MF59 Mediates Its B Cell Adjuvanticity by Promoting T Follicular Helper Cells and Thus Germinal Center Responses in Adult and Early Life

Beatris Mastelic Gavillet, Christiane S. Eberhardt, Floriane Auderset, Flora Castellino, Anja Seubert, John S. Tregoning, Paul-Henri Lambert, Ennio de Gregorio, Giuseppe Del Giudice and Claire-Anne Siegrist

J Immunol published online 13 April 2015
http://www.jimmunol.org/content/early/2015/04/11/jimmunol.1402071

The Journal of Immunology is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Published April 13, 2015, doi:10.4049/jimmunol.1402071

MF59 Mediates Its B Cell Adjuvanticity by Promoting T Follicular Helper Cells and Thus Germinal Center Responses in Adult and Early Life

Beatris Mastelic Gavillet,* Christiane S. Eberhardt,* Floriane Auderset,* Flora Castellino,†,[1] Anja Seubert,† John S. Tregoning,‡ Paul-Henri Lambert,* Ennio de Gregorio,† Giuseppe Del Giudice,† and Claire-Anne Siegrist*

The early life influenza disease burden calls for more effective vaccines to protect this vulnerable population. Influenza vaccines including the MF59 oil-in-water adjuvant induce higher, broader, and more persistent Ab responses in adults and particularly in young, through yet undefined mechanisms. In this study, we show that MF59 enhances adult murine IgG responses to influenza hemagglutinin (HA) by promoting a potent T follicular helper cells (T_{FH}) response, which directly controls the magnitude of the germinal center (GC) B cell response. Remarkably, this enhancement of T_{FH} and GC B cells is already fully functional in 3-wk-old infant mice, which were fully protected by HA/MF59 but not HA/PBS immunization against intranasal challenge with the homologous H1N1 (A/California/7/2009) strain. In 1-wk-old neonatal mice, MF59 recruits and activates APCs, efficiently induces $CD4^+$ effector T cells and primes for enhanced infant responses but induces few fully functional T_{FH} cells, which are mostly follicular regulatory T cells, and poor GC and anti-HA responses. The B cell adjuvanticity of MF59 appears to be mediated by the potent induction of T_{FH} cells which directly controls GC responses both in adult and early life, calling for studies assessing its capacity to enhance the efficacy of influenza immunization in young infants. ***The Journal of Immunology*****, 2015, 194: 000–000.**

Influenza has a high disease burden with annual incidences of 5–10% in adults and 20–30% in children, resulting in 3–5 million cases of severe illness and up to 500,000 deaths/year (1). Hospitalization for influenza-related complications is most frequent in the age group 0–12 months, with excess hospitalization rates of 50–100/10,000 children/year (2–4), and children up to five years remain at risk for developing serious complications (3, 5, 6). Consequently, the World Health Organization and most national health authorities recommend influenza vaccination for individuals at high risk for hospitalization, severe illness, or death, especially patients at both extremes of age (1). As both young children and elderly patients have weakened immune responses due to their immature or senescent immune systems (7), large efforts are undertaken to design more efficient influenza vaccines for these vulnerable populations (8).

The MF59 oil-in-water adjuvant (Adj) emulsion enhances influenza responses in the elderly and is licensed for immunization of individuals aged 65 years and older (9–11). In infants, a first trial evaluating MF59-adjuvanted versus nonadjuvanted monovalent A/H1N1 influenza vaccines between 6 and 36 months of age showed that MF59-adjuvanted formulations generated higher response rates with persistent seroprotection (12). Moreover, MF59-adjuvanted trivalent influenza vaccine (TIV) exhibited higher immunogenicity and efficacy compared with nonadjuvanted TIV (79 versus 40% between 6 and 36 months and 92 versus 45% between 36 and 72 months of age) (13).

The mode of action of MF59 is not yet completely deciphered: this oil-in-water emulsion enhances the recruitment of innate immune cells at the injection site, involving the transient release of ATP (14), and increases Ag uptake and delivery to the draining lymph nodes (dLNs) (15). Compared with other Adjs (such as Alum) or nonadjuvanted vaccines, MF59 appears to influence Ag processing/presentation, generating Abs recognizing a broader repertoire of epitopes (16, 17). Whether MF59 directly or indirectly activates B cells to increase Ab responses is yet unknown. Recent studies with nonadjuvanted TIV indicated that influenza immunization induces in humans the expansion of circulating T cells with a T follicular helper (T_{FH}) phenotype that associates and predicts the Ab and plasma cell (PC) response (18–21). As the induction of T_{FH} and germinal center (GC) B cells may not be readily assessed in humans, especially in infants and neonates, we used validated murine models (22) to study the Adj influence of MF59 on T_{FH} and GC B cells in adult and early life.

*Department of Pathology-Immunology, World Health Organization Collaborating Center for Vaccinology and Neonatal Immunology, University of Geneva, CH-1211 Geneva 4, Switzerland; †Novartis Vaccines and Diagnostics, 53100 Siena, Italy; and ‡Mucosal Infection and Immunity Group, Section of Virology, St. Mary's Campus, Imperial College London, London W2 1PG, United Kingdom

[1]Current address: Trans-NIH Center for Human Immunology, Autoimmunity and Inflammation, National Institutes of Health, Bethesda, MD.

Received for publication August 13, 2014. Accepted for publication March 6, 2015.

This work was supported by the European Commission of the Seventh Framework Program (Advanced Immunization Technologies) Grant 280873 and the Swiss National Science Foundation Grant 310000-111926/1 (to C.-A.S.) and by research grants from the Center for Vaccinology and Neonatal Immunology.

Address correspondence and reprint requests to Prof. Claire-Anne Siegrist, World Health Organization Collaborating Center for Vaccinology and Neonatal Immunology, Department of Pathology-Immunology, University of Geneva, CH-1211 Geneva 4, Switzerland. E-mail address: Claire-Anne.Siegrist@unige.ch

The online version of this article contains supplemental material.

Abbreviations used in this article: Adj, adjuvant; ASC, Ab-secreting cell; Bcl6, B cell lymphoma 6; cDC, conventional DC; DC, dendritic cell; dLN, draining LN; GC, germinal center; HA, hemagglutinin; LN, lymph node; MBC, memory B cell; MFI, mean fluorescence intensity; MoDC, monocyte-derived DC; Mono, inflammatory monocyte; PC, plasma cell; PD1, programmed death-1; p.i., postimmunization; PMN, polymorphonuclear neutrophil; PNA, peanut lectin (agglutinin); T_{FH}, T follicular helper cell; T_{FR}, T follicular regulatory cell; TIV, trivalent-inactivated influenza vaccine.

Materials and Methods

Mice

CB6F1/OlaHsd mice were purchased from Harlan (Horst, the Netherlands) and used at 6–8 wk (adults) of age. A cross between BALB/cOlaHsd female and C57BL/6JOlaHsd male mice purchased from Harlan (Horst, the Netherlands) were bred and kept in pathogen-free animal facilities in accordance with local guidelines. The F_1 hybrid mice were used at 1 wk (neonates) or 3 wk (infants) of age. All animal experiments were approved by the Geneva Veterinary Office and conducted under relevant Swiss and European guidelines. Challenge experiments were performed in parallel at Imperial College London and were carried out in accordance with Animals (Scientific Procedures) Act 1986.

Ags, Adjs, and immunization

One-week-old, 3-wk-old, and adult $CB6F_1$ mice were immunized s.c. at the scruff of the neck with 50 μl vaccine including 1 μg purified hemagglutinin (HA) subunit from influenza strain H1N1 A/California/7/2009 (Novartis, Siena, Italy) mixed 1:1 (v/v) with research grade MF59 emulsion (5% squalene, 0.5% Tween 80, and 0.5% Span 85 in citrate buffer [v/v]) (Novartis, Siena, Italy). For adjuvanticity experiments, groups of five to eight animals were immunized s.c. (scruff of the neck) on days 0 and 21 with 50 μl HA/MF59 vaccine or HA/PBS as control. Serum samples of individual mice were collected on days 0, 20 (preboost), and 35 (14 d after the last immunization on day 21) and evaluated for HA-specific total IgG and for IgG1 and IgG2a Ab isotype titers by ELISA.

Cell recruitment into dLNs

For Adj^+/Ag^+ cell tracking experiments, groups of five to eight mice were immunized s.c. in both hind legs with 25 μl/leg (50 μl total/mouse) of OVA-FITC (10 μg/mouse; Molecular Probes, Life Technologies) and MF59-Vybrant DiD (Molecular Probes, Life Technologies) vaccine or PBS as control. Labeling with the lipophilic dye DiD was performed by gently mixing the dye (1/150 dilution) to MF59 emulsion, followed by 20 min of incubation at 37˚C. MF59-DiD was mixed with an equal volume of buffer and Ag prior to injection. For activation markers and costimulatory molecules assessment, mice were immunized s.c. in both hind legs with 25 μl/leg (50 μl total/mouse) of 1 μg HA and MF59-Vybrant DiD vaccine or HA/PBS as control. Mice were killed 24 h or 4 d postimmunization (p.i.), and the inguinal LNs were harvested and placed into 24-well tissue culture plates (Nunc) containing serum-free DMEM. LNs were cut into small pieces and digested for 20 min at 37˚C with 0.4 mg/ml Liberase CI (Roche) and 1 mg/ml DNase (Sigma-Aldrich) in serum-free DMEM. EDTA (0.1 M) was added, and single-cell suspensions were obtained by homogenization. Cells were stained with combinations of the following Abs: Ly6G, B220, CD11c, Ly6C, CD86, CD80 (all from BD Biosciences), CD40 (produced in-house), CD11b, and F4/80 (all from BioLegend).

In vitro restimulation of Ag-specific T cell responses

The brachial and axillary LNs were harvested and pooled per mouse after s.c. immunization at the scruff of the neck to assess the frequency and phenotype of Ag-specific $CD3^+$,$CD4^+CD44^+$ T cells. Single-cell suspensions were cultured with HA (1 μg/ml) or medium alone as well as anti-CD28 and anti-CD49d Abs (BD Biosciences) for 2 h, before the addition of brefeldin A (Sigma-Aldrich). Following additional 5 h of incubation at 37˚C, 5% CO_2, cells were stained with conjugated Abs against CD4, CD3 (from BD Biosciences), CD8α, and CD44 (from BioLegend) and then fixed and permeabilized with Cytofix/Cytoperm solution (from BD Biosciences). For determination of Ag-specific cytokine responses by intracellular staining, cells were stained with conjugated Abs against IFN-γ, IL-2, IL-17, IL-5, IL-4 (all from BD Biosciences), TNF-α, and IL-10 (both from eBioscience). Data were acquired on the Gallios (Beckman Coulter) cytometer and analyzed using FlowJo Software (Tree Star).

Flow cytometric analysis

The brachial and axillary LNs were harvested and pooled per mouse after s.c. immunization at the scruff of the neck. LN single-cell suspensions were prepared by homogenization and analyzed by flow cytometry using mAbs to GL7, CD8 (both from BD Biosciences), programmed death-1 (CD279, known as PD1), B220, Ter119, Gr-1, CD11c (all from eBioscience), and CD4 (BioLegend). CXCR5 staining was done using purified anti-CXCR5 (BD Biosciences), followed by FITC anti-rat IgG (Southern Biotechnology Associates), and normal rat serum (eBioscience). For GC B cell and PC staining, cells were preincubated with blocking 2.4G2 (CD16/32), and stained for B220, TCRβ, CD95 (Fas) (all from eBioscience), and GL7 (BD Biosciences) or with CD4, CD8 (produced in-house), IgD (BioLegend), B220 (eBioscience), and CD138 (BD Biosciences), respectively. Each staining step was done in PBS + 2% FCS + 0.05% azide on ice; samples were acquired without fixation. Intracellular Bcl6 (BD Biosciences) and Foxp3 (eBioscience) staining were performed with the Foxp3 Staining Set (eBioscience). Data were acquired on the Gallios (Beckman Coulter) cytometer and analyzed using FlowJo Software (Tree Star).

Real-time quantitative PCR

RNA and cDNA were prepared as previously described (23). Briefly, RT-PCRs were performed on a SDS 7900 HT instrument (Applied Biosystems). Each reaction was performed in three replicates, with *ActinG*, *EEf1*, and *MmRPS9* as internal controls genes for data normalization. Raw cycle threshold values obtained with SDS 2.2 (Applied Biosystems) were imported in Microsoft Excel, and normalization factor and fold changes were calculated using the GeNorm method (24). Primer sequences used for internal control genes and for *il-21* are stated in Refs. 23 and 25, respectively.

Immunohistochemistry

dLNs from mice immunized s.c. with HA/MF59 or HA/PBS (five to eight mice per group) were frozen in Tissue-Tek OCT compound (Sakura) and cut into 6-μm frozen sections, thaw mounted on glass slides, air-dried, fixed in acetone, and stored at −20˚C until staining. One section from each mouse (five to eight mice per time points) was rehydrated in 1% BSA in PBS for 10 min and blocked with 10% horse serum in PBS for 30 min at room temperature. For B cell follicle enumeration, slides were stained for 1 h with control or anti-IgD purified Ab (BioLegend) and then with a goat-anti-rat IgG (H+L)-Alexa 488 (Invitrogen). For $CD4^+$ T cells enumeration within GC, slides were stained with control or anti–CD4-Alexa 647 Abs (Biolegend) and biotinylated-peanut lectin (agglutinin) (PNA) (Vector Laboratories) for 30 min, followed by Streptavidin-Cy3 (Invitrogen). Sections were visualized and photographed with a Zeiss LSM700 confocal microscope (objective, ×20) at room temperature, and images were acquired with Zeiss LSM image browser software (Zeiss). The numbers of GC per LN were counted under the microscope, and the average of GC per LN was calculated for each LN so as to correct for difference in LN size between mice of different ages.

Quantification of Ag-specific Abs in serum

Serum samples were obtained on days 20 and 35 and Ag-specific total IgG, IgG1, or IgG2a measured by ELISA. For HA-specific ELISA, ELISA plates (96-well flat bottom plates; Nunc Maxisorp) were coated with 2 μg/ml purified HA subunit from influenza strain H1N1 A/California/7/2009 and blocked with 1% BSA (Sigma-Aldrich) in PBS for 1 h at 37˚C and then washed three times and incubated for 2 h at 37˚C with individual mouse sera. Serum was added in an initial dilution of 1:100, after which 1:2 serial dilutions were performed in 0.05% Tween and 0.01% BSA in PBS. A standard serum was included in each assay as positive control. Plates were washed and incubated for 1 h at 37˚C with secondary HRP-conjugated anti-mouse total IgG (Invitrogen Life Technologies) or the different subclasses IgG1 (BD Pharmingen) or IgG2a (Southern Biotechnology Associates). Plates were washed four times and developed with tetramethylbenzidine (Invitrogen Life Technologies) for 10 min and then stopped with 1 N HCl. The OD of each well was determined by using a SpectraMax (Molecular Devices) reader at 450 nm, and end-point titers were calculated. Prior to challenge, IgG Abs were detected by incubation for 2 h at 37˚C with HRP-conjugated goat anti-mouse IgG (AbD Serotec). Plates were washed and developed with 50 μl tetramethylbenzidine/E substrate and the reaction was terminated by the addition of 50 μl 2 M H_2SO_4 prior to reading at 450 nm. A dilution series of recombinant murine IgG was used on each plate as a standard to quantify the influenza-specific Abs.

ELISPOT assay for HA-specific PCs

HA-specific PCs were quantified by direct ex vivo ELISPOT assay. Ninety-six-well Multiscreen HA Nitrocellulose filtration plates (Millipore) were coated with 50 μl 4 μg/ml HA diluted in PBS. As a positive control for total IgG-secreting cells, some wells on each plate were coated with goat anti-mouse IgG (Southern Biotechnology Associates). The plates were incubated at 4˚C overnight, washed twice in PBS, and then blocked with 200 μl complete RPMI 1640 medium for 1 h at 37˚C. The plates were then washed twice with PBS, and cell suspensions were added at an initial number of 2×10^6 cells, after which 1:2 serial dilutions were performed in 100 μl complete RPMI 1640 medium. The plates were incubated at 37˚C, 5% CO_2 for 5 h and then washed twice in PBS and twice with PBS with 0.1% Tween (PBS-T). A total of 100 μl goat anti-mouse IgG- HRP conjugated Ab (Abcam) diluted 1:1000 in PBS-T containing 1% FCS was

added, and the plates were incubated overnight at 4°C. Plates were washed three times with PBS-T, followed with three washes with PBS. Detection was carried out by adding 100 μl/well 3-amino-9-ethylcarbazole substrate buffer and incubating in the dark for 30 min until spots appeared. The reaction was stopped by thorough washing with cold tap water and air-dried. Spots were counted by eye under the microscope.

ELISPOT assay for HA-specific memory B cells

Memory B cells were detected using a modification of the PC ELISPOT. In brief, 1 ml 2.5×10^6 cell suspensions of spleen were distributed on flat-bottom 24-well plates (Nunc) and cultured for 6 d in 1 ml complete RPMI 1640 medium containing 2 μg R595 LPS (Enzo Life sciences), 0.8×10^6 irradiated (1200 rad) naive congenic splenocytes and 20 μl supernatant from Con A–stimulated splenocytes, prepared as previously described (26). Cells were harvested, washed with RPMI 1640 medium containing 2.5 mM EDTA, and counted in complete RPMI 1640 medium. Cell suspensions were added to 96-wells Multiscreen HA Nitrocellulose filtration plates coated with 50 μl 4 μg/ml HA diluted in PBS at an initial number of 4×10^5 cells, after which 1:2 serial dilutions were performed in 100 μl complete RPMI 1640 medium. The ELISPOT assay was then performed as described above for HA-specific PC detection. The frequencies of HA-specific memory B cells were counted by eye under the microscope.

Challenge against influenza

A mouse-adapted H1N1 Influenza strain (A/California/7/2009) was grown in Madin–Darby canine kidney cells in serum-free DMEM supplemented with 1 μg/ml trypsin. The virus was harvested 3 d after inoculation and stored at −80°C. Viral titer and lung viral load were determined by plaque assay as previously described (27). Thirty-five days p.i., 3×10^4 PFU in 100 μl were instilled intranasally under inhalation anesthesia with isoflurane. Mice were observed daily until day 7 to monitor body weight.

Statistical analysis

Statistical analysis between results obtained from various group of mice were performed using the Mann–Whitney *U* test (*p = 0.01–0.05, **p = 0.001–0.01, ***$p < 0.001$, and ****$p < 0.0001$). Differences with $p > 0.05$ were considered to be insignificant. Spearman correlation coefficients (r) were assessed between the numbers of T_{FH} and of GC B cells at each age; $r > 0.8$ was considered as a strong correlation using a significance level of 0.05 for all analyses. Differences in weight loss following challenge were assessed by ANOVA test with Bonferroni correction.

Results

MF59 adjuvantation elicits adult-like influenza HA serum IgG responses in infant mice

To define the influence of MF59 adjuvantation on Ab responses to influenza HA, infant (3 wk old) and adult (6–8 wk old) mice were immunized s.c. at days 0 and 21 with HA/MF59 or HA/PBS. Serum IgG, IgG1, and IgG2a Ab responses were assessed on days 0, 10, 20, and 35 after priming. As expected (28), in adult mice, MF59 significantly enhanced IgG anti-HA primary and secondary responses compared with PBS (day 20: 4.71 ± 0.06 versus 3.65 ± 0.21 $\log_{10}$; day 35: 6.02 ± 0.22 versus 4.81 ± 0.17 $\log_{10}$, $p \leq 0.001$), primarily through the induction of IgG1 Abs (Fig. 1A). Remarkably, HA/MF59 induced slightly delayed (day 10 IgG; 3 wk: 3.16 ± 0.09 versus adult: 3.96 ± 0.06 $\log_{10}$, $p < 0.0001$) but otherwise adult-like primary and secondary IgG and IgG1 HA responses in infant mice (Fig. 1A).

In accordance with these results, similar numbers of HA-specific IgG PCs were detected by ELISPOT in the spleen of adult and infant mice (2.2 ± 0.28 versus 1.43 ± 0.49 ASC/10^6 splenocytes, respectively) (Supplemental Fig. 1A). The frequency of spleen HA-memory B cells (MBCs) measured by ELISPOT 8 wk after immunization was also similar in adult and infant mice (1.49 ± 0.93 versus 0.77 ± 0.25 MBC/10^5 splenocytes, respectively) (Supplemental Fig. 1B). Thus, MF59 adjuvantation proved as efficient in infant as in adult mice, increasing IgG1-secreting PCs and MBCs to adult levels.

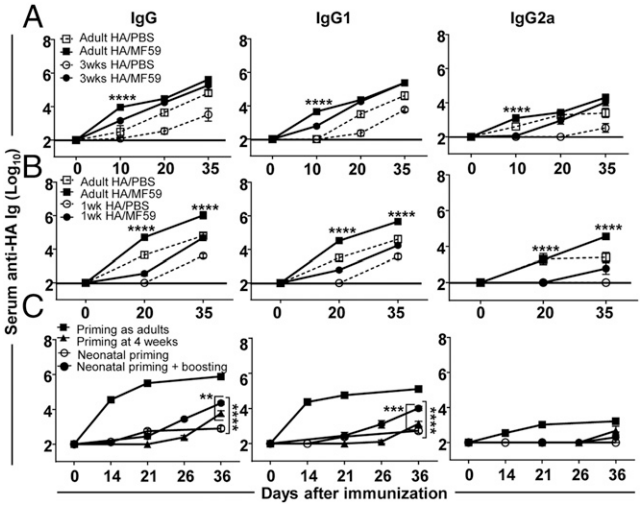

FIGURE 1. MF59 adjuvantation elicits adult-like IgG/IgG1 anti-HA responses in infant but not in neonatal mice. Naive $CB6F_1$ mice were immunized s.c. at days 0 and 21 with HA/MF59 or HA/PBS as adults (6–8 wk of age) (**A** and **B**), infant (3-wk-old) (A), or neonates (1-wk-old) (B). In (**C**), HA/MF59 was administered to 4-wk-old mice either primed as neonates (prime-boost on days 0 and 21, respectively) with HA/MF59 (●) or not (▲) (prime on day 21), control mice being primed only on day 0 as neonates (○), or as adults (■). Mice were bled on indicated time points for determination of serum IgG, IgG1, and IgG2a HA–specific Ab responses. Data are represented as mean ± SEM and representative of one of two independent experiments, five to eight mice per group (**p = 0.001–0.01, ***$p < 0.001$, ****$p < 0.0001$).

MF59 adjuvanticity: potent induction of germinal center responses in adult and infant mice

The mechanisms through which MF59 adjuvantation enhances B cell responses have not yet been described. Germinal center (GC) reactions are essential determinants of the magnitude and the quality of Ab responses and humoral memory (29–31), which may not be readily assessed in humans. We first used confocal microscopy to study the influence of MF59 on GC responses. Ten days after a single HA/MF59 (or HA/PBS) immunization, frozen dLN sections were stained for follicular B cells (IgD, blue), GC B cells (PNA, red), and T cells (CD4, green) (Fig. 2A). PNA^+ GCs were easily identified in HA/MF59-immunized adult and infant mice (Fig. 2A, 2B), whereas they were very rare (0–1 per LN sections) in adult and absent in infant HA/PBS-immunized mice (Fig. 2A; data not shown). A trend toward lower average numbers of PNA^+ GCs per LN was observed in HA/MF59-immunized infant mice (Fig. 2B), mirroring their reduced day 10 Ab response (Fig. 1A). However, the number of GCs was not statistically different even at this early time point after immunization (Fig. 2B). To more precisely quantify the GC response, GL-7^+CD95^+ GC B cells were assessed by flow cytometry in the dLNs 10 d after immunization. Compared with HA/PBS, MF59 adjuvantation markedly increased adult GC responses to HA (Fig. 3A, 3C) and elicited similar numbers of GC B cells in adult and infant mice, both in proportion (data not shown) and numbers (Fig. 3D).

MF59 adjuvanticity is mediated through the induction of T_{FH} cells

In recent years, T_{FH} cells have emerged as critical for GC formation and for the generation of PCs and MBCs (32–35). To define whether MF59 enhanced serum IgG and GC responses through an increase of T_{FH} cells, infant and adult mice were immunized with HA/MF59, and $CXCR5^{high}PD\text{-}1^{high}CD4^+$ T_{FH} cells

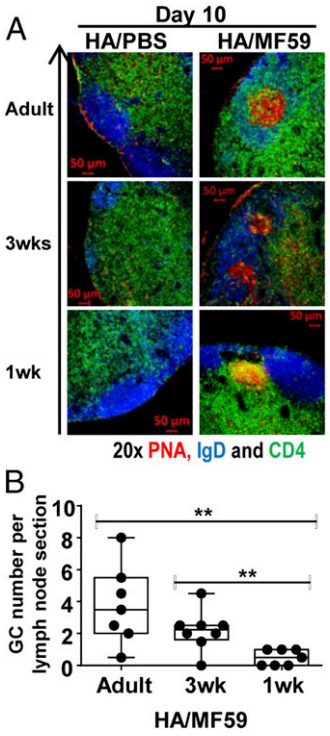

FIGURE 2. MF59 enhances GC responses in adult and infant mice. dLNs from adult, infant, and neonatal mice (five to eight mice per group) were harvested 10 d after one dose of HA/MF59 or HA/PBS and were analyzed by immunohistochemistry. (**A**) One representative section of dLN immunohistochemical analysis for IgD (blue), PNA (red), and CD4 (green) in adult, infant, and 1-wk-old mice. (**B**) The number of GCs per section in HA/MF59-immunized mice was enumerated by confocal microscopy. Results are expressed as mean ± SEM obtained from five to eight LNs sections (**p = 0.001–0.01).

were assessed 10 d later in their dLNs (Fig. 3B). MF59 adjuvantation significantly increased $CXCR5^{high}PD\text{-}1^{high}CD4^+$ T_{FH} cell responses (Fig. 3B–D), and T_{FH} cell expansion was as strong following infant as adult HA/MF59 immunization (Fig. 3B, 3D).

GC B cell and T_{FH} cell responses may be qualified by their functional markers. The B cell lymphoma 6 (Bcl6) transcriptional repressor is a master regulator for both the GC B cell program (36) and for the T_{FH} cell lineage (37–39). In GC B cells, Bcl6 enables and maintains the GC phenotype, whereas it promotes the differentiation of $CD4^+$ T cells into T_{FH} cells. To define whether infant HA/MF59 immunization elicited fully-differentiated GC B cells and T_{FH} cells, we assessed Bcl6 expression by $GL\text{-}7^+$ $CD95^+$ GC B cells and $CXCR5^{high}PD\text{-}1^{high}CD4^+$ T_{FH} cells following HA/MF59 immunization. In both age groups, the mean fluorescence intensity (MFI) of Bcl6 was significantly higher on GC B cells than on control $GL7^-CD95^-B220^+$ follicular B cells and on T_{FH} cells than on control $CXCR5^-PD\text{-}1^-CD4^+$ T cells, respectively (data not shown). The MFI ratio of $Bcl6^+$ on GC B/follicular B cells and on $T_{FH}/CXCR5^-PD\text{-}1^-CD4^+$ T cells was similarly high in infant and adult mice (Fig. 3E). This also applies to GL7, a marker for fully differentiated T_{FH} cells preferentially located within GCs (data not shown) (40). These results demonstrate that the MF59 adjuvantation of influenza vaccines induces fully-functional adult-like GC B cells and T_{FH} cells in both adult and infant mice.

GC responses are controlled by a large number of determinants. To define the relative contribution of the induction of T_{FH} cells to MF59-elicited GC responses, the correlations between T_{FH} and GC B cells numbers were assessed in individual mice 10 d after HA/MF59 immunization. A strong positive direct correlation was observed between adult T_{FH} and GC B cell numbers (Fig. 3F), indicating that the increase of the number of T_{FH} cells by MF59 adjuvantation directly translates to increased GC responses, leading to higher Ab-secreting PCs and MBCs. Despite their ongoing immune maturation, a similarly strong correlation between T_{FH} and GC B cells number was observed in infant mice (Fig. 3F), indicating that the MF59 formulation meets all the requirements of a potent Adj for influenza HA infant immunization.

MF59 is required for the protection of infant mice against H1N1 viral challenge

To assess the influence of MF59 adjuvantation on protection against challenge, infant mice previously injected with HA/PBS, HA/MF59 or PBS alone were challenged intranasally with homologous H1N1 virus. Body weight loss was monitored as sign of progressive infection. The body weight of unvaccinated (PBS) or HA/PBS-immunized mice declined below 80% within 6–7 d after challenge (Fig. 3G). In contrast, all infant and adult control mice immunized with HA/MF59 only showed a small (<10%) and transient decrease in body weight (Fig. 3G). This complete protection was associated to comparable Ab responses in HA/MF59-immunized adult and infant mice (Fig. 3G).

MF59 adjuvantation fails to enhance IgG Ab responses in neonates

As young age is a critical risk factor for severe influenza infection, we next asked whether MF59 might circumvent the limitations of B cell responses earlier in life (i.e., in neonates [1-wk-old mice]). Serum IgG responses to two doses of HA/MF59 (or HA/PBS) were assessed at various times after neonatal priming and compared with those elicited in adult mice. Neonates did not raise detectable primary IgG responses to HA/PBS and their secondary responses remained weak (Fig. 1B). MF59 adjuvantation slightly increased primary IgG and IgG1 responses, although not significantly. Secondary responses were also not significantly higher after HA/PBS or HA/MF59 immunization and remained significantly lower than adult responses (day 35: 1wk: 4.68 ± 0.17 $\log_{10}$; adult: 6.02 ± 0.22 $\log_{10}$, $p < 0.0001$) (Fig. 1B). Accordingly, 8 wk after priming, HA-specific IgG-secreting PCs were significantly fewer in the neonatal spleen (0.4 ± 0.36 $ASC/10^6$ splenocytes) than in adults (2.2 ± 0.28 $ASC/10^6$ splenocytes) (Supplemental Fig. 1A).

Because two doses of HA/MF59 were required for 1-wk-old mice to reach Ab titers elicited by a single dose in adult mice, we asked whether MF59 adjuvantation did induce neonatal priming. The frequency of spleen HA-MBC measured by ELISPOT 8 wk after a single immunization was significantly lower in neonatally primed than in adult mice (mean frequencies 0.13 ± 0.13 versus 1.49 ± 0.93 $MBC/10^5$ splenocytes) (Supplemental Fig. 1B). Nevertheless, some MBCs were elicited by a single neonatal dose of HA/MF59. Furthermore, IgG/IgG1 responses of 4-wk-old mice primed as neonates with HA/MF59 and boosted at 4 wk of age were significantly higher than in age-matched mice having received a single dose of HA/MF59 at 1 or 4 wk of age (day 36; IgG1: neonatal priming and boosting: 4.01 ± 0.11 $\log_{10}$ versus priming at 4 wk: 3.13 ± 0.14 $\log_{10}$ ($p < 0.001$); versus neonatal priming: 2.73 ± 0.13 $\log_{10}$ [$p < 0.0001$]) (Fig. 1C). Thus, MF59 adjuvantation failed to induce neonatal anti-HA IgG responses but primed for enhanced infant responses.

MF59 adjuvantation elicits weak germinal center responses in neonates

Because IgG and memory responses are primarily elicited through GC reactions, PNA^+ GCs were quantified by confocal microscopy

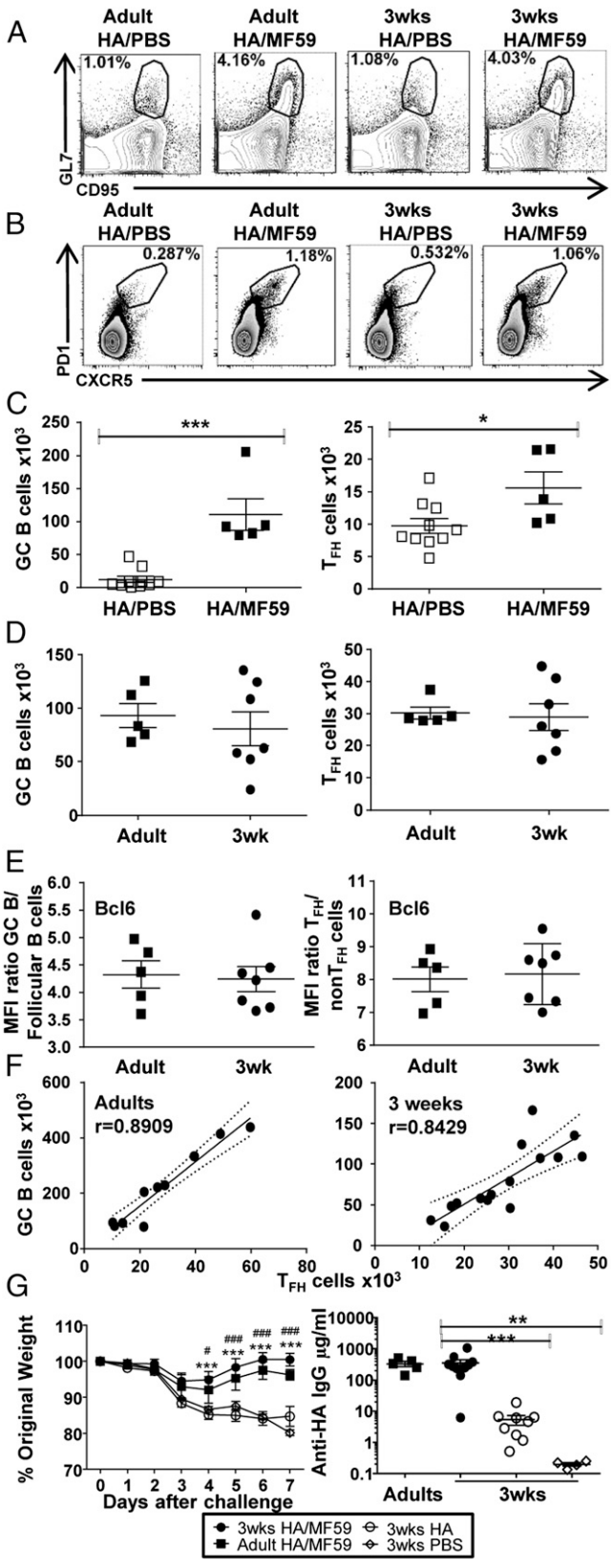

FIGURE 3. MF59 similarly elicits functional GC B cells and T_{FH} cells in adult and infant mice. (**A**–**F**) dLNs harvested 10 d after HA/MF59 or HA/PBS immunization of adult and infant mice (five to eight mice per group) were analyzed by flow cytometry to determine the percent and number of GL-7$^+$ Fas$^+$ (CD95) GC B cells and CXCR5^{high}PD1^{high} T_{FH} cells. Representative dot plots of adult and infant immunized mice, gated on B220$^+$ B cells (A) and CD4$^+$ T cells (B). (C) Number of GC B cells and T_{FH} cells in adult mice immunized with HA/MF59 or HA/PBS (*p = 0.01–0.05, ***$p < 0.001$). (D) Number of GC B cells and T_{FH} cells in adult and infant mice immunized with HA/MF59. (E) Bcl6 MFI on GC B and T_{FH} cells from adult and infant mice day 10 p.i. The MFI ratio was calculated by dividing the Bcl6 MFI of GC B cells by that of GL7$^-$CD95$^-$B220$^+$ follicular B cells and by dividing the Bcl6 MFI of T_{FH} cells by the MFI of CXCR5$^-$PD-1$^-$CD4$^+$ T cells. Results are expressed as mean ± SEM. (F) Correlations (day 10 p.i.) between T_{FH}/GC B cells in individual mice immunized at 3 and 6–8 wk of age with HA/MF59. Spearman correlation coefficients (r) between the numbers of T_{FH} and of GC B cells are indicated at each age. Data are representative of one of two independent experiments. (**G**) Groups of six to eight 3-wk-old and adult $CB6F_1$ mice were immunized s.c. with 50 µl HA/MF59, HA/PBS, or PBS. Thirty-four days after injection (1 d prior to infection), serum samples were collected and evaluated for HA-specific total IgG titers by ELISA (*right panel*). A total of 3 × 10^4 PFU of mouse-adapted H1N1 Cal/7/2009 strain was instilled intranasally under inhalation anesthesia. Mice were observed daily until day 7 to monitor body weight (*left panel*). Results are expressed as mean ± SEM. Data are representative of one of two independent experiments. Infant mice: HA/MF59 versus HA alone (**p = 0.001–0.01, ***$p < 0.001$); HA/MF59 versus PBS ($^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$).

10 d after neonatal HA/MF59 immunization. Despite MF59 adjuvantation, GCs were rarely detectable in B cell follicles after neonatal immunization (1 wk: 0.5 ± 0.19; adult: 3.8 ± 0.9; p = 0.0047) (Fig. 2A, 2B). The quantification of GL-7$^+$CD95$^+$ GC B cells by flow cytometry (Fig. 4A, 4C) identified a weak GC B cell response 10 d after neonatal HA/MF59 immunization, but GC B cells remained 15 times lower in neonates compared with adults (1 wk: 7.1 ± 1.2 × 10^3 versus adult: 110.9 ± 23.9 × 10^3; p = 0.001).

This weak neonatal GC response was paralleled by that of CXCR5^{high}PD-1^{high}CD4$^+$ T_{FH} cells, which was significantly reduced in neonatal compared with adult HA/MF59-immunized mice (1 wk: 5.8 ± 0.5 × 10^3 versus adult: 15.6 ± 2.5 × 10^3; p = 0.001) (Fig. 4B, 4C). As observed in adult and infant mice, the MFI of Bcl6 was significantly higher on the few neonatal GC B and T_{FH} cells than on GL7$^-$CD95$^-$B220$^+$ follicular B cells or CXCR5$^-$PD-1$^-$ CD4$^+$ T cells, respectively (data not shown). However, the Bcl6$^+$ MFI ratio of GC B/follicular B cells, the intensity of Bcl6 expression in T_{FH} cells and that of GL7, which identifies GC T_{FH} cells were all significantly lower in neonates (Fig. 4D). The proportion of T_{FH} cells expressing Foxp3 was significantly higher in neonates (Fig. 4E), suggesting a preferential induction of T follicular regulatory (T_{FR}) cells (41–43). Furthermore, IL-21 mRNA transcripts were expressed at lower levels in neonatal than infant or adult activated CD3$^+$CD8$^-$CD4$^+$CD44$^+$ cells (Fig. 4F). These results indicate that in neonates MF59 adjuvantation is not sufficient to induce fully-functional Foxp3$^-$IL-21$^+$ T_{FH} cells and the T_{FH} – GC B cells cross-talk, which is essential for the optimal formation of GCs and Ab responses (44–46).

MF59 induces a similar recruitment pattern of Adj- and Ag-loaded cells in adult and early life dLNs

Because MF59 adjuvanticity is primarily mediated through enhanced innate cell recruitment and activation at the site of immunization and in the dLNs (47, 48), we asked whether the limited T_{FH} and thus GC B cell neonatal responses resulted from impaired cell recruitment and/or activation. Using fluorescently labeled OVA (OVA-FITC) and MF59 (MF59-DiD), we assessed the number and percentages of Adj- and Ag-loaded cells (Fig. 5A) and the percentages of polymorphonuclear neutrophils (PMNs; CD11c$^-$, CD11b$^+$, Ly6G$^+$, Ly6C$^+$), inflammatory monocytes (Mono; CD11c$^-$, CD11b$^+$, Ly6G$^-$, Ly6C^{high}), macrophages (CD11c$^-$, CD11b$^+$, Ly6G$^-$, Ly6C$^-$, F4/80$^+$), B cells (CD11b$^-$, CD11c$^-$, B220$^+$), plasmacytoid dendritic cells (DCs) (CD11b$^-$,

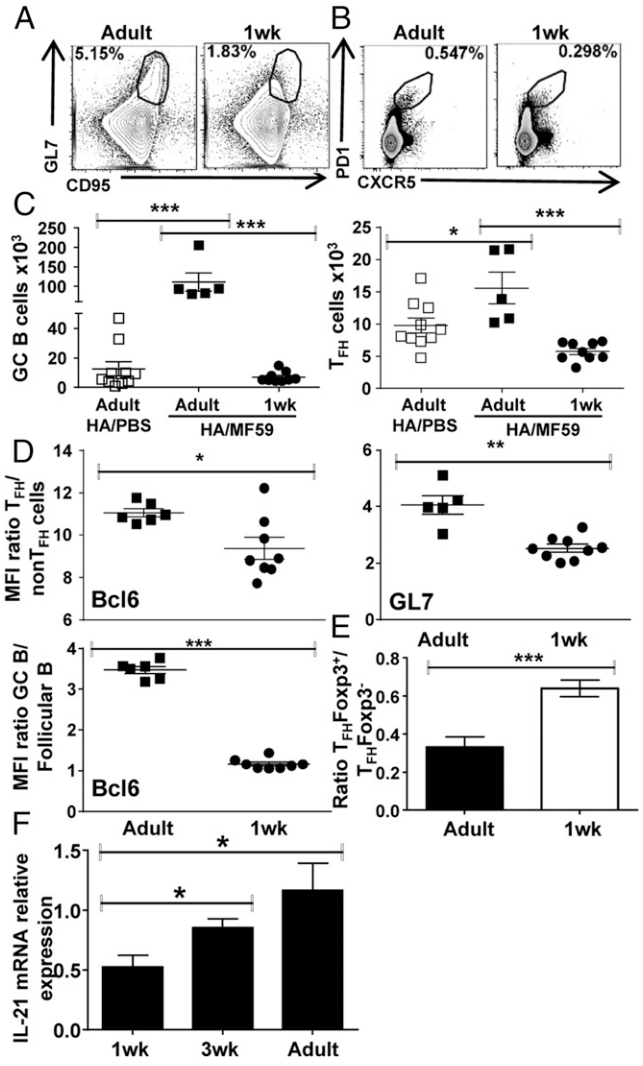

FIGURE 4. MF59 adjuvantation induces a limited expansion of functional T_{FH} cells and thus GC responses in neonates. (**A**–**D**) dLNs from day 10 adult and neonatal mice immunized (five to eight mice per group) with HA/MF59 (or HA/PBS as control) were analyzed by flow cytometry to determine the percent and number of GL-7$^+$ Fas$^+$ (CD95) GC B cells and CXCR5^{high}PD1^{high} T_{FH} cells. Representative dot plots of adult and 1-wk-old immunized mice and gated on B220$^+$ B cells (A) and CD4$^+$ T cells (B). (C) Number of GC B cells and T_{FH} cells. (D) Bcl6 and GL7 MFI on GCB and T_{FH} cells from adult and neonatal mice day 10 p.i. The MFI ratio was calculated by dividing the Bcl6 MFI of GCB cells by that of GL7$^-$CD95$^-$ B220$^+$ follicular B cells and by dividing the Bcl6 or GL7 MFI of T_{FH} cells by the corresponding MFI of CXCR5$^-$PD-1$^-$CD4$^+$ T cells. (**E**) Foxp3 expression on gated T_{FH} cells from adult and 1-wk-old mice day 10 p.i. The ratio was calculated by dividing the number of Foxp3$^+$ T_{FH} cells by the number of Foxp3$^-$ T_{FH} cells. Results are expressed as mean ± SEM. Data are representative of one of two independent experiments (*p = 0.01–0.05, **p = 0.001–0.01, ***p < 0.001). (**F**) CD3$^+$CD8$^-$CD4$^+$CD44$^+$ T cells were sorted on day 10 after HA/MF59 immunization of 1-wk-old, infant, and adult mice. Pooled LNs of 5 adults, 8 infants, and 16 neonatal mice from three independent experiments were analyzed in triplicates by real-time PCR for IL-21 expression (*p = 0.01–0.05).

B220$^+$, CD11c$^+$, Ly6C$^+$), conventional DCs (cDCs; CD11c$^+$, CD11b^{hi}, Ly6C$^-$) and monocyte-derived DCs (MoDCs; CD11c$^+$, CD11b^{hi}, Ly6C^{hi}) among the Adj- and Ag-loaded cells recruited to the dLNs 24h after neonatal, infant or adult immunization (Fig. 5B, gating strategy in Supplemental Fig. 2). As previously described (48), adult OVA-MF59 immunization led to the recruitment of Ag-loaded cells to the dLNs (Fig. 5A). In the adult dLNs, Adj$^+$ and Ag$^+$ cells essentially included monocytes, cDCs, MoDCs, and to a lesser extent B cells (Fig. 5B). Among each APC population, the highest proportion of Ag$^+$Adj$^+$ cells was observed for MoDCs (41.77 ± 2.687%), followed by PMNs (31.6 ± 4.135%), monocytes (23.72 ± 2.106%) and macrophages (14.35 ± 1.318%) (Fig. 5C). A similar influx of Ag$^+$Adj$^+$ cells (Fig. 5A) and distribution of APCs among the Ag$^+$Adj$^+$ cells (Fig. 5B) were observed in mice immunized at 3 wk of age.

In neonates, the number of Ag$^+$Adj$^+$ APCs was lower but their proportion was similar to that of infant and adult mice (Fig. 5A), indicating efficient draining into the neonatal dLNs. The proportion of each APC population among Ag$^+$Adj$^+$ cells included a smaller proportion of cDCs (11.25 ± 1.23 versus 17.31 ± 0.71%) and, to a lesser extent, of Mono than in adults (Fig. 5B), a difference that persisted for cDCs up to 4 d after neonatal immunization (data not shown). However, the proportion of Ag$^+$Adj$^+$ cells among each neonatal APC population was similar to that of infant mice that respond well to MF59 adjuvantation (Fig. 5C), except for a lower proportion of Ag$^+$Adj$^+$ PMNs. Thus, the limitations of T_{FH} neonatal responses do not result from critical differences of APC targeting by MF59.

To investigate whether the lower recruitment of vaccine-loaded cDCs and inflammatory monocytes (Mono) in neonates was associated to a distinct activation status, we compared the expression of CD40, CD80, and CD86 on neonatal and infant Adj-loaded cDCs and Mono, following s.c. immunization with HA and labeled-MF59 (Fig. 5D). At 24h after HA/MF59 immunization, MF59 induces a marked upregulation of CD40, CD80, and CD86 costimulatory molecules by Adj$^+$ cDC and monocytes, with few differences among age groups (Fig. 5D). Thus, MF59 induced a similar pattern of immune cells recruitment and activation into neonatal dLNs, although vaccine-loaded cDCs were fewer and slightly less activated in neonates than in infant mice.

MF59 induces comparable effector T cell priming in neonates and infant mice

The observation that reduced numbers of Adj$^+$Ag$^+$ cDCs reached the neonatal dLNs following HA/MF59 immunization suggested that this may impact the priming of HA-specific CD4$^+$ T cells, including T_{FH} cells. To address this hypothesis, mice were immunized s.c. with HA/MF59 or HA/PBS as neonates or infants, an age at which adult-like innate cell and B cell responses are elicited. Ten days later, dLN cells were restimulated in vitro with HA, and the cytokine (IL-2, IL-4, IL-13, IL-5, TNF-α, and IFN-γ) of HA-specific T cells was assessed by flow cytometry. Gating on CD3$^+$CD8$^-$CD4$^+$CD44$^+$ Ag-experienced T cells, a larger expansion of CD4$^+$ T cells was measured following HA/MF59 compared with HA/PBS immunization (Fig. 6A, 6B). Importantly, neonatal and infant cytokine responses of HA-specific CD4$^+$ T_H cells were comparable and similarly dominated by IL-2, TNF-α, and IL-4 (Fig. 6A, 6B). Thus, MF59 adjuvantation similarly primes CD4$^+$ T_H effector responses in infant and neonatal mice but not fully functional Foxp3$^-$IL-21$^+$ T_{FH} cells.

Discussion

In this study, we demonstrate that the MF59 Adj mediates its influence on B cell responses through the induction of potent T_{FH} responses that directly control the magnitude of the GC B cell reaction, sparking new insights into its mode of action and identifying T_{FH} cells as biomarkers of GC and Ab responses to MF59-adjuvanted vaccines. Remarkably, this T_{FH} promoting influence is already observed in infant mice in which MF59 elicits adult-like responses and affords 100% protection against influenza virus challenge. In neonates, MF59 adjuvantation primes for enhanced

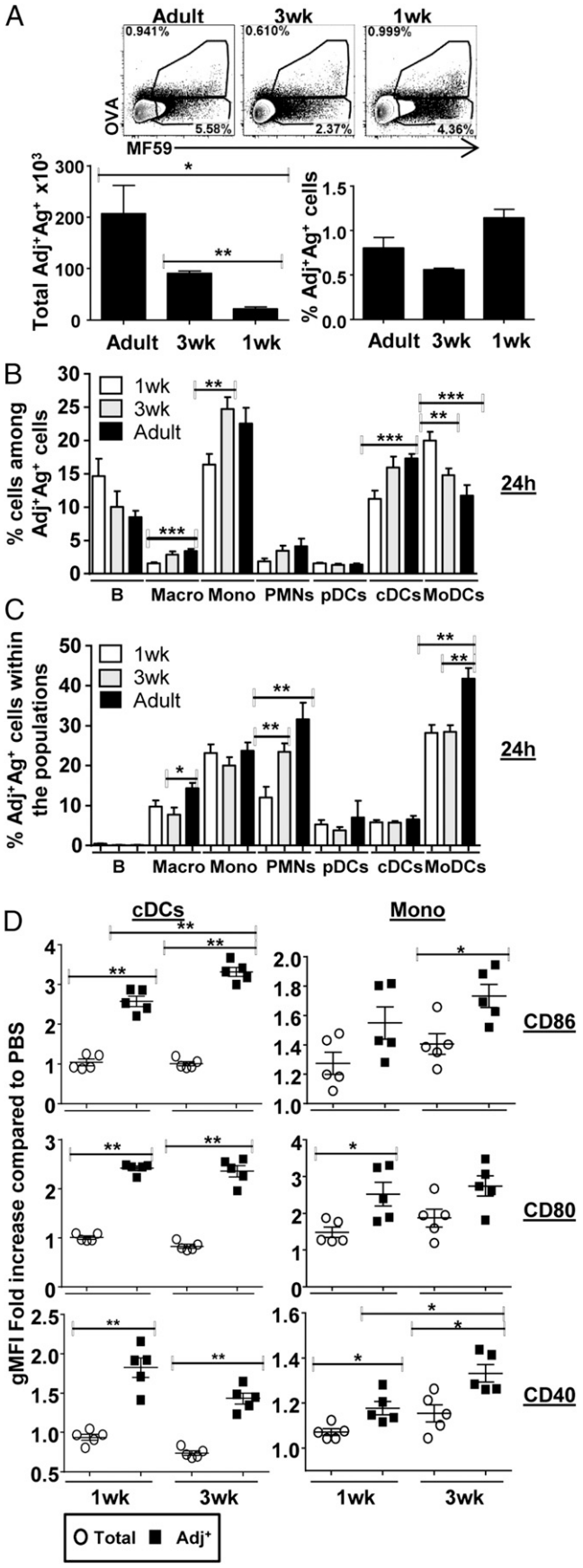

FIGURE 5. MF59 increases the recruitment and activation of Ag-loaded cells in the neonatal dLNs. Adult, infant and neonatal mice (five to eight mice per group) were immunized s.c. with 25 μl OVA-FITC/MF59-DID, or PBS as control, in both hind legs. Following enzymatic digestion, single-cell suspensions of dLNs were analyzed by flow cytometry at 24 h. (**A**) Representative dot plots of total Adj^+ (MF59-DiD) and Ag^+ (OVA-FITC) cells in adult, infant, and 1-wk-old immunized mice. Numbers and percentage of total $OVA^+/MF59^+$ cells in all age groups. (**B** and **C**) Comparison of cell recruitment into the dLN in response to immunization with MF59 in different age groups. Several cell types identified by their specific markers were analyzed by flow cytometry at 24 h. (B) Histograms show the percentage of each cell population among Adj^+ (MF59-DiD) and Ag^+ (OVA-FITC) cells for each age group. (C) Histograms show the percentage of Adj^+ (MF59-DiD) and Ag^+ (OVA-FITC) cells within each cell population of adult, infant, and neonatal immunized mice. Results are expressed as mean ± SEM (*p = 0.01–0.05, **p = 0.001–0.01, ***$p < 0.001$). (**D**) cDCs and Mono were further analyzed for their expression of CD40, CD80, and CD86 24 h p.i. with HA and labeled MF59. Data are presented as the fold increase compared with the mean geometric MFI of PBS control-immunized mice and are representative of one of two independent experiments (*p = 0.01–0.05, **p = 0.001–0.01).

infant responses by eliciting a modest memory B cell response but does not promote $Foxp3^-IL\text{-}21^+CD4^+$ T_{FH} cells, GC, and Ab responses.

A key feature of MF59 adjuvantation is to increase the breadth, the magnitude, and the persistence of Ab responses in humans (49). In mice, this is mediated by the transient release of endogenous ATP at the site of injection (14), triggering the preferential recruitment and activation of PMNs and monocytes, which take up and transport Ags and Adj molecules into the dLNs (14, 15, 47, 48). This APC recruitment/activation process directly primes for potent $CD4^+$ T cell effector responses characterized by a mixed T_{h1}/T_{h2} profile (15). Exactly how MF59 expands B cell responses is yet unknown. In this study, we show that MF59 is a potent inducer of the T_{FH} cell differentiation program (Fig. 3C) and that suggests that this feature controls its B cell adjuvanticity: each additional 1000 T_{FH} cells induced by HA/MF59 in adult mice results into the differentiation of 7000 additional GC B cells (Fig. 3F) in a strong positive linear correlation indicating that additional mechanisms only exert a limited contribution to GC responses. Given the critical role of the GC reaction for PC generation, memory induction, and affinity maturation (31), this identifies T_{FH} cells as biomarkers of GC responses to MF59-adjuvanted vaccines and provides a mechanistic explanation for the higher magnitude, breadth, and durability of Ab responses observed following MF59 adjuvantation (49). This is in accordance with recent studies demonstrating the importance of T_{FH} cell help in human responses to unadjuvanted influenza vaccines (18, 19, 50). Given the importance of optimizing influenza vaccine responses in high-risk group patients, studies should now identify the risk groups in whom MF59 elicits potent T_{FH} cell responses or fails to do so.

Infants are a major risk group for influenza complications (51). We show in this study that MF59 adjuvantation is as effective at inducting T_{FH} cells in infant as in adult mice and that this similarly translates into increased GC B cells numbers and Ab responses. That each additional 1000 T_{FH} cell results into an increase of 2500 GC B cells indicates that the MF59-induced ATP-mediated APC recruitment/activation/presentation process is already fully functional in early life. Although murine observations may not always be translated to humans, numerous similarities have been described between the early life patterns of responses of these two species (reviewed in Ref. 7). In accordance with the protective efficacy of influenza vaccines in young children, we show in this study that MF59 adjuvantation is required to protect infant mice. This suggests that the enhanced and more persistent Ab responses to MF59-adjuvanted influenza vaccines in older

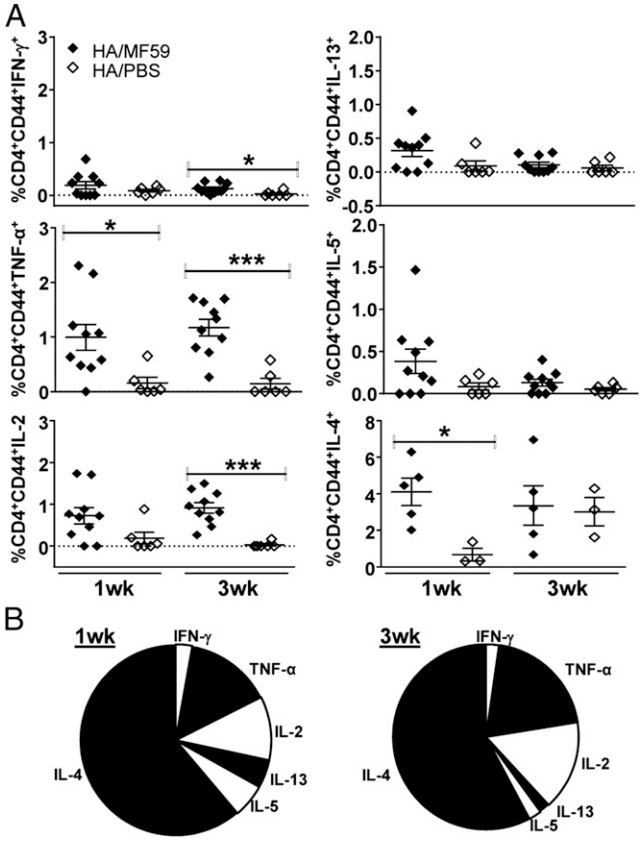

FIGURE 6. MF59 elicits comparable effector T cell priming to influenza HA in neonates and infant mice. dLNs from neonatal and infant mice (five to eight mice per group) were taken 10 d following s.c. immunization with HA/MF59 or HA/PBS, and HA-specific $CD4^+$ T cells were reactivated by in vitro stimulation. Cells were gated on $CD3^+CD8^-CD4^+CD44^+$ T cells for the analysis of Ag-experienced $CD4^+$ T cells. (**A**) The percentage of their intracellular cytokines expression of IFN-γ, TNF-α, IL-2, IL-13, IL-5, and IL-4. (**B**) Pie charts represent the percentage of cytokine expression in HA-specific $CD4^+$ T cells from 1-wk-old or infant-immunized mice. Results are expressed as mean ± SEM (*p = 0.01–0.05, ***$p < 0.001$). Data pool of two independent experiments, except for IL-4; data are representative of one of two independent experiments.

infants and young children (12, 13) are mediated by enhanced T_{FH}/GC responses conferring higher protective efficacy. Whether this T_{FH}/GC promoting influence applies to other Ags is biologically probable and deserves prompt investigations given the generically limited magnitude and persistence of infant responses to Adj free or alum-based vaccines.

Neonates and young infants are at even higher risks of acute cardiorespiratory failure following influenza (51, 52), and vaccines proven effective before 6 mo of age could markedly reduce this disease burden. We thus assessed the influence of MF59 adjuvantation on B cell responses earlier in ontogeny in neonates. In contrast to the adult-like responses of 3-wk-old mice, anti-HA IgG and GC responses remained weak in 1-wk-old neonates despite MF59 adjuvantation. This was associated with a limited induction of T_{FH} cells (Fig. 4): few neonatal T_{FH} cells were elicited, and they expressed lower levels of key molecules such as Bcl6 (a master switch of the T_{FH} cell differentiation program (37–39)), GL7 (40), and IL-21 (53) while expressing higher levels of Foxp3. This preferential differentiation toward $Foxp3^+$ T_{FR} cells may contribute to impede T_{FH} cells development and thus GC B cell differentiation and survival (41–43).

Why are T_{FH} (and thus GC) cell responses so readily elicited in infancy but not in neonates? Following immunization with MF59-adjuvanted vaccines, Ag and Adj molecules are readily transported to the neonatal draining LNs (Fig. 5). A lower proportion of $Ag^+/MF59^+$ cDCs was observed in neonatal than infant mice, both early and several days after immunization (Fig. 5). cDCs are critical to initiate the T_{FH} cell differentiation program (54), suggesting a limited cDC recruitment/activation/presentation process. However, neonatal cDCs readily respond to MF59 adjuvantation, as reflected by their increased expression of CD40, CD80, and CD86, and their recruitment/activation is sufficient to induce similar $CD4^+$ T cell effector responses as in infant mice (Fig. 6). This demonstrates a specific limitation of the T_{FH} cell program and its critical contribution to neonatal B cell responses (23).

The induction of T effector and T_{FH} cell responses is fine-tuned by specific subsets of DCs expressing cytokines that promote (such as IL-6 or IL-21) or prevent (such as IL-2) T_{FH} cell differentiation (as reviewed in Ref. 54), and further studies will focus on the relative T effector versus T_{FH} cell activating capacities of neonatal DCs. Another hypothesis is that T_{FH} cell neonatal differentiation is effectively initiated by MF59 but that this process is abortive, neonatal T_{FH} cells failing to expand as previously observed with Alum (23). CpG-ODNs increase adult T_{FH} cell responses when combined with water-in-oil and oil-in-water emulsions (55) and act synergistically with aluminum salts to enhance T_{FH} and GC B neonatal responses (23). However, the coadministration of CpG-B ODNs and HA/MF59 had a very modest effect on neonatal T_{FH} cell responses and did not increase GC B cells nor Ab responses (Supplemental Fig. 3). Thus, the limitation of neonatal T_{FH} cell differentiation may be circumvented by the synergistic influence of CpG-B with aluminum salts but not with MF59. Further studies will define whether this results from distinct influences on neonatal T_{FH} cell priming or expansion and delineate the requirements for vaccine Adjs to elicit stronger GC responses already in neonates.

That MF59 adjuvantation did not significantly enhance T_{FH}/GC B cell responses in neonatal mice does not imply that it will not prove effective in very young infants. Indeed, neonatal priming with HA/MF59 induced an MBC response and higher Ab responses to infant boosting (Fig. 1). This neonatal priming/infant boosting pattern is similar to that observed in human infants immunized at birth with HIVgp120/MF59 in whom adjuvantation did not increase neonatal Ab responses but primed for higher responses to infant boosting (56). MBCs may be elicited within or outside of GCs, even in the absence of T_{FH} cell help (57), and it will be interesting to address whether neonatal immunization essentially induces GC-independent MBCs.

In summary, the potent induction of the T_{FH} cell differentiation program is a key feature of MF59 adjuvanticity that is already effective early in life despite distinct and specific T_{FH} cell differentiation requirements at various stages of ontogeny and calls for the evaluation of MF59-adjuvanted influenza vaccines in young infants.

Acknowledgments

We thank Novartis Vaccines (Siena, Italy) for providing the reagents and particularly Grazia Galli and Cecilia Buonsanti. We thank particularly Jacqueline U. McDonald (Imperial College London) for setting up the challenge model. We thank Stephane Grillet for key contributions to the complex experimental work required by this study, Chantal Tougne for the immunohistology analyses, Paolo Quirighetti for excellent assistance with animal care, Olivier Brun and François Prodon from the Bioimaging Platform for help with confocal microscopy, and Jean-Pierre Aubry-Lachainaye and Cécile Gameiro from the flow cytometry platform.

Disclosures

The authors have no financial conflicts of interest.

References

1. World Health Organization. 2014. *Influenza (Seasonal) Fact sheet No. 211*. World Health Organization, Geneva, Switzerland.
2. Poehling, K. A., K. M. Edwards, G. A. Weinberg, P. Szilagyi, M. A. Staat, M. K. Iwane, C. B. Bridges, C. G. Grijalva, Y. Zhu, D. I. Bernstein, et al. 2006. The underrecognized burden of influenza in young children. *N. Engl. J. Med.* 355: 31–40.
3. Coffin, S. E., T. E. Zaoutis, A. B. Rosenquist, K. Heydon, G. Herrera, C. B. Bridges, B. Watson, R. Localio, R. L. Hodinka, and R. Keren. 2007. Incidence, complications, and risk factors for prolonged stay in children hospitalized with community-acquired influenza. *Pediatrics* 119: 740–748.
4. Neuzil, K. M., B. G. Mellen, P. F. Wright, E. F. Mitchel, Jr., and M. R. Griffin. 2000. The effect of influenza on hospitalizations, outpatient visits, and courses of antibiotics in children. *N. Engl. J. Med.* 342: 225–231.
5. Mertz, D., T. H. Kim, J. Johnstone, P. P. Lam, M. Science, S. P. Kuster, S. A. Fadel, D. Tran, E. Fernandez, N. Bhatnagar, and M. Loeb. 2013. Populations at risk for severe or complicated influenza illness: systematic review and meta-analysis. *BMJ* 347: f5061.
6. Fraaij, P. L., and T. Heikkinen. 2011. Seasonal influenza: the burden of disease in children. *Vaccine* 29: 7524–7528.
7. Siegrist, C. A., and R. Aspinall. 2009. B-cell responses to vaccination at the extremes of age. *Nat. Rev. Immunol.* 9: 185–194.
8. Leroux-Roels, I., and G. Leroux-Roels. 2009. Current status and progress of prepandemic and pandemic influenza vaccine development. *Expert Rev. Vaccines* 8: 401–423.
9. Della Cioppa, G., U. Nicolay, K. Lindert, G. Leroux-Roels, F. Clement, F. Castellino, C. Galli, N. Groth, Y. Levin, and G. Del Giudice. 2014. A dose-ranging study in older adults to compare the safety and immunogenicity profiles of MF59-adjuvanted and non-adjuvanted seasonal influenza vaccines following intradermal and intramuscular administration. *Hum. Vaccin. Immunother.* 10: 1701–1710.
10. Van Buynder, P. G., S. Konrad, J. L. Van Buynder, E. Brodkin, M. Krajden, G. Ramler, and M. Bigham. 2013. The comparative effectiveness of adjuvanted and unadjuvanted trivalent inactivated influenza vaccine (TIV) in the elderly. *Vaccine* 31: 6122–6128.
11. Puig-Barberà, J., A. Natividad-Sancho, J. Calabuig-Pérez, J. A. Lluch-Rodrigo, E. Pastor-Villalba, S. Martínez-Úbeda, S. Pérez-Vilar, and J. Díez-Domingo. 2013. MF59-adjuvanted and virosomal influenza vaccines for preventing influenza hospitalization in older people: comparative effectiveness using the Valencia health care information system. *Vaccine* 31: 3995–4002.
12. Block, S. L., G. M. Ruiz-Palacios, M. L. Guerrero, J. Beygo, V. Sales, and S. J. Holmes. 2012. Dose-range study of MF59-adjuvanted versus nonadjuvanted monovalent A/H1N1 pandemic influenza vaccine in six- to less than thirty-six-month-old children. *Pediatr. Infect. Dis. J.* 31: e92–e98. Available at: http://journals.lww.com/pidj/Fulltext/2012/07000/Dose_range_Study_of_MF59_adjuvanted_Versus.18.aspx.
13. Vesikari, T., M. Knuf, P. Wutzler, A. Karvonen, D. Kieninger-Baum, H. J. Schmitt, F. Baehner, A. Borkowski, T. F. Tsai, and R. Clemens. 2011. Oil-in-water emulsion adjuvant with influenza vaccine in young children. *N. Engl. J. Med.* 365: 1406–1416.
14. Vono, M., M. Taccone, P. Caccin, M. Gallotta, G. Donvito, S. Falzoni, E. Palmieri, M. Pallaoro, R. Rappuoli, F. Di Virgilio, et al. 2013. The adjuvant MF59 induces ATP release from muscle that potentiates response to vaccination. *Proc. Natl. Acad. Sci. USA* 110: 21095–21100.
15. O'Hagan, D. T., G. S. Ott, E. De Gregorio, and A. Seubert. 2012. The mechanism of action of MF59 - an innately attractive adjuvant formulation. *Vaccine* 30: 4341–4348.
16. Khurana, S., N. Verma, J. W. Yewdell, A. K. Hilbert, F. Castellino, M. Lattanzi, G. Del Giudice, R. Rappuoli, and H. Golding. 2011. MF59 adjuvant enhances diversity and affinity of antibody-mediated immune response to pandemic influenza vaccines. *Sci. Transl. Med.* 3: 85ra48.
17. Bihari, I., G. Pánczél, J. Kovacs, J. Beygo, and E. Fragapane. 2012. Assessment of antigen-specific and cross-reactive antibody responses to an MF59-adjuvanted A/H5N1 prepandemic influenza vaccine in adult and elderly subjects. *Clin. Vaccine Immunol.* 19: 1943–1948.
18. Bentebibel, S. E., S. Lopez, G. Obermoser, N. Schmitt, C. Mueller, C. Harrod, E. Flano, A. Mejias, R. A. Albrecht, D. Blankenship, et al. 2013. Induction of $ICOS^+CXCR3^+CXCR5^+$ TH cells correlates with antibody responses to influenza vaccination. *Sci. Transl. Med.* 5: 176ra132. Available at: http://stm.sciencemag.org/content/5/176/176ra32.
19. Spensieri, F., E. Borgogni, L. Zedda, M. Bardelli, F. Buricchi, G. Volpini, E. Fragapane, S. Tavarini, O. Finco, R. Rappuoli, et al. 2013. Human circulating influenza-$CD4^+ICOS1^+IL\text{-}21^+$ T cells expand after vaccination, exert helper function, and predict antibody responses. *Proc. Natl. Acad. Sci. USA* 110: 14330–14335.
20. Ma, C. S., and E. K. Deenick. 2014. Human T follicular helper (Tfh) cells and disease. *Immunol. Cell Biol.* 92: 64–71.
21. Mastelic, B., N. Garcon, G. Del Giudice, H. Golding, M. Gruber, P. Neels, and B. Fritzell. 2013. Predictive markers of safety and immunogenicity of adjuvanted vaccines. *Biologicals* 41: 458–468.
22. Siegrist, C. A. 2001. Neonatal and early life vaccinology. *Vaccine* 19: 3331–3346.
23. Mastelic, B., A. T. Kamath, P. Fontannaz, C. Tougne, A. F. Rochat, E. Belnoue, C. Combescure, F. Auderset, P. H. Lambert, F. Tacchini-Cottier, and C. A. Siegrist. 2012. Environmental and T cell-intrinsic factors limit the expansion of neonatal follicular T helper cells but may be circumvented by specific adjuvants. *J. Immunol.* 189: 5764–5772.
24. Vandesompele, J., K. De Preter, F. Pattyn, B. Poppe, N. Van Roy, A. De Paepe, and F. Speleman. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* 3: RESEARCH0034. Available at: http://genomebiology.com/content/3/7/RESEARCH0034.
25. Chen, G., K. Hardy, K. Bunting, S. Daley, L. Ma, and M. F. Shannon. 2010. Regulation of the IL-21 gene by the NF-κB transcription factor c-Rel. *J. Immunol.* 185: 2350–2359.
26. Kelso, A., and A. L. Glasebrook. 1984. Secretion of interleukin 2, macrophage-activating factor, interferon, and colony-stimulating factor by alloreactive T lymphocyte clones. *J. Immunol.* 132: 2924–2931.
27. Elleman, C. J., and W. S. Barclay. 2004. The M1 matrix protein controls the filamentous phenotype of influenza A virus. *Virology* 321: 144–153.
28. Dormitzer, P. R., R. Rappuoli, D. Casini, D. O'Hagan, C. Runham, E. Montomoli, B. Baudner, J. J. Donnelly Iii, G. Lapini, and A. Wack. 2009. Adjuvant is necessary for a robust immune response to a single dose of H1N1 pandemic flu vaccine in mice. *PLoS Curr.* 1: RRN1025. Available at: http://currents.plos.org/influenza/article/adjuvant-is-necessary-for-a-robust-immune-response-to-a-single-dose-of-h1n1-pandemic-flu-vaccine-in-mice/.
29. MacLennan, I. C. 1994. Germinal centers. *Annu. Rev. Immunol.* 12: 117–139.
30. Tarlinton, D. M. 2008. Evolution in miniature: selection, survival and distribution of antigen reactive cells in the germinal centre. *Immunol. Cell Biol.* 86: 133–138.
31. Victora, G. D., and M. C. Nussenzweig. 2012. Germinal centers. *Annu. Rev. Immunol.* 30: 429–457.
32. Crotty, S. 2011. Follicular helper CD4 T cells (TFH). *Annu. Rev. Immunol.* 29: 621–663.
33. King, C. 2009. New insights into the differentiation and function of T follicular helper cells. *Nat. Rev. Immunol.* 9: 757–766.
34. McHeyzer-Williams, M., S. Okitsu, N. Wang, and L. McHeyzer-Williams. 2012. Molecular programming of B cell memory. *Nat. Rev. Immunol.* 12: 24–34.
35. Vinuesa, C. G., M. A. Linterman, C. C. Goodnow, and K. L. Randall. 2010. T cells and follicular dendritic cells in germinal center B-cell formation and selection. *Immunol. Rev.* 237: 72–89.
36. Basso, K., C. Schneider, Q. Shen, A. B. Holmes, M. Setty, C. Leslie, and R. Dalla-Favera. 2012. BCL6 positively regulates AID and germinal center gene expression via repression of miR-155. *J. Exp. Med.* 209: 2455–2465.
37. Johnston, R. J., A. C. Poholek, D. DiToro, I. Yusuf, D. Eto, B. Barnett, A. L. Dent, J. Craft, and S. Crotty. 2009. Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation. *Science* 325: 1006–1010.
38. Nurieva, R. I., Y. Chung, G. J. Martinez, X. O. Yang, S. Tanaka, T. D. Matskevitch, Y. H. Wang, and C. Dong. 2009. Bcl6 mediates the development of T follicular helper cells. *Science* 325: 1001–1005.
39. Yu, D., S. Rao, L. M. Tsai, S. K. Lee, Y. He, E. L. Sutcliffe, M. Srivastava, M. Linterman, L. Zheng, N. Simpson, et al. 2009. The transcriptional repressor Bcl-6 directs T follicular helper cell lineage commitment. *Immunity* 31: 457–468.
40. Yusuf, I., R. Kageyama, L. Monticelli, R. J. Johnston, D. Ditoro, K. Hansen, B. Barnett, and S. Crotty. 2010. Germinal center T follicular helper cell IL-4 production is dependent on signaling lymphocytic activation molecule receptor (CD150). *J. Immunol.* 185: 190–202.
41. Linterman, M. A., W. Pierson, S. K. Lee, A. Kallies, S. Kawamoto, T. F. Rayner, M. Srivastava, D. P. Divekar, L. Beaton, J. J. Hogan, et al. 2011. $Foxp3^+$ follicular regulatory T cells control the germinal center response. *Nat. Med.* 17: 975–982.
42. Chung, Y., S. Tanaka, F. Chu, R. I. Nurieva, G. J. Martinez, S. Rawal, Y. H. Wang, H. Lim, J. M. Reynolds, X. H. Zhou, et al. 2011. Follicular regulatory T cells expressing Foxp3 and Bcl-6 suppress germinal center reactions. *Nat. Med.* 17: 983–988.
43. Wollenberg, I., A. Agua-Doce, A. Hernández, C. Almeida, V. G. Oliveira, J. Faro, and L. Graca. 2011. Regulation of the germinal center reaction by $Foxp3^+$ follicular regulatory T cells. *J. Immunol.* 187: 4553–4560.
44. Qi, H., J. L. Cannons, F. Klauschen, P. L. Schwartzberg, and R. N. Germain. 2008. SAP-controlled T-B cell interactions underlie germinal centre formation. *Nature* 455: 764–769.
45. Vogelzang, A., H. M. McGuire, D. Yu, J. Sprent, C. R. Mackay, and C. King. 2008. A fundamental role for interleukin-21 in the generation of T follicular helper cells. *Immunity* 29: 127–137.
46. Choi, Y. S., R. Kageyama, D. Eto, T. C. Escobar, R. J. Johnston, L. Monticelli, C. Lao, and S. Crotty. 2011. ICOS receptor instructs T follicular helper cell versus effector cell differentiation via induction of the transcriptional repressor Bcl6. *Immunity* 34: 932–946.
47. Seubert, A., E. Monaci, M. Pizza, D. T. O'Hagan, and A. Wack. 2008. The adjuvants aluminum hydroxide and MF59 induce monocyte and granulocyte chemoattractants and enhance monocyte differentiation toward dendritic cells. *J. Immunol.* 180: 5402–5412.
48. Calabro, S., M. Tortoli, B. C. Baudner, A. Pacitto, M. Cortese, D. T. O'Hagan, E. De Gregorio, A. Seubert, and A. Wack. 2011. Vaccine adjuvants alum and MF59 induce rapid recruitment of neutrophils and monocytes that participate in antigen transport to draining lymph nodes. *Vaccine* 29: 1812–1823.
49. Galli, G., K. Hancock, K. Hoschler, J. DeVos, M. Praus, M. Bardelli, C. Malzone, F. Castellino, C. Gentile, T. McNally, et al. 2009. Fast rise of broadly cross-reactive antibodies after boosting long-lived human memory B cells primed by an MF59 adjuvanted prepandemic vaccine. *Proc. Natl. Acad. Sci. USA* 106: 7962–7967.
50. Pallikkuth, S., A. Parmigiani, S. Y. Silva, V. K. George, M. Fischl, R. Pahwa, and S. Pahwa. 2012. Impaired peripheral blood T-follicular helper cell function in HIV-infected nonresponders to the 2009 H1N1/09 vaccine. *Blood* 120: 985–993.

51. Fiore, A. E., D. K. Shay, K. Broder, J. K. Iskander, T. M. Uyeki, G. Mootrey, J. S. Bresee, and N. J. Cox, Centers for Disease Control and Prevention. 2010. Prevention and control of seasonal influenza with vaccines: recommendations of the Advisory Committee on Immunization Practices (ACIP), 2009. [Published erratum appears in 2009 *MMWR Recomm. Rep.* 58: 896-897.] *MMWR Recomm. Rep.* 58: 1–52.
52. Monto, A. S. 2009. The risk of seasonal and pandemic influenza: prospects for control. *Clin. Infect. Dis.* 48(Suppl. 1): S20–S25.
53. Nurieva, R. I., Y. Chung, D. Hwang, X. O. Yang, H. S. Kang, L. Ma, Y. H. Wang, S. S. Watowich, A. M. Jetten, Q. Tian, and C. Dong. 2008. Generation of T follicular helper cells is mediated by interleukin-21 but independent of T helper 1, 2, or 17 cell lineages. *Immunity* 29: 138–149.
54. Ballesteros-Tato, A., and T. D. Randall. 2014. Priming of T follicular helper cells by dendritic cells. *Immunol. Cell Biol.* 92: 22–27.
55. Chakarov, S., and N. Fazilleau. 2014. Monocyte-derived dendritic cells promote T follicular helper cell differentiation. *EMBO Mol. Med.* 6: 590–603.
56. McFarland, E. J., W. Borkowsky, T. Fenton, D. Wara, J. McNamara, P. Samson, M. Kang, L. Mofenson, C. Cunningham, A. M. Duliege, et al. 2001. Human immunodeficiency virus type 1 (HIV-1) gp120-specific antibodies in neonates receiving an HIV-1 recombinant gp120 vaccine. *J. Infect. Dis.* 184: 1331–1335.
57. Kaji, T., A. Ishige, M. Hikida, J. Taka, A. Hijikata, M. Kubo, T. Nagashima, Y. Takahashi, T. Kurosaki, M. Okada, et al. 2012. Distinct cellular pathways select germline-encoded and somatically mutated antibodies into immunological memory. *J. Exp. Med.* 209: 2079–2097.