and optical density at 405 nm using a microplate reader (Bio-Rad). Serum samples were tested in duplicate. Titers were expressed as the reciprocal of the highest dilution giving an absorbance of at least 0.4 relative to the negative control. Data points were excluded if they were >2 standard deviations from the mean.

**Confocal microscopy**

LNs were collected under dry conditions at the appropriate time points, immediately frozen using liquid nitrogen, and stored at ~80°C until processing. Cryosections of LNs were obtained with the Leica CM1950 cryostat and stained using the following Abs: anti-CD21/35-Pacific Blue (BioLegend) to identify follicular DC; anti-GL-7–FITC (eBioscience) to identify GC B cells; and anti–IgD–PE (BD Pharmingen) to identify follicular B cells. The cryosections were collected under dry conditions at the appropriate time points, immediately frozen using liquid nitrogen, and stored at ~80°C until processing. Cryosections of LNs were obtained with the Leica CM1950 cryostat and stained using the following Abs: anti-CD21/35-Pacific Blue (BioLegend) to identify follicular DC; anti-GL-7–FITC (eBioscience) to identify GC B cells; and anti–IgD–PE (BD Pharmingen) to identify follicular B cells. The cryosections (8 µm thick) were cut along the entire organ to analyze all the planes of the organs. The cryosections were fixed immediately frozen using liquid nitrogen, and stored at ~80°C until processing. Cryosections of LNs were obtained with the Leica CM1950 cryostat and stained using the following Abs: anti-CD21/35-Pacific Blue (BioLegend) to identify follicular DC; anti-GL-7–FITC (eBioscience) to identify GC B cells; and anti–IgD–PE (BD Pharmingen) to identify follicular B cells. The cryosections were collected under dry conditions at the appropriate time points, immediately frozen using liquid nitrogen, and stored at ~80°C until processing. Cryosections of LNs were obtained with the Leica CM1950 cryostat and stained using the following Abs: anti-CD21/35-Pacific Blue (BioLegend) to identify follicular DC; anti-GL-7–FITC (eBioscience) to identify GC B cells; and anti–IgD–PE (BD Pharmingen) to identify follicular B cells. The cryosections (8 µm thick) were cut along the entire organ to analyze all the planes of the organs. The cryosections were fixed immediately frozen using liquid nitrogen, and stored at ~80°C until processing. Cryosections of LNs were obtained with the Leica CM1950 cryostat and stained using the following Abs: anti-CD21/35-Pacific Blue (BioLegend) to identify follicular DC; anti-GL-7–FITC (eBioscience) to identify GC B cells; and anti–IgD–PE (BD Pharmingen) to identify follicular B cells. The cryosections were collected under dry conditions at the appropriate time points, immediately frozen using liquid nitrogen, and stored at ~80°C until processing. Cryosections of LNs were obtained with the Leica CM1950 cryostat and stained using the following Abs: anti-CD21/35-Pacific Blue (BioLegend) to identify follicular DC; anti-GL-7–FITC (eBioscience) to identify GC B cells; and anti–IgD–PE (BD Pharmingen) to identify follicular B cells. The cryosections (8 µm thick) were cut along the entire organ to analyze all the planes of the organs. The cryosections were fixed immediately frozen using liquid nitrogen, and stored at ~80°C until processing. Cryosections of LNs were obtained with the Leica CM1950 cryostat and stained using the following Abs: anti-CD21/35-Pacific Blue (BioLegend) to identify follicular DC; anti-GL-7–FITC (eBioscience) to identify GC B cells; and anti–IgD–PE (BD Pharmingen) to identify follicular B cells. The cryosections were collected under dry conditions at the appropriate time points, immediately frozen using liquid nitrogen, and stored at ~80°C until processing. Cryosections of LNs were obtained with the Leica CM1950 cryostat and stained using the following Abs: anti-CD21/35-Pacific Blue (BioLegend) to identify follicular DC; anti-GL-7–FITC (eBioscience) to identify GC B cells; and anti–IgD–PE (BD Pharmingen) to identify follicular B cells. The cryosections (8 µm thick) were cut along the entire organ to analyze all the planes of the organs. The cryosections were fixed immediately frozen using liquid nitrogen, and stored at ~80°C until processing.
Results

MF59 enhances Hla-specific functional Ab titers regardless of the vaccination schedule

Hla-specific humoral responses were characterized in mice immunized with MF59- or alum-formulated HlaH35L. BALB/c mice were immunized twice, i.m., with 10 μg HlaH35L alone, HlaH35L plus MF59, or HlaH35L plus alum. Three different vaccination schedules, 2-wk, 1-mo, or 4-mo interval between first and second dose, were compared (Fig. 1A). Ten days after the second immunization, Hla-specific total IgG titers, Ab avidity, and Hla-neutralizing titers were determined (Fig. 1B–D). Both alum and MF59 formulations increased Hla-specific IgG titers compared with the unadjuvanted HlaH35L; furthermore, HlaH35L plus MF59 induced statistically significant higher Hla-specific IgG titers compared with HlaH35L plus alum (p < 0.001) (Fig. 1B) in all three schedules of immunization tested. Hla-specific titers in the MF59 group were higher in the 1-mo interval schedule, compared with the shorter 2-wk boosting interval (5.51 ± 0.08 LogEC50 and 4.98 ± 0.08 LogEC50, respectively; p < 0.001); no statistical differences were observed between the 1- and 4-mo boosting intervals. Avidity of Hla-specific IgG was further measured with a modified ELISA comprising a washing step with ammonium thiocyanate (NH4SCN) before the addition of the secondary Ab. IgG avidity was expressed as the ratio between LogEC50 (+thiocyanate) and (–thiocyanate) treated sera, respectively.

Statistical analyses

Data are presented as the mean ± SEM. Statistical analyses were performed using GraphPad Prism 5 software (GraphPad Software). Unpaired Student t test or one-way ANOVA statistical tests followed by Tukey’s or Bonferroni’s posttests were applied as reported in the legend of each figure. Hla-neutralizing titers were calculated using a log(agonist) versus normalized response nonlinear regression curve with Excel XLFit (Microsoft).

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Characterization of CD19^+IgM^2IgD^+Hla-specific B cells. BALB/c mice were immunized twice, 1 mo apart, with HlaH35L plus MF59 and HlaH35L plus alum. B cell responses were measured 10 d after the last immunization by flow cytometry. (A) Combinatorial expression of CD38 and GL-7 markers on CD19^+IgM^+IgD^+Hla-specific B cells from proximal-, distal-draining LN, and spleen; numbers show the percentage of the gated populations. (B) Frequencies of HlaH35L-specific CD38^+GL-7^+ GC B cells in inguinal proximal-axillary distal-draining LN, and spleen, as gated in (A). (C) Number of HlaH35L-specific CD38^+GL-7^+ GC B cells/10^6 cells in inguinal proximal-draining LN, as gated in (A). (D) Combinatorial expression of CD73 and CD80 on Hla-specific CD38^+GL-7^+ memory and CD38^+GL-7^+ GC B cells, as gated in (A); numbers show the percentage of the gated populations. Each organ from each mouse was processed and acquired separately. Fcs files from mice of the same group of immunization (four to five mice) were concatenated with the FlowJo software: (A) and (D) show one representative experiment, and (B) and (C) show three independent experiments, where each dot is one experiment. Unpaired Student t test was used for statistical analyses: ^p < 0.05.

FIGURE 4.
cyanate) and LogEC50 (−thiocyanate) values. Hla-specific IgG with statistically significant higher avidity were induced by HlaH35L plus MF59 compared with HlaH35L plus alum in the 1-mo interval schedule (0.884 and 0.782, respectively; *p < 0.05), with a positive trend to higher avidity Abs induced by the MF59 formulation also in the 2-wk and 4-mo schedules (Fig. 1C). Ab avidity increased with the increase of time between the two immunizations, with the 4-mo boosting interval inducing Hla-specific IgG with the highest avidity. Finally, we characterized the functionality of these Abs by measuring their ability to neutralize the activity of the Hla toxin. HlaH35L plus MF59 outperformed HlaH35L plus alum in inducing functional Hla-neutralizing Abs (Fig. 1D), and neutralizing titers were positively correlated with total IgG titers and avidity (Fig. 1E, 1F). The enhancement in serum-neutralizing activity observed in the MF59 group may reflect a greater magnitude of the Ab responses (Fig. 1B) or an intrinsic increase in Ab affinity. Based on the serological data, the 1-mo immunization schedule was chosen for further characterization of B cells at the single-cell level.

Ag-specific B cells are expanded in bone marrow, PBMC, and lymphoid organs of HlaH35L plus MF59–immunized mice

We first determined the number of Hla-specific plasma cells and the frequencies of Hla-specific memory B cells recovered in immunized mice from femoral bone marrows and spleens, respectively. HlaH35L plus MF59 induced the highest number of Ag-specific plasma cells (98.5 ± 14.1 per 10⁶ cells; *p < 0.01) and frequency of Hla-specific memory B cells (2.12 ± 0.41% of total IgG; *p < 0.05) in the 1-mo interval schedule, as measured by ELISPOT (Fig. 2). In particular, there was a 2.23-fold increase in the number of Ag-specific bone marrow plasma cells and a 2.27-fold increase in the frequencies of Ag-specific memory B cells in the HlaH35L plus MF59 versus HlaH35L plus alum groups, hence confirming the enhanced adjuvant effect of MF59 on B cell responses. Higher responses in the HlaH35L plus MF59 group still persisted 1 mo postimmunization (Supplemental Fig. 1A, 1B).

To further characterize Hla-specific B cells, HlaH35L was conjugated to A488 and used to stain Hla-specific BCR-bearing memory B cells ex vivo for flow cytometry analyses. Switched CD19⁺IgM⁻IgD⁻A488⁺ Hla-specific B cells were detected in mice immunized with HlaH35L plus MF59, but not in mice treated with MF59 alone (Supplemental Fig. 2A), and Hla-staining specificity was confirmed on sorted cells by memory B cell ELISPOT: 16 of 19 (84%) memory B cells selected by FACS were Hla positive also on the ELISPOT assay (Supplemental Fig. 2B). Taking advantage of this new staining protocol, frequencies of switched CD19⁺IgM⁻IgD⁻A488⁺ Hla-specific B cells were determined in PBMC and secondary lymphoid organs from mice immunized twice, 1 mo apart, with HlaH35L, HlaH35L plus MF59, HlaH35L plus alum, and saline formulation as negative control (Supplemental Fig. 2C).

The analyses of inguinal proximal-draining LNs from mice receiving HlaH35L plus MF59 showed a markedly expanded total CD19⁺IgM⁻IgD⁻ compartment 10 d after the second immuni-
zation (Fig. 3A); Hla-specific B cells represented up to 7.3% of the total IgM IgD− B cell subset, with no statistical differences observed among the three groups of immunization (Fig. 3B). We also identified a population of IgM−IgD− Hla-specific B cells, likely to be Ag-specific IgM+ memory B cells, that was expanded in the HlaH35L plus MF59 group (Supplemental Fig. 3A, 3B). CD19+ IgM+IgD− Hla-specific B cells were also detected in axillary distal LNs, with the highest frequency in the HlaH35L plus MF59 group (4.47 ± 0.65%; p < 0.05). Hla-specific B cells were also expanded in spleen and PBMC from mice receiving HlaH35L plus MF59, and represented the 0.81 and 0.76% of Ig-switched B cells, respectively. Lower frequencies were observed in mice immunized with HlaH35L plus alum (in spleen, p < 0.01) or HlaH35L alone, and this trend was in agreement with our previous observations by ELISPOT analyses in the same experimental settings.

Overall, these results suggest that MF59 exerts its adjuvant effects beyond the injection site and proximal-draining LNs, and differs from alum by inducing higher frequencies of Ag-specific IgM−IgD− memory B cells in distal LNs, spleen, and blood; moreover, MF59 induces higher frequencies of Ag-specific IgM+ memory B cells in draining LNs compared with the other formulations.

**MF59 promotes expanded IgM−IgD− Hla-specific GC B cells in proximal- and distal-draining LNs**

To further determine the phenotype of CD19+IgM−IgD− Hla-specific B cells, we measured the expression of CD38 and GL-7, markers of memory and GC, respectively. CD38 is a cyclic ADP ribose hydrolase expressed by naive and memory B cells, and downregulated on GC B cells; GL-7 is commonly used as a marker for murine GC B cells (26). Combinatorial expression of these two markers in the IgM+IgD− compartment defines memory (CD38−GL-7−) and GC B cells (CD38−GL-7+), respectively, whereas naive B cells only express CD38 (Supplemental Fig. 3C).

In inguinal proximal-draining LN, most of the IgM−IgD− Hla-specific B cells were CD38−GL-7− (>75%) corresponding to a GC phenotype (Fig. 4A, 4B); no differences in the percentage were observed between MF59 and alum formulations, but the number of the IgM−IgD− Hla-specific B cells/106 cells was higher in the MF59 group (Fig. 4C), possibly associated with the expansion of the total CD19+IgM+IgD− cells in this site (Fig. 3A). Axillary distal-draining LNs from HlaH35L plus MF59–immunized mice contained both GC CD38+GL-7− and memory CD38−GL-7− IgM−IgD− Hla-specific B cells (54 and 34%, respectively), whereas only memory CD38−GL-7− IgM−IgD− Hla-specific B cells were observed in HlaH35L plus alum–immunized animals (>85%) (Fig. 4A, 4B). In the spleen, IgM−IgD− Hla-specific B cells predominantly showed a CD38−GL-7− memory phenotype (>76%) without differences observed between MF59 and alum formulations (Fig. 4A, 4B).

We further characterized the IgM+IgD− Hla-specific memory and GC B cells for the expression of CD80 and CD73 activation markers. CD80 is a ligand for CD28 and CTLA-4 expressed on T cells, and its expression on B cells is critical for a correct development of T-dependent B cell responses. A greater proportion of memory B cells upregulates CD80 expression (27, 28). CD73 is a nucleoside triphosphate-diphosphohydrolase that converts AMP in adenosine, is associated with high frequencies of mutation of the Ig genes, and plays a key role in Ig class switch recombination (28–30). The combinatorial expression of CD80 and CD73 is not associated with a clear functional phenotype, but a double-positive population is considered more memory-like (hence, more differentiated) than a single-positive one (30, 31). In this study, we showed that IgM+IgD− Hla-specific CD38−GL-7− B cells upregulated the expression of both CD80 and CD73, whereas most of the IgM+IgD− Hla-specific CD38−GL-7− GC B cells expressed mainly CD73, but not CD80 (Fig. 4D). No differences were observed between the two immunization groups. Similarly, IgM+IgD− Hla-specific CD38−GL-7− memory B cells upregulated the expression of both CD80 and CD73 (Supplemental Fig. 3D), whereas naive IgM+IgD− B cells expressed low levels of both CD80 and CD73 markers (Supplemental Fig. 3C).

**FIGURE 6.** Long-lasting Hla-specific functional humoral and B cell responses. BALB/c mice (n = 10) were immunized twice, 1 mo apart, with HlaH35L, HlaH35L plus MF59, HlaH35L plus alum, or saline formulation (ctrl). Humoral and B cell responses were measured 4 mo after the last immunization. (A) Treatment schedules; (B) Hla-specific IgG titer measured by ELISA; (C) avidity of Hla-specific IgG expressed as the ratio between the LogEC50 values obtained by ELISA and with and without thioctane treatments; (D) Hla-neutralizing titers; (E) number of Hla-specific bone marrow plasma cells measured by ELISPOT; (F) frequencies of Hla-specific memory B cells in spleen measured by ELISPOT. Nonadjuvanted HlaH35L group reported only in (B). Data show the merge of two independent experiments; each dot in (E) and (F) represents one mouse. Unpaired Student t test (C and D) and one-way ANOVA followed by Tukey’s posttest (B, E, and F) were used for statistical analyses: *p < 0.05, **p < 0.01.
To visualize GC structures in proximal-draining LNs of immune mice, we performed immunostaining of tissue sections with anti-CD21/35 (follicular DC), anti-IgD (naive B cells), and anti–GL-7 (GC) Abs, followed by confocal microscopy analyses. Mice that received HlaH35L plus MF59 or HlaH35L plus alum formulations showed characteristic GL-7⁺IgD⁻ GC structures 10 d after the last immunization (Fig. 5A, 5B). At the 1-mo time point, GL-7⁺ areas were reduced in size compared with day 10 in both vaccine formulation groups; however, LNs from mice that had received HlaH35L plus MF59 retained a higher number and larger GC structures (Fig. 5A, 5B).

A hallmark of GC responses is the presence of T\textsubscript{FH} cells that sustain proliferation and differentiation of Ag-specific GC B cells; hence, we characterized the frequencies of T\textsubscript{FH} cells in inguinal-draining LNs of mice immunized with HlaH35L, HlaH35L plus alum, or HlaH35L plus MF59. LN cell suspensions were stained

FIGURE 7. MF59 promotes persistent Ag-specific GC B cells. BALB/c mice were immunized twice, 1 mo apart, with HlaH35L plus MF59, HlaH35L plus alum, or saline formulation (ctrl). CD19⁺IgM⁺IgD⁻ Hla-specific B cells were characterized by flow cytometry in inguinal-draining LNs 4 mo after the last immunization. (A and B) Frequencies of Hla-specific B cells; (C and D) combinatorial expression of CD38 and GL-7 memory and GC markers on Hla-specific B cells; (E) combinatorial expression of CD73 and CD80 activation markers on Hla-specific CD38⁺GL-7⁻ memory and CD38⁺GL-7⁺ GC populations, as gated in (C). (A, C, and E) Numbers show the percentage of the gated populations. Each pair of inguinal lymph nodes from each mouse was processed and acquired separately. Fcs files from mice of the same group of immunization (four to five mice) were concatenated with the FlowJo software: (A), (C), and (E) show one representative experiment, and (B),(D) show three independent experiments where each dot is one experiment. Unpaired Student t test was used for statistical analyses: *p < 0.05. (F) Confocal microscopy analysis of inguinal-draining LNs of mice immunized with HlaH35L plus MF59 or HlaH35L plus alum, 4 mo after vaccination. LN sections were stained to detect follicular DC (green) and GC formation through upregulation of GL-7 (orange) and downregulation of IgD (blue). For each treatment are reported the images of IgD staining of whole LN sections (left, original magnification ×5) or the images of CD21/35, GL-7, and IgD stainings of selected follicles shown separately or merged (center and right, original magnification ×40). The image of one section is shown as example of consecutive sections of a whole LN, which is representative of three organs. Scale bar, 100 μm.
with PD-1, CXCR5, and ICOS markers to identify T<sub>FH</sub> cells in the CD3<sup>+</sup>CD4<sup>+</sup> T cell compartment. Immune mice showed expanded frequencies of T<sub>FH</sub> cells compared with mice that received the saline formulation; furthermore, mice immunized with HlaH<sub>35L</sub> plus MF59 had higher frequencies of CD4<sup>+</sup>PD-1<sup>+</sup>CXCR5<sup>+</sup>ICOS<sup>+</sup> T cells compared with the alum or nonadjuvanted group (Fig. 5C).

Overall, these data suggest that IgM<sup>+</sup>IgD<sup>+</sup>Hla-specific B cells are Ag-experienced B cells that, with the help of T<sub>FH</sub> cells, form strong GC responses in draining LN. Moreover, only MF59 induced Ag-specific GC B cells in proximal- and distal-draining LNs.

**HlaH<sub>35L</sub> plus MF59 induces long-lasting functional Ab titers and persistent GC B cells**

Induction of persistent immunity is desirable from each successful vaccine formulation; therefore, humoral and B cell responses were measured 4 mo after the second immunization in the 1-mo interval schedule (Fig. 6A). HlaH<sub>35L</sub> plus MF59 promoted higher Hla-specific IgG titers with higher avidity, and, most importantly, sera with higher functional titers compared with the HlaH<sub>35L</sub> plus alum group, 4 mo after the last immunization (Fig. 6B–D). In addition, HlaH<sub>35L</sub> plus MF59 were associated with higher number of Hla-specific bone marrow plasma cells (Fig. 6E) that could translate in the higher functional titers detected in this group compared with the HlaH<sub>35L</sub> plus alum group. Comparing these results with the 10-d time point in the 1-mo schedule (Fig. 2A), the number of Ag-specific bone marrow plasma cells increased by 2.3- and 2.6-fold for HlaH<sub>35L</sub> plus MF59 and HlaH<sub>35L</sub> plus alum, respectively. Although the overall frequencies of splenic Hla-specific memory B cells measured by ELISPOT decreased over time in all groups, HlaH<sub>35L</sub> plus MF59–immunized mice retained higher frequencies of Hla-specific memory B cells at all time points (Figs. 2B, 6F).

As memory B cells and bone marrow plasma cells mostly derive from GC, and both cell populations are maintained at higher levels in HlaH<sub>35L</sub> plus MF59–immunized mice, the persistence of Ag-specific GC B cells in these mice was analyzed by flow cytometry. HlaH<sub>35L</sub> plus MF59–immunized mice showed higher frequencies of IgM<sup>+</sup>IgD<sup>+</sup> Hla-specific B cells in inguinal-draining LNs compared with HlaH<sub>35L</sub> plus alum–treated mice (Fig. 7A, 7B). Most IgM<sup>+</sup>IgD<sup>-</sup> Hla-specific B cells showed a CD38<sup>+</sup>GL-7<sup>+</sup> memory phenotype in both immunization groups (67 and 89%, respectively). However, a persistent population of CD38<sup>+</sup>GL-7<sup>+</sup> Hla-specific GC B cells (22%) was still present in mice immunized with HlaH<sub>35L</sub> plus MF59 4 mo later (Fig. 7C, 7D). In agreement with the observations at the 10-d time point, splenic IgM<sup>+</sup>IgD<sup>-</sup> Hla-specific B cells were mainly CD38<sup>+</sup>GL-7<sup>+</sup> memory regardless of the adjuvant formulation (data not shown). Ag-specific memory and GC B cells were CD73<sup>+</sup>CD80<sup>+</sup> and CD73<sup>+</sup>CD80<sup>-</sup>, respectively (Fig. 7E), as already observed for those two populations at the 10-d time point.

Finally, immunostaining of tissue sections from proximal-draining lymph nodes showed that mice that received HlaH<sub>35L</sub> plus MF59 had persistent GC structures detected up to 4 mo after the last immunization (3 GC structures identified on 27 follicles analyzed); instead, GC structures were not observed in the HlaH<sub>35L</sub> plus alum group at the same time point (0 GC structures identified on 38 follicles analyzed) (Fig. 7F). Altogether, these data suggest that MF59 promotes more persistent Ag-specific GC B cell populations with high expression of CD73 possibly associated with ongoing Ig somatic hypermutations (Fig. 8).

**Discussion**

Characterization of the Ag-specific B cell compartment in previously published studies has involved the use of BCR-transgenic mice or Ag/adjuvant models not immediately transferable to human applications (26, 28, 32). In this study, we characterized Ag-specific B cell responses in wild-type mice immunized with HlaH<sub>35L</sub>, a S. aureus Ag known to induce protective Ab responses (22, 23, 25), formulated with adjuvants licensed in human vaccines. Altogether, our findings demonstrate that MF59 induces higher and more persistent functional Ab titers compared with alum in three different vaccination schedules. ELISPOT analyses revealed that HlaH<sub>35L</sub> plus MF59 also induced higher Hla-specific bone marrow plasma cells and memory B cells compared with the alum formulation in the 1-mo schedule. We performed a comprehensive characterization of HlaH<sub>35L</sub>-specific B cells by flow cytometry and demonstrated that the oil-in-water emulsion MF59 not only induced increased number of Ag-specific GC B cells in proximal-draining LNs compared with the alum formulation 10 d after the immunization, but also promoted new GC formation in the distal-draining ones. In parallel to increasing the number of GC B cells in the draining LN, MF59 formulation also induced higher frequencies of T<sub>FH</sub> cells. Most importantly, MF59 promoted persisting GC structures and Ag-specific GC B cells detected up to 4 mo after immunization by confocal microscopy and flow cytometry, respectively. In this study, we provide mechanistic insights of the B cell compartment associated with functional Ab responses following vaccination; we suggest that the same methodology can be extended to other vaccines or to response to infection.

In two seminal studies, Jenkins et al. (26, 33) reported that mice immunized with PE adjuvanted with CFA had Ag-specific GC B cells in distal-draining LN, long-lasting GC, and IgM<sup>+</sup> memory B cells. CFA is a water-in-oil emulsion plus microbial products derived from *Mycobacterium tuberculosis*, whereas MF59 is an oil-in-water emulsion that may share some mechanisms of action, but still maintaining very different inflammatory profiles. Remarkably, MF59 also induced higher frequencies of Ag-specific IgM<sup>+</sup> memory B cells compared with the other formulations; this population has the unique feature to further differentiate in Ag-specific GC B cells after Ag re-exposure, and could play a critical role in the context of MF59-adjuvanted vaccines, as previous clinical evidence (19–21, 34) and this work have demonstrated the strong impact that MF59 has on GC outcomes.

**FIGURE 8.** Oil-in-water emulsion MF59 mechanisms of action. Altogether, we know that MF59 (I) enhances the recruitment of phagocytic cells at the site of injection 24 h after immunization (11–13, 15); (II) induces cross-protective neutralizing Abs and memory B cells (19–21); (III) expands Ag-specific T cell subsets that are predictive of protective levels of neutralizing Abs (17, 18); and in the present work, we learned that (IV) MF59 increases the persistence of Ag-specific GC B cells.
Differences between MF59 and alum in shaping the GC responses may reflect their different mechanisms of action to activate the innate immune system [extensively reviewed by De Gregorio et al. (11)]. Whereas alum efficiently delivers the Ag to conventional DC (9, 35), MF59 generates an immunocompetent environment at the injection site that favors the trafficking and activation of monocytes (14, 15, 36). Monocyte trafficking and differentiation toward a DC phenotype have already been associated with enhanced GC responses induced by TLR agonists (39–41), and we speculate that monocyte-derived DC, rather than conventional DC, play a crucial role in MF59 adjutancy.

Previous publications have reported a correlation between the magnitude of GC B cells and TFH cells in response to increasing amount of Ag or to TLR agonists in the adjutant formulation (39, 42, 43); in addition, a recent study from Siegrist and colleagues (44) has shown that MF59 promotes potent TFH and GC B cell responses in adult mice, whereas younger infant mice, which develop only few functional TFH cells, have poor GC and Ab responses. In the present work, we show higher frequencies of TFH cells in proximal-draining LNs of mice that received the MF59 formulation compared with the alumn group, hence confirming the importance of the adjutant in shaping TFH cell responses and providing a possible explanation to the sustained Ag-specific GC B cell responses observed with MF59 formulation.

To our knowledge, for the first time, we show that a human-licensed adjuvant promotes long-lasting Ag-specific GC B cells, suggesting that Ig-gene hypermutation and affinity maturation may still be ongoing up to 4 mo after immunization. Most importantly, our findings suggest that persisting GC reactions contribute to the generation of broadly neutralizing high-affinity Ab responses, typically associated with MF59-adjutanted vaccines. Strategies to develop next-generation vaccines include Ag-adjuvant combinations that preferentially drive long-lasting and broad B cell and Ab responses, by promoting persistent GC reactions and improving the breadth of the Ig repertoire (45, 46). As example, structural vaccinology targets immunization strategies based on rationally designed Ags to drive B cell lineages toward protective epitopes. MF59 might be an ideal candidate to meet this purpose as it is safe, is already licensed in humans, expands the Ab repertoire, and, as reported in this work, promotes strong and persistent GC B cells that can be targeted to enhance their affinity maturation fate.

Adjuvants have been traditionally used to increase the magnitude of licensed vaccines by enhancing the functional Ab responses. Today, we need that selected adjuvants guide the type of adaptive responses to produce the most effective forms of immunity against each specific pathogen. To our knowledge, this is the first time that an adjuvant used in human licensed vaccines is reported to induce strong and persistent GC B cell responses. Our work provides key insights on the mechanisms of action of MF59 (Fig. 8) and strongly supports the use of oil-in-water emulsions for the rational design of vaccine formulations to promote potent and persistent GC and high-avidity humoral responses.

Acknowledgments

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Disclosures

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