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Research Paper

MF59 Emulsion Is an Effective Delivery System for a Synthetic TLR4 Agonist (E6020)

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Purpose. The effectiveness of vaccines depends on the age and immunocompetence of the vaccinee. Conventional non-adjuvanted influenza vaccines are suboptimal in the elderly and vaccines with improved ability to prevent influenza are required. The TLR4 agonist E6020, either given alone or co-delivered with MF59, was evaluated and compared to MF59 and the TLR9 agonist CpG. Its ability to enhance antibody titres and to modulate the quality of the immune response to a subunit influenza vaccine was investigated.
Methods. Mice were immunized with either antigens alone, with MF59 or with the TLR agonists alone, or with a combination thereof. Serum samples were assayed for IgG antibody titres and hemagglutination inhibition (HI) titres. Th1/Th2 type responses were determined by titrating IgG subclasses in serum samples and by T-cell cytokine responses in splenocytes.
Results. MF59 was the best single adjuvant inducing HI and T-cell responses in comparison to all alternatives. The co-delivery of E6020 or CpG with MF59 did not further increase antibody titres however shifted towards a more Th1 based immune response.
Conclusion. Combining adjuvants like E6020 and MF59 allowed a finer tuning of the immune response towards a particular Th bias, thus have significant implications for the development of improved influenza vaccines.

KEY WORDS: adjuvants; influenza vaccine delivery; MF59; toll like receptor agonists; T-cell cytokine response.

INTRODUCTION

The possibility to select among classical and new generation adjuvants, to use them alone or in combination with the potential to act synergistically opens new ways to improve vaccine efficacy, optimally adapted to the target population and to the known or presumed mechanism of protection against specific infectious diseases.

Vaccination is the principal measure for preventing influenza and reducing the impact of epidemics. In annual influenza epidemics, 5–15% of the population are affected with upper respiratory tract infections. In the very young, the elderly and people suffering from medical conditions such as lung diseases, diabetes, cancer, kidney or heart problems, influenza poses a serious risk. In these groups, the infection may lead to severe sequelae or opportunistic superinfections, potentially resulting in pneumonia and death (http://www.who.int/mediacentre/factsheets/fs211/en/ (accessed 12/17/08)). The effectiveness of influenza vaccines depends primarily on the age and immunocompetence of the vaccine recipient and the degree of similarity between the virus strains used for the vaccine and those in circulation (http://www.who.int/mediacentre/factsheets/fs211/en/ (accessed 12/17/08)). In the elderly, influenza vaccines are less effective at preventing influenza, mainly due to senescence-related impaired immune responses. Both antibody (Ab) titres and cell-mediated immunity in response to influenza are reduced in the elderly (1,2). Thus, conventional non-adjuvanted influenza vaccines are far from optimal, and vaccines with higher ability to prevent influenza are clearly required. Vaccine adjuvants are an attractive option to overcome impaired immune responses in the elderly and may offer the opportunity to enhance influenza vaccine efficacy (3,4).

Immunological adjuvants have been classified in a number of ways and can be divided broadly into two categories: delivery systems and immune potentiators. Delivery systems may optimize antigen exposure to the immune system or are capable of targeting antigen to specific physiological locations, whereby uptake by different populations of professional antigen-presenting cells can result in enhanced immunity. Alternatively immune potentiators act directly on immune cells by activating pathways important in the induction of adaptive immunity (5,6).

The adjuvant MF59 is an oil in water emulsion with a low oil content, resulting in a non-viscous formulation that is easy to

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inject. The use of a microfluidizer in the preparation process allows the generation of a droplet size in the nanoscale range. The small droplet size is crucial for the potency of the adjuvant, enhances its stability and allows the formulation to be sterile filtered for clinical use (7,8). The MF59 adjuvant has been included in a licensed influenza vaccine (Fluad®) for a decade, therefore a significant amount of clinical data to establish its potency and safety is available. Compared with conventional influenza vaccines, MF59 adjuvanted subunit vaccine gives an improved immune response and is effective even when the match between vaccine and circulating strains is not perfect (9). In addition, MF59 has been clinically evaluated with a range of alternative vaccines such as herpes simplex virus (HSV-2), HIV, CMV, HBV and hepatitis C virus (HCV) (10–14). Thus MF59 has broad potential to be used as a safe and effective vaccine adjuvant for a wide range of vaccine types, and presents an ideal vehicle to co-deliver lipid-based immune potentiators, which have the potential to strongly enhance or modulate the quality of the immune response.

Increased appreciation that activation of the innate immune system initiates, amplifies and drives antigen-specific immune responses has provided a multitude of new targets for the development of novel adjuvants. Relationships between Toll-like receptors (TLRs) and innate and adaptive immunity have been demonstrated. Signal transduction pathways activated by TLR agonists regulate antigen-presenting cell (APC) function and production of cytokines and chemokines and shape the magnitude and quality of the adaptive immune response (15–17). A number of TLR agonists have been identified and some are being evaluated as vaccine immune potentiators. In the current study we focus on the synthetic TLR4 agonist E6020, and compare it to the TLR9 agonist CpG.

While the cell wall lipopolysaccharide (LPS) of gram-negative bacteria is a potent TLR4 agonist, the toxicity profile of the natural product precludes its use in humans. Molecules mimicking lipid A, the simplest form of LPS, have been widely reported. Such molecules, for example monophosphoryl lipid A (MPL), are effective vaccine adjuvants in animal models and humans, with suppressed toxicity while maintaining the ability to bind to TLR4 (18–20). Importantly, for use in humans, MPL is always combined with delivery systems like alum or others (21,22) (www.gsk.com/media/flu/flu-adjuvant.pdf (accessed 12/17/08)). Recently, a novel synthetic TLR4 agonist, E6020 was developed. E6020 is chemically well defined, has a promising safety profile based on investigations with animal models (23), and has a single mechanism of action. Structurally, E6020 consists of a simple hexa-acylated acyclic backbone, which allows for a more direct preparation of high-purity material than other synthetic TLR4 agonists (24,25).

Recently we compared a number of adjuvants for influenza vaccine in mice and showed that MF59 significantly outperforms various alternatives, for both antibody and T-cell responses. Additionally, the ability of MF59 to deliver a CpG oligonucleotide adjuvant showed that the combination provided a potent synergy while strongly biasing the immune response toward a Th1 profile (26).

In the current study, the TLR4 agonist E6020, given alone or co-delivered with MF59 emulsion, was evaluated as a potential new adjuvant and compared to MF59, a licensed adjuvant for influenza vaccines, and the TLR9 agonist CpG, which is known to strongly bias the immune response (26). The efficacy of E6020 to increase hemagglutination inhibition (HI) titres to seasonal subunit influenza vaccine and its ability to bias the immune response towards a desired Th profile was investigated. We assessed the same parameters for E6020 co-delivered with the MF59 emulsion and compared effects to those reported for the combination CpG and MF59.

MATERIALS AND METHODS

Materials

Trivalent influenza vaccine composed of equal amounts of hemagglutinin (HA) from influenza strains H1N1 A/Solomon/3/2006, H3N2 A/Wisconsin/67/2005 and B/Malaysia /2506/2004 (Novartis Vaccines, Siena, Italy) was used in all experiments. The trivalent vaccine contains purified subunit antigens and is standardized for HA content by single-radial-immunodiffusion as recommended by regulatory authorities.

MF59 emulsion was obtained from Novartis Vaccines, Marburg, Germany. The oil in water emulsion MF59 was manufactured as previously described (8).

The synthetically produced TLR4 agonist E6020 was obtained from the Eisai Research Institute (Andover, MA).

The CpG oligonucleotide (5′-TCC ATG ACG TTC CTG ACG TT-3′), previously described as 1826 was synthesized with a phosphorothioate backbone by Oligos Etc. (Wilsonville, OR), ethanol precipitated, and re-suspended in 10 mM Tris (pH 7.0) 1 mM EDTA for storage at −80°C.

Preparation of MF59 Emulsion Containing E6020

MF59, consisting of 4.3% squalene, 0.5% Tween 80, 0.5% Span 85 (Sigma, St. Louis, MO) in citrate buffer, was prepared as previously described (8). In brief the emulsion was prepared by homogenization at 12,000 psi with a microfluidizer (Microfluidics, Newton, MA). The mean particle size of the emulsion droplets was determined with a Mastersizer X (Malvern Instruments, Southborough, MA). The emulsion was made sterile by passage through a polysulfone filter (220 nm pore size; Gelman Sciences, Ann Arbor, MI) and then stored at 4°C (27). In the formulation containing the immune potentiator E6020 in MF59, E6020 was dissolved at 1 mg/ml in $CHCl_3$, and added to the squalene fraction. Before homogenization $CHCL_3$ was completely evaporated from the squalene fraction. The final concentration of E6020 within the emulsion was 200 μg/ml, and the mean particle size of the emulsion was 162 ± 20 nm with a polydispersity index (PDI) of 0.11. The presence of E6020 or CpG neither influenced the particle size nor the PDI of MF59 (size 167 ± 20 nm, PDI 0.09).

Individual Vaccine Adjuvant Formulations

For MF59 adjuvanted vaccine formulations influenza vaccine was prepared by mixing MF59 (*v*/*v*) 1:1 with trivalent antigen to a final concentration of 0.3 μg/dose trivalent antigen (0.1 μg each antigen) and Phosphate Buffered Saline (D-PBS 1X, Gibco). In formulations containing either E6020 or CpG, immune potentiators were added to the formulations prior to immunizations at 10 μg/dose. In formulations containing both MF59 and E6020, E6020 formulated into MF59 (see above) was mixed with trivalent antigen at respective doses.

Mice and Immunizations

For the immunogenicity studies, groups of eight BALB/c female mice, 7–8 weeks-old, obtained from Charles River were used. Animals were immunized two times at 3-week intervals in the tibialis anterior muscles in the two hind legs of each animal with 50 μl/leg (100 μl total per mouse). Doses were 0.3 μg (0.1 μg each antigen) of either influenza soluble trivalent egg-derived antigen alone; antigen mixed with 10 μg E6020, or 10 μg CpG; antigen mixed with MF59 alone; and antigen mixed with MF59+E6020, MF59+CpG. Samples blood (all mice) and spleen (of three mice per group) were collected at 2 weeks following the first and second immunizations.

Immunogenicity was measured in serum samples using the hemagglutination inhibition assay, additionally total immunoglobulin G (IgG) antibodies were determined by ELISA. Th1/Th2 type responses were measured by titration of HA-specific IgG subclasses 1 and 2a in serum samples by ELISA and by monitoring antigen-specific T-cell cytokine responses in splenocytes.

Immunoassays

Determination of Antibodies by Hemagglutination Inhibition Assay

The HI assay was carried out on individual sera taken 2 weeks after the first and the second immunization. To inactivate non-specific inhibitors in serum samples, aliquots of each serum were separately treated with receptor destroying enzyme (RDE) prior to being tested with a final serum dilution of 1:10 (starting dilution for the assays). Samples were serially diluted two-fold into V-bottom 96 well microtiter plates. Briefly, 25 μl of two-fold serially diluted samples were incubated with 25 μl of strain-specific influenza antigen (whole virus, containing four hemagglutinating units) for 60 min at room temperature. A 0.5% *v*/*v* suspension of red blood cells obtained from adult turkeys were added and the mixture was incubated for another 60 min. Reactions were followed through visual inspection: a red dot formation indicates a positive reaction (inhibition) and a diffuse patch of cells a negative reaction (hemagglutination). As a negative control and in order to determine the background values of the assay serum samples of mice immunized with buffer were tested in parallel. Serum response to vaccine antigens was considered positive if a rise in antibody titres >4-fold compared to background was detectable. All sera were run in duplicate. The HI titre is defined as the serum dilution in which the last complete agglutination inhibition occurs. The antibody concentration corresponds to the reciprocal value of the titre. Geometric mean titres (GMT) of five mice per group are shown.

Determination of Antigen-Specific Antibody Subclasses by ELISA

Titration of HA-specific immunoglobulin G (IgG) total and subclasses 1 and 2a was performed on individual sera 2 weeks after the last immunization. Maxisorp plates (Nunc, Roskilde, Denmark) were coated overnight at 27–30°C with 0.2 μg/well with H1N1, H3N2 or B antigens in PBS and blocked for 1 h at room temperature with 300 μl of 3% poly vinyl pyrolidine. Serum samples and serum standard were initially diluted 1:5,000–1: 20,000 in PBS, 1% BSA, 0.05% Tween-20, transferred into coated-blocked plates and serially diluted. Antigen-specific IgG, IgG1 and IgG2a was revealed with alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma Chemical Co., SA Louis, Mo.), IgG1 or IgG2a, respectively (Southern Biotechnology Associates, Birmingham, AL). Antibody titres are those dilutions that gave an optical density (OD) higher than the mean plus five times the standard deviation of the average OD obtained in the pre-immune sera. The titres were normalized with respect to the reference serum assayed in parallel. Geometric mean titres (GMT) 2 weeks after the first immunization (post-1) of eight mice per group and 2 weeks after the second immunization (post-2) of five mice per group were calculated. IgG1:IgG2a titre ratios were calculated using respective geometric mean titres.

Antigen-Specific T-Cell Cytokine Response

Three mice per treatment were sacrificed, spleens were collected, pooled, and single cell suspensions were obtained. Red blood cells were lysed and splenocytes cultured in RPMI (Gibco) containing 2.5% FCS (Hyclone), beta-mercaptoethanol and antibiotics. Splenocytes were stimulated in the presence of anti-CD28 (1 μg/ml) (Becton–Dickinson) and a mix of the three influenza Ags (1 μg/ml each), or with anti-CD28 alone (unstimulated, <0.1% total cytokine-positive cells), or with anti-CD28 plus anti-CD3 (1 μg/ml) (Becton–Dickinson). After 4 h of stimulation, Brefeldin A (5 μg/ml) was added for additional 12 h. Cells were washed, fixed and permeabilized using the Cytofix/Cytoperm Kit (BD Biosciences Pharmingen) and stained with the following mAbs: Pacific Blue-conjugated anti-CD4, PerCP-Cy5.5-conjugated anti-CD3, FITC-conjugated anti-IFN-γ, Alexa 700-conjugated anti-TNF-α, PE-conjugated anti-IL5 and APC-conjugated anti-IL2 (all Becton–Dickinson). Cells were acquired on a LSR-II (Becton–Dickinson) and analyzed using FlowJo software (Tree Star). Values displayed represent the response of splenocytes from three pooled spleens. For each treatment, percentages of unstimulated samples were subtracted from the Ag-stimulated sample.

Statistical Analysis

Serum antibody titres are reported as geometric mean titre. Significant differences among groups were ascertained using the ANOVA factorial test at the 95% confidence interval (StatView 4.4 software; Abacus Concepts, Inc.). Tukey–Kramer HSD tests were used for post-hoc comparison.

RESULTS

TLR Agonists Do Not Further Increase Antibody Titres when Compared to MF59

After one immunization, E6020 was less potent compared to MF59 adjuvanted influenza vaccine with respect to antibodies induced against H1N1 A/Solomon ($p<0.001$) and B/Malaysia ($p<0.01$). However after two vaccine doses, although MF59 when compared to E6020 induced five-fold

higher antibody titres for all three vaccine strains, significant differences between E6020 and MF59 adjuvanted vaccine were only detectable for antibody titres against the influenza B/Malaysia strain ($p<0.001$). Generally MF59 was the most potent single adjuvant post-1 dose and post-2 doses and induced enhanced IgG titres compared to non-adjuvanted, E6020 or CpG adjuvanted vaccine to all of the three influenza antigens included in the vaccine (Fig. 1). The co-delivery of E6020 with MF59 adjuvanted influenza vaccine did not lead to a significant increase of total IgG antibody titres but was comparable to titres induced by MF59 adjuvanted vaccine ($p>0.05$). The same was observed for co-delivery of CpG with MF59 ($p>0.05$). Interestingly total IgG titres post-2 anti H1N1 A/Solomon induced by non-adjuvanted influenza vaccine were significantly lower when compared to all given adjuvanted formulations, ($p<0.05$) when compared to CpG

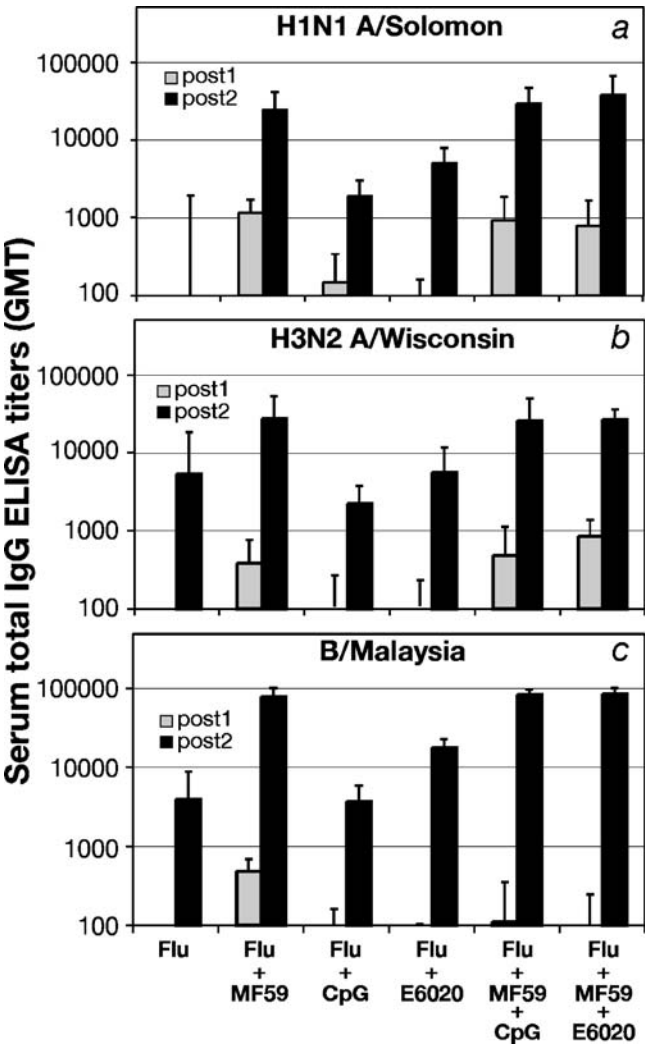

Fig. 1. Enhanced total IgG antibody responses to adjuvanted influenza vaccine. Groups of eight Balb/c mice were immunized intramuscularly at weeks 0 and 3 with influenza vaccine containing 0.1 μg of each antigen derived from influenza strain H1N1 A/Solomon, H3N2 A/Wisconsin and B/Malaysia, either alone (Flu) or adjuvanted as indicated. Shown are the geometric means (and standard error) of serum IgG titres against H1N1 (**a**), H3N2 (**b**) and against B (**c**) 2 weeks post-1 (eight mice/group) and second dose (five mice/group).

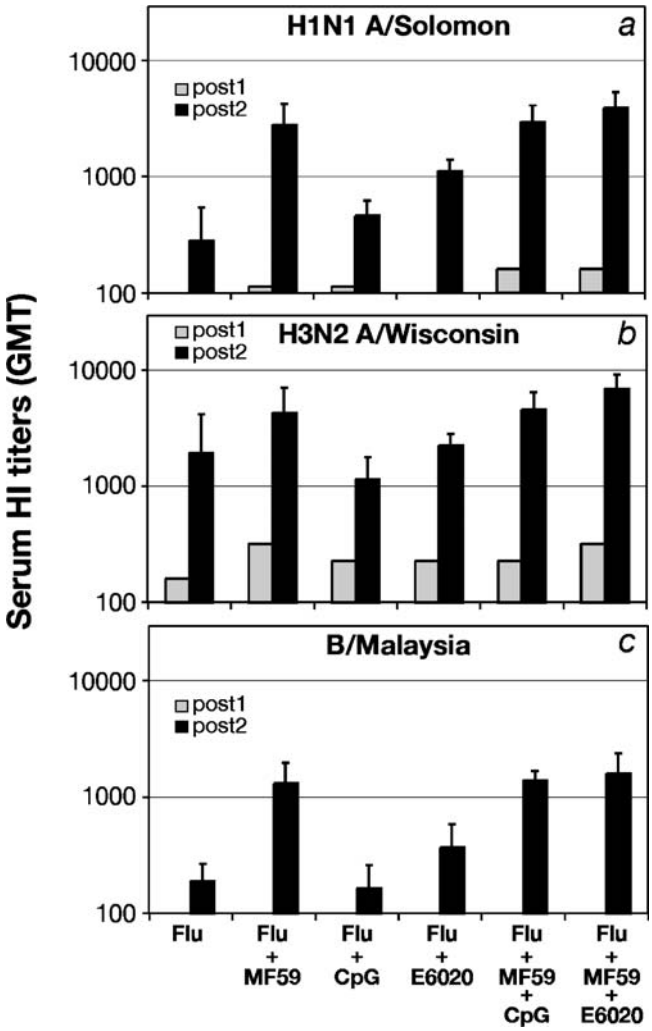

Fig. 2. Addition of adjuvants (MF59, CpG or E6020) to influenza vaccines enhances HI antibody responses. Mice were immunized intramuscularly twice at weeks 0 and 3 with vaccine containing 0.1 μg of each antigen derived from influenza strain H1N1 A/Solomon, H3N2 A/Wisconsin and B/Malaysia, adjuvanted with the following combinations: none (Flu), MF59, CpG or E6020, either alone or in combination with MF59, as indicated. Shown are the geometric means (and standard error) of serum HI titres against H1N1 (**a**), H3N2 (**b**) and against B (**c**) 2 weeks post-1 (pool of eight mice/group) and second dose (five mice/group).

adjuvanted influenza vaccine, ($p<0.01$) when compared to E6020 adjuvanted influenza vaccine and ($p<0.001$) if MF59 was present in the formulation (Fig. 1a). Whereas, post-2 total IgG titres anti H3N2 A/Wisconsin induced by non-adjuvanted vaccine were not significantly increased in the presence of any adjuvant, and comparable to those induced by E6020 adjuvanted formulations and little higher than those induced in the presence of CpG (Fig. 1b). Anti B/Malaysia antibody titres were similar for non-adjuvanted vaccine formulations and CpG adjuvanted formulations ($p>0.05$) whereas E6020 adjuvanted formulations induced four times higher antibody titres ($p<0.001$) (Fig. 1c). All anti B/Malaysia antibody titres were significantly increased in the presence of MF59 ($p<0.001$) when compared to non-adjuvanted vaccine.

Similar results were found for the induction of hemagglutination inhibition (HI) titres. The HI assay is the most

widely used serological assay for monitoring influenza immunity and is the accepted standard for measuring functional influenza-specific serum antibodies to the hemagglutinin following vaccination (28,29). Fig. 2 shows the evaluation of HI antibody titres to each of the three influenza strains included in the influenza vaccine.

E6020 was significantly less potent when compared to MF59 adjuvanted influenza vaccine ($p<0.05$ for H1N1 A/Solomon, and $p<0.01$ for influenza B/Malaysia strain). Furthermore MF59, which was the most potent single adjuvant, induced significantly enhanced HI titres post-2 doses against all three vaccine strains when compared to CpG adjuvanted influenza vaccine ($p<0.001$ for H1N1 A/Solomon, $p<0.05$ for H3N2 A/Wisconsin and $p<0.001$ for B/Malaysia). For H1N1, MF59 induced a ten-fold increase in post-2 dose HI titres as compared to non-adjuvanted vaccine post-2 dose ($p<0.001$), while the other adjuvants induced a 2–5-fold increase ($p<0.01$ for E6020 and $p>0.05$ for CpG) (Fig. 2a). Against H3N2 A/Wisconsin and B/Malaysia influenza virus the use of new generation immune potentiators, E6020 or CpG, was not very effective and induced only marginally if any enhanced HI antibody responses over influenza vaccine alone ($p>0.05$) (Fig. 2b, c). Against H3N2 A/Wisconsin and B/Malaysia influenza virus no enhancement of responses over that achieved with MF59 was induced by the co-delivery of either TLR agonist formulated with MF59 ($p>0.05$) (Fig. 2).

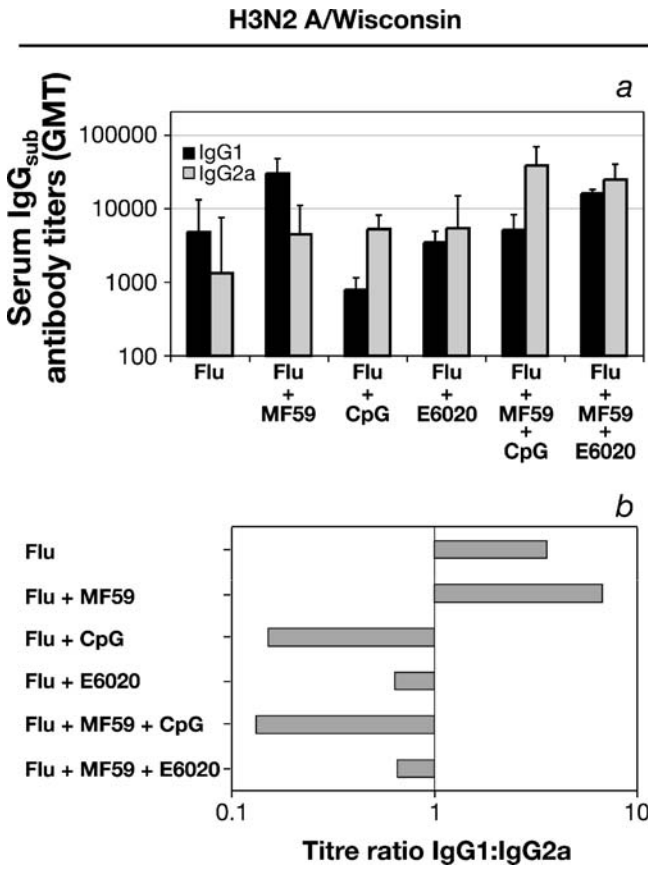

Fig. 3. Addition of CpG or E6020 to MF59 adjuvanted influenza vaccines increases IgG2a antibody responses. Mice were immunized intramuscularly twice at weeks 0 and 3 with influenza vaccine alone or with MF59, CpG or E6020 or various combinations with MF59. IgG1 and IgG2a titres were determined by ELISA. **a** Geometric mean titres (and standard error) against H3N2 2 weeks after the second immunization are shown (five mice/group). **b** Furthermore the titre ratios of IgG1:IgG2a isotype geometric mean titres against H3N2 were calculated.

MF59 Potentiates Th1 Biased Immune Responses Induced by TLR Agonists

The addition of either TLRs agonist, E6020 or CpG, to the vaccine alone or to MF59 adjuvanted vaccine enhanced H3N2 A/Wisconsin-specific IgG2a antibody isotype as measured by ELISA (Fig. 3a). Interestingly the addition of MF59 adjuvant to influenza vaccine either formulated without immune potentiator or with E6020 or CpG did not influence the quality of the immune response but significantly enhanced both IgG1 and IgG2a isotype antibody titres maintaining the titre ratio IgG1:IgG2a (Fig. 3b) unchanged ($p>0.05$). TLR 9 agonist CpG was more potent in shifting the immune response towards a more Th1 biased when compared to E6020.

TLR Agonists Induce a More Th1 Biased Cytokine Production by Ag-Specific T-Cells

The Ag-specific T-cell response was measured by the frequency of CD4 T-cells producing the T-cell growth factor IL-2, the cytokine TNF-α, which can be produced both by Th1 and Th2 cells, the Th1 indicator cytokine IFN-γ, the Th2 indicator cytokine IL-5 or combinations thereof after re-stimulation with vaccine antigens. Analysis of this combination of cytokines allows a detailed understanding of the Th1 *versus* Th2 bias of the T-cell response. Results in Fig. 4 show that MF59 strongly enhances the magnitude of the T cell response post-2 without altering the quality, i.e. the composition of cytokines produced, found already in the IL-5 dominated response induced by vaccine alone. The addition of either TLR agonist E6020 or CpG to MF59 induced a higher response post-1 and a shift towards a more pronounced Th1 profile, dominated by IFN-γ, often produced by the same cell in combination with TNF-α and IL-2 (Fig. 4). When comparing the post-2 responses, we found that MF59 + CpG leads to a strongly Th1 biased profile, whereas the addition of E6020 to MF59 induces both IFN-γ and IL-5 producing T cells, which represents a cytokine profile that is more balanced between Th1 and Th2. This is shown in Fig. 4b as the ratio between the frequency of IL-5 and IFN-γ positive T cells. Addition of E6020 or CpG alone to the vaccine Ag shifted the response towards Th1 but did not increase the overall magnitude of the T cell response above that to non-adjuvanted vaccine. Vaccine plus E6020 induced both IFN- γ and IL-5 producing T cells, similar to what was found in combination with MF59. Overall, MF59 induces strong CD4 T-cell responses, and the addition of E6020 shifts this response to a mixed Th1/Th2 profile composed of IFN-γ and IL-5 producing T cells.

DISCUSSION

Growing consideration of the importance of cell-mediated (Th1) immunity in the protection against intracellular pathogens like influenza virus has substantiated the benefit from an immune response beyond antibody production and B-cell memory in order to prevent disease (30–32). This,

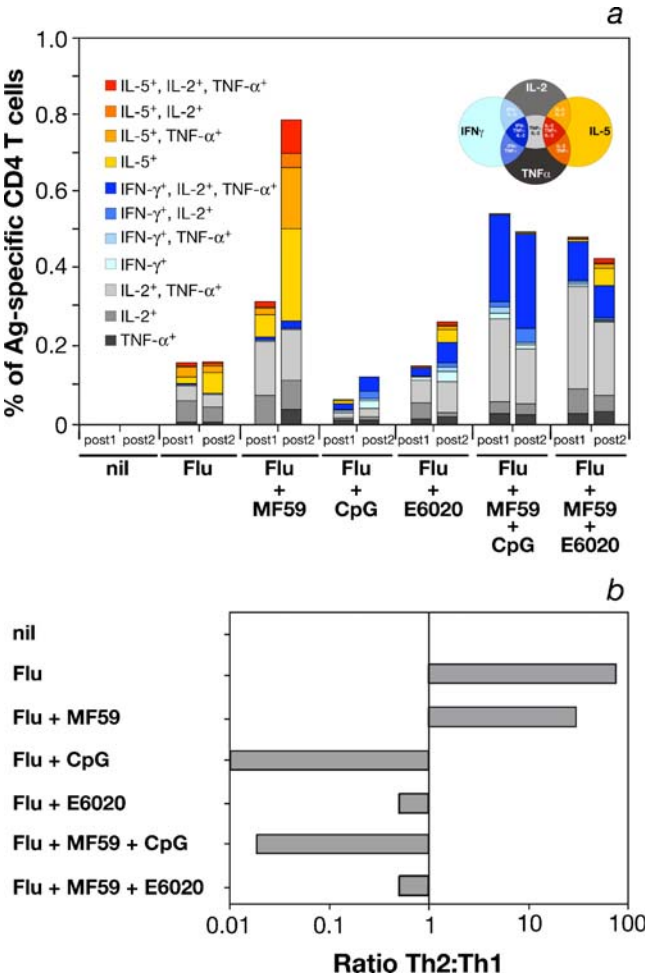

Fig. 4. MF59 alone and in combination with CpG or E6020 induces strong Ag-specific CD4 T-cell responses. Mice were vaccinated with influenza adjuvanted as indicated, spleens were removed and splenocytes restimulated *in vitro* with influenza Ag, and intracellular cytokine staining was performed. Through appropriate gating, cells expressing single cytokines or combinations thereof can be identified. The indicator cytokines for Th1 is IFN-γ, for Th2 is IL-5, and a colour coding was used to show the fraction of cells expressing either one or the other of these cytokines. No IL-5/IFN-γ double positive cells are found in these experiments. **a** Histogram showing CD4 T-cell cytokine responses 2 weeks post-1 and second dose of the vaccine. **b** The ratio of IL-5 positive divided by IFN-γ positive CD4 T cells is shown. Each *bar* represents the response of splenocytes from three pooled spleens. A total of three experiments with similar outcome were performed.

together with a better understanding of the immune system, especially regarding the impact of innate and adaptive immunity and their close interaction, has allowed for a more rational approach in the design of new vaccines including the use of adjuvants. For almost one century, aluminium hydroxide (alum) has been the only vaccine adjuvant approved for use in humans worldwide. Only in the last decade three additional adjuvants, the oil-in-water emulsions MF59 and AS03, and the TLR4 agonist monophosphoryl lipid A (MPL) formulated in alum (AS04), have been licensed by the European Medicinal Evaluation Agency (33). MPL adjuvant has been used extensively in clinical trials with more than 33,000 doses administered. The clinical evaluations of MPL demonstrated the efficacy of an attenuated TLR4 agonist as a vaccine adjuvant. Presently, two TLR4 agonist containing vaccines are approved for use in humans, namely Fendrix® for the prevention of hepatitis B and the cervical cancer vaccine Cervarix™ (19–21,34,35). In both cases MPL is formulated with a delivery system. Furthermore adjuvant systems have also been tested in various influenza vaccine programmes (22) (www.gsk.com/media/flu/flu-adjuvant.pdf (accessed 12/17/08)).

Novel lipid A mimetics that lack a disaccharide backbone, nevertheless retaining TLR4 stimulatory activity were recently described by Eisai (23–25). The simplified agonist structures ease compound preparation and yield highly purified products in abundant quantities, thus providing potential improvements in safety and cost. One of these synthetic compounds, E6020, was found to be more potent than MPL, but preclinically safe at the dosing levels required for vaccine adjuvanticity (25).

Although MF59 is a more potent adjuvant when compared to E6020 or CpG (this study), and other adjuvants like alum, calcium phosphate and the delivery system poly-(lactide co-glycolide) as shown recently (26), it is mostly effective at enhancing antibody and T-cell proliferative responses (8,36), but it is not a powerful adjuvant for the induction of Th1 cellular immune responses, confirming results obtained in various preclinical models (8,11,36,37). Since Influenza virus induces Th1 responses and IFN-γ and TNF-α have been shown to have some antiviral effect (38) it may be desirable to induce a Th1 response against influenza and in other viral infections. In addition, reduced protection in the elderly appears to correlate better with IFN-γ production from T cells than with HI titres (32,39), the commonly accepted correlate of protection (40).

The finding that the co-delivery of MF59 adjuvant to plain influenza vaccine formulated with E6020 or CpG immune potentiator did not modify the quality of the immune response but significantly amplified both IgG1 and IgG2a isotype antibody titres, while maintaining the ratio IgG1: IgG2a unchanged, lead to the conclusion that MF59 can be more precisely defined as a neutral adjuvant, which enhances whichever response is present, without biasing the profile. In other, more Th1 prone experimental settings, such as in certain mouse strains or in mice pre-exposed to influenza virus, MF59 simply increased the magnitude of the pre-existing Th1 response, further indicating that MF59 enhances immune responses in an essentially neutral manner (unpublished data). This ''neutrality'' of MF59 may make it an ideal vehicle to deliver adjuvants, which have the potential to strongly bias the immune response.

Among immune potentiators able to bias the immune response, the synthetic TLR4 agonist E6020 and the TLR9 agonist CpG have been tested preclinically as admixed adjuvant with a number of protein antigens (23,41,42). E6020 showed enhanced total IgG to the same degree as alum, and enhanced IgG2a, which is associated with Th1 activation in mice. Splenocytes from immunized mice restimulated *in vitro* showed significant suppression of IL-5, a Th2 associated cytokine (23), and thus addition of E6020 to traditional vaccine formulations might enhance Th1 or IFN-γ responses. Knowing the lipid-based structure of E6020, it is

rational to explore incorporation of E6020 into lipid-based delivery systems, such as MF59 emulsions. Recent work showed improved *Neisseria meningitidis* group B vaccine efficacy after formulation of MPL analogs with poly-(lactide co-glycolide) delivery system (43), and after the formulation of E6020 within MF59 (manuscript in preparation). The use of emulsions like MF59 may facilitate and favour formulations comprising both lipophilic compounds like E6020 and hydrophilic compounds as various antigens. In the present study, E6020 was incorporated within the oil phase prior to homogenization; however it still needs to be evaluated whether the pre-formulation of E6020 within the oil phase prior to homogenization of the emulsion is beneficial when compared to the more simple approach of just adding the immune potentiator to the ready MF59 emulsion as such. Interference of Tween 80 in the E6020 detection assay did not allow determining the exact location of E6020 in relation to the emulsion droplet, or in the aqueous phase. With respect to influence on immunogenicity no differences could be seen between the two approaches using freshly prepared formulations, results previously also observed for CpG (26). Nevertheless it needs to be evaluated if long term stability studies, or more detailed distribution studies, for the E6020 are able to highlight a difference and an advantage of one over the other formulation.

Recent studies (44,45) revealed that, MF59 enhances the immune response at a range of points, including the induction of chemokines to increase recruitment of immune cells to the injection site, enhanced antigen uptake by monocytes at the injection site and enhanced differentiation of monocytes into DCs, important for priming naive T cells. An important feature of MF59 is that it facilitates the migration of DCs into draining lymph nodes where they can trigger the adaptive immune response specific to the vaccine (44,45). On the other hand, the direct activation effects on DCs is very bland compared to that by immunostimulants such as CpG or LPS, which might be the reason for the lack of bias imposed on the ensuing T cell and Ab response and thus may explain why MF59 is a rather neutral adjuvant with respect to Th1–Th2 bias. In contrast, it is conceivable that E6020 engages TLR4 on DCs and thus activates them to produce higher levels of IL-12 and other factors promoting Th1 induction. Alternatively, other TLR4 expressing cells present at the injection site or co-migrating to the draining lymph node may be induced by E6020 to produce cytokines that direct the T cell priming by DCs towards IFN-γ secreting Th1 cells.

As individual adjuvant, MF59 induced optimal HI titres, whereas neither E6020 nor CpG when administered as single adjuvant with the influenza vaccine were able to induce significantly increased HI titres as MF59 does. On the other hand, MF59 was not able to induce effective Th1 responses, which could be achieved by the addition of E6020 or CpG. This confirms the ability of E6020 and CpG to shift immune responses towards a Th1 profile when combined with conventional vaccines (23,25,41,42).

Although CpG might be a more potent adjuvant in inducing Th1 biased immune responses when compared to E6020, to date no TLR9 agonist is approved for its use in humans. In contrast, the adjuvant capacity of AS04 has been evaluated during the development of several candidate vaccines, including hepatitis B, herpes simplex and HPV16/18 L1 VLPs (35,46–49). Both efficacy data and immunogenicity data have demonstrated the benefit of TLR4 agonist in vaccine formulations. Importantly, a TLR4 agonist enhanced the initiation of the immune response through the activation of innate immunity, leading to an improved cellular and humoral adaptive immune response, able to boost the immune system response for a longer period of time.

For influenza the anti-viral role of interferons is well established, and experiments *in vitro* show anti-viral activity of IFN-γ as well as TNF-α against human influenza viruses on lung epithelial cells (50). Given the synergistic antiviral effect of these two cytokines (38), it is an important observation that a high proportion of T-cells induced by MF59/E6020 or MF59/CpG produce both TNF-α and IFN-γ. In addition, IL-2 is a central autocrine T cell growth factor important for the maintenance of T cells and therefore, for memory. In influenza infections cytokine responses are known to be involved in the early and crucial stages of host defence (51). More generally, the induction of multi-cytokine producing T cells through vaccination has been associated with increased protection (52–55), and the majority of IFN-γ producing CD4 cells found here also secrete IL-2 and TNF-α thus responding to the requirement of inducing multifunctional cells as suggested in these studies.

Importantly, only the co-delivery of E6020 or CpG with MF59 allowed the induction of both, substantial HI titres and a potent Th1 response, as measured by the induction of IFN-γ. Altering the ratio between MF59 and the immune potentiator may allow to control and direct the quality of the immune response induced by the vaccine formulation.

As an adjuvant for a potential pandemic vaccine, MF59 allowed a significant reduction in the antigen dose, while maintaining the potency of the vaccine, a finding that might be important to allow an increase in the number of people immunized when an influenza pandemic occurs, assuming vaccine is available (56). MF59 significantly enhanced antibody responses in human subjects (56–58), whereas alum did not appear to be a potent adjuvant for a potential pandemic vaccine (59).

The use of adjuvants as MF59 or alum and their combinations with immune potentiators possibly allow a further reduction of the antigen dose required to stimulate the appropriate immune response able to create effective immunity against specific diseases (21,22,56,60,61). Here, the post-1 dose T cell responses induced by combination adjuvants were higher than those induced by MF59 alone, suggesting a more rapid onset of the adaptive immune response, a factor that may be of crucial importance in a pandemia. Accumulated data clearly establish that MF59 is a more potent adjuvant than Alum for a range of vaccines, while having a similarly acceptable safety profile in humans (8,10,11,62–64). Also in the context of paediatric vaccines, MF59 was shown to be a well-tolerated and potent adjuvant (11,13,14,65,66). E6020, on the other hand, was able to perform as vaccine adjuvant for the trivalent subunit influenza vaccine evaluated here, in particular when used in combination with MF59. Therefore we can conclude that the oil in water emulsion MF59 offers an attractive, practical and potent approach for the delivery of adjuvant active TLR4 agonist E6020.

CONCLUSIONS

The use of a combination of adjuvants with additive and sometimes synergistic effects, provides a potential advantage over the conventional use of a single adjuvant. The immune potentiator E6020, like other TLR agonists, presents an attractive tool for disease targets such as influenza and other infections with virus or intracellular pathogens that require enhanced Th1 immune responses, including the induction of IFN-γ and TNF-α. Importantly, the co-delivery of E6020 with MF59 emulsion allows a finer tuning towards a particular Th bias likely improving the overall efficacy of the vaccine. It remains to be evaluated if respective combinations retain their remarkable potency while also being safe and well tolerated in humans.

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REFERENCES

1. I. Kang, M. S. Hong, H. Nolasco, S. H. Park, J. M. Dan, J. Y. Choi, and J. Craft. Age-associated change in the frequency of memory CD4+ T cells impairs long term CD4+ T cell responses to influenza vaccine. *J. Immunol.* **173**:673–681 (2004).
2. K. Goodwin, C. Viboud, and L. Simonsen. Antibody response to influenza vaccination in the elderly: a quantitative review. *Vaccine*. **24**:1159–1169 (2006).
3. K. G. Nicholson, J. M. Wood, and M. Zambon. Influenza. *Lancet*. **362**:1733–1745 (2003).
4. A. Banzhoff, P. Nacci, and A. Podda. A new MF59-adjuvanted influenza vaccine enhances the immune response in the elderly with chronic diseases: results from an immunogenicity meta-analysis. *Gerontology*. **49**:177–184 (2003).
5. D. T. O'Hagan, and N. M. Valiante. Recent advances in the discovery and delivery of vaccine adjuvants. *Nat. Rev. Drug Discov.* **2**:727–735 (2003).
6. B. Guy. The perfect mix: recent progress in adjuvant research. *Nat. Rev. Microbiol.* **5**:505–517 (2007).
7. D. T. O'Hagan. MF59 is a safe and potent vaccine adjuvant that enhances protection against influenza virus infection. *Expert Rev. Vaccines*. **6**:699–710 (2007).
8. G. Ott, R. Radhakrishnan, J. H. Fang, and M. Hora. The adjuvant MF59: A 10-year perspective. *Methods Mol. Med.* **42**:211–228 (2000).
9. S. De Donato, D. Granoff, M. Minutello, G. Lecchi, M. Faccini, M. Agnello, F. Senatore, P. Verweij, B. Fritzell, and A. Podda. Safety and immunogenicity of MF59-adjuvanted influenza vaccine in the elderly. *Vaccine*. **17**:3094–3101 (1999).
10. A. Podda, G. Del Giudice, and D. T. O'Hagan. MF59: a safe and potent adjuvant for human use. In V. Schijns, and D. T. O'Hagan (eds.), *Immunopotentiators in Modern Vaccines*, Academic, Oxford, 2006, pp. 149–159.
11. A. Podda, and G. Del Giudice. MF59-adjuvanted vaccines: increased immunogenicity with an optimal safety profile. *Expert Rev. Vaccines*. **2**:197–203 (2003).
12. A. Podda, and G. Del Giudice. MF59 adjuvant emulsion. In M. M. Levine, J. B. Kaper, R. Rappuoli, M. A. Liu, and M. F. Good (eds.), *New Generation Vaccines*, 3Marcel Dekker, New York, 2004, pp. 225–236.
13. D. K. Mitchell, S. J. Holmes, R. L. Burke, A. M. Duliege, and S. P. Adler. Immunogenicity of a recombinant human cytomegalovirus gB vaccine in seronegative toddlers. *Pediatr. Infect. Dis. J.* **21**:133–138 (2002).
14. E. J. McFarland, W. Borkowsky, T. Fenton, D. Wara, J. McNamara, P. Samson, M. Kang, L. Mofenson, C. Cunningham, A. M. Duliege, F. Sinangil, S. A. Spector, E. Jimenez, Y. Bryson, S. Burchett, L. M. Frenkel, R. Yogev, F. Gigliotti, K. Luzuriaga, R. A. Livingston, and AIDS Clinical Trials Group 230 Collaborators. 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 (2001).
15. A. Iwasaki, and R. Medzhitov. Toll-like receptor control of the adaptive immune responses. *Nat. Immunol.* **5**:987–995 (2004).
16. T. Kaisho, and S. Akira. Regulation of dendritic cell function through Toll-like receptors. *Curr. Mol. Med.* **3**:373–385 (2003).
17. D. M. Underhill. Toll-like receptors: networking for success. *Eur. J. Immunol.* **33**:1767–1775 (2003).
18. J. T. Evans, C. W. Cluff, D. A. Johnson, M. J. Lacy, D. H. Persing, and J. R. Baldridge. Enhancement of antigen-specific immunity via the TLR4 ligands MPL adjuvant and Ribi.529. *Expert Rev. Vaccines*. **2**:219–229 (2003).
19. CERVARIX® Product Information Human Papillomavirus Vaccine Types 16 and 18 (Recombinant, AS04 adjuvanted) GlaxoSmithKline Australia Pty Ltd. (2007).
20. M. Kundi. New hepatitis B vaccine formulated with an improved adjuvant system. *Expert Rev. Vaccines*. **6**:133–140 (2007).
21. N. Garçon, P. Chomez, and M. Van Mechelen. GlaxoSmithKline Adjuvant Systems in vaccines: concepts, achievements and perspectives. *Expert Rev. Vaccines*. **6**:723–739 (2007).
22. C. K. Fraser, K. R. Diener, M. P. Brown, and J. D. Hayball. Improving vaccines by incorporating immunological coadjuvants. *Expert Rev. Vaccines*. **6**:559–578 (2007).
23. M. Przetak, J. Chow, H. Cheng, J. Rose, L. D. Hawkins, and S. T. Ishizaka. Novel synthetic LPS receptor agonists boost systemic and mucosal antibody responses in mice. *Vaccine*. **21**:961–970 (2003).
24. L. D. Hawkins, S. T. Ishizaka, P. McGuinness, H. Zhang, W. Gavin, B. DeCosta, Z. Meng, H. Yang, M. Mullarkey, D. W. Young, H. Yang, D. P. Rossignol, A. Nault, J. Rose, M. Przetak, J. C. Chow, and F. Gusovsky. A novel class of endotoxin receptor agonists with simplified structure, Toll-like receptor 4-dependent immunostimulatory action, and adjuvant activity. *J. Pharmacol. Exp. Ther.* **300**:655–661 (2002).
25. S. T. Ishizaka, and L. D. Hawkins. E6020: a synthetic Toll-like receptor 4 agonist as a vaccine adjuvant. *Expert Rev. Vaccines*. **6**:773–784 (2007).
26. A. Wack, B. C. Baudner, A. K. Hilbert, I. Manini, S. Nuti, S. Tavarini, H. Scheffczik, M. Ugozzoli, M. Singh, J. Kazzaz, E. Montomoli, G. Del Giudice, R. Rappuoli, and D. T. O'Hagan. Combination adjuvants for the induction of potent, long-lasting antibody and T-cell responses to influenza vaccine in mice. *Vaccine*. **26**:552–561 (2008).
27. M. Dupuis, D. M. McDonald, and G. Ott. Distribution of adjuvant MF59 and antigen gD2 after intramuscular injection in mice. *Vaccine*. **18**:434–439 (1999).
28. Session 1, R.B. Couch. Correlates of protection against seasonal influenza and Summary of 1. Day. http://www.fda.gov/CbER/pandemic/panflu121007.htm (accessed 12/17/08).
29. R. A. Bright, D. M. Carter, C. J. Crevar, F. R. Toapanta, J. D. Steckbeck, K. S. Cole, N. M. Kumar, P. Pushko, G. Smith, T. M. Tumpey, and T. M. Ross. Cross-clade protective immune responses to influenza viruses with H5N1 HA and NA elicited by an influenza virus-like particle. *PLoS ONE*. **3**(1):e1501 (2008).
30. P. G. Thomas, R. Keating, D. J. Hulse-Post, and P. C. Doherty. Cell-mediated protection in influenza infection. *Emerg. Infect. Dis.* **12**:48–54 (2006).
31. P. C. Doherty, S. J. Turner, R. G. Webby, and P. G. Thomas. Influenza and the challenge for immunology. *Nat. Immunol.* **7**:449–455 (2006).
32. J. Bennink. Cell mediated immunity to influenza in mice: T-cell specific responses that correlate with protection. http://www.fda.gov/CbER/pandemic/panflu121007.htm (accessed 12/17/08).
33. E. De Gregorio, E. Tritto, and R. Rappuoli. Alum adjuvanticity: Unraveling a century old mystery. *Eur. J. Immunol.* **38**:2068–2071 (2008).
34. M. E. Pichichero. Improving vaccine delivery using novel adjuvant systems. *Hum. Vaccin.* **4**:262–70 (2008).
35. N. Thönes, A. Herreiner, L. Schädlich, K. Piuko, and M. Müller. A direct comparison of human papillomavirus type 16 L1 particles reveals a lower immunogenicity of capsomeres than

viruslike particles with respect to the induced antibody response. *J. Virol.* **82**:5472–5485 (2008).
36. G. Ott, G. L. Barchfeld, D. Chernoff, R. Radhakrishnan, P. van Hoogevest, and G. Van Nest. Design and evaluation of a safe and potent adjuvant for human vaccines. In M. F. Powell, and M. J. Newman (eds.), *Vaccine Design: The Subunit and Adjuvant Approach*, Plenum, New York, 1995.
37. M. Singh, M. Ugozzoli, J. Kazzaz, J. Chesko, E. Soenawan, D. Mannucci, F. Titta, M. Contorni, G. Volpini, G. Del Guidice, and D. T. O'Hagan. A preliminary evaluation of alternative adjuvants to alum using a range of established and new generation vaccine antigens. *Vaccine*. **10**:1680–1686 (2006).
38. G. H. Wong, and D. V. Goeddel. Tumour necrosis factors alpha and beta inhibit virus replication and synergize with interferons. *Nature*. **323**:819–822 (1986).
39. J. E. McElhaney, D. Xie, W. D. Hager, M. B. Barry, Y. Wang, A. Kleppinger, C. Ewen, K. P. Kane, and R. C. Bleackley. T cell responses are better correlates of vaccine protection in the elderly. *J. Immunol.* **176**:6333–6339 (2006).
40. A. S. Evans. Serologic studies of acute respiratory infections in military personnel. *Yale J. Biol. Med.* **48**:201–209 (1975).
41. T. Sugai, M. Mori, M. Nakazawa, M. Ichino, T. Naruto, N. Kobayashi, Y. Kobayashi, M. Minami, and S. Yokota. A CpG-containing oligodeoxynucleotide as an efficient adjuvant counterbalancing the Th1/Th2 immune response in diphtheria–tetanus–pertussis vaccine. *Vaccine*. **23**:5450–5456 (2005).
42. C. Coban, K. J. Ishii, A. W. Stowers, D. B. Keister, D. M. Klinman, and N. Kumar. Effect of CpG oligodeoxynucleotides on the immunogenicity of Pfs25, a Plasmodium falciparum transmission-blocking vaccine antigen. *Infect. Immun.* **72**:584–588 (2004).
43. J. Kazzaz, M. Singh, M. Ugozzoli, J. Chesko, E. Soenawan, and D. T. O'Hagan. Encapsulation of the immune potentiators MPL and RC529 in PLG microparticles enhances their potency. *J. Control Release*. **110**:566–573 (2006).
44. A. Seubert, E. Monaci, M. Pizza, D. T. O'Hagan, and A. Wack. The adjuvants aluminum hydroxide and MF59 induce monocyte and granulocyte chemoattractants and enhance monocyte differentiation toward dendritic cells. *J. Immunol.* **180**:5402–5412 (2008).
45. F. Mosca, E. Tritto, A. Muzzi, E. Monaci, F. Bagnoli, C. Iavarone, D. O'Hagan, R. Rappuoli, and E. De Gregorio. Molecular and cellular signatures of human vaccine adjuvants. *Proc. Natl. Acad. Sci. U. S. A.* **105**:10501–10506 (2008).
46. N. K. Tong, J. Beran, S. A. Kee, J. L. Miguel, C. Sánchez, J. M. Bayas, A. Vilella, J. R. de Juanes, P. Arrazola, F. Calbo-Torrecillas, E. L. de Novales, V. Hamtiaux, M. Lievens, and M. Stoffel. Immunogenicity and safety of an adjuvanted hepatitis B vaccine in pre-hemodialysis and hemodialysis patients. *Kidney Int.* **68**:2298–2303 (2005).
47. N. Bourne, G. N. Milligan, L. R. Stanberry, R. Stegall, and R. B. Pyles. Impact of immunization with glycoprotein D2/AS04 on herpes simplex virus type 2 shedding into the genital tract in guinea pigs that become infected. *J. Infect. Dis.* **192**:2117–2123 (2005).
48. T. J. Kemp, A. García-Piñeres, R. T. Falk, S. Poncelet, F. Dessy, S. L. Giannini, A. C. Rodriguez, C. Porras, R. Herrero, A. Hildesheim, L. A. Pinto, and Costa Rica Vaccine Trial (CVT) Group. Evaluation of systemic and mucosal anti-HPV16 and anti-HPV18 antibody responses from vaccinated women. *Vaccine*. **26**:3608–3616 (2008).
49. S. L. Giannini, E. Hanon, P. Moris, M. Van Mechelen, S. Morel, F. Dessy, M. A. Fourneau, B. Colau, J. Suzich, G. Losonksy, M. T. Martin, G. Dubin, and M. A. Wettendorff. Enhanced humoral and memory B cellular immunity using HPV16/18 L1 VLP vaccine formulated with the MPL/aluminium salt combination (AS04) compared to aluminium salt only. *Vaccine*. **24**:5937–5949 (2006).
50. S. H. Seo, and R. G. Webster. Tumor necrosis factor alpha exerts powerful anti-influenza virus effects in lung epithelial cells. *J. Virol.* **76**:1071–1076 (2002).
51. F. G. Hayden, R. Fritz, M. C. Lobo, W. Alvord, W. Strober, and S. E. Straus. Local and systemic cytokine responses during experimental human influenza A virus infection. Relation to symptom formation and host defense. *J. Clin. Invest.* **101**:643–649 (1998).
52. M. R. Betts, M. C. Nason, S. M. West, S. C. De Rosa, S. A. Migueles, J. Abraham, M. M. Lederman, J. M. Benito, P. A. Goepfert, M. Connors, M. Roederer, and R. A. Koup. HIV nonprogressors preferentially maintain highly functional HIV-specific CD8+ T cells. *Blood*. **107**:4781–4789 (2006).
53. S. C. De Rosa, F. X. Lu, J. Yu, S. P. Perfetto, J. Falloon, S. Moser, T. G. Evans, R. Koup, C. J. Miller, and M. Roederer. Vaccination in humans generates broad T cell cytokine responses. *J. Immunol.* **173**:5372–5380 (2004).
54. G. Pantaleo, and R. A. Koup. Correlates of immune protection in HIV-1 infection: what we know, what we don't know, what we should know. *Nat. Med.* **10**:806–810 (2004).
55. A. Harari, V. Dutoit, C. Cellerai, P. A. Bart, R. A. Du Pasquier, and G. Pantaleo. Functional signatures of protective antiviral T-cell immunity in human virus infections. *Immunol. Rev.* **211**:236–254 (2006).
56. K. G. Nicholson, A. E. Colegate, A. Podda, I. Stephenson, J. Wood, E. Ypma, and M. C. Zambon. Safety and antigenicity of non-adjuvanted and MF59-adjuvanted influenza A/Duck/Singapore/97 (H5N3) vaccine: a randomised trial of two potential vaccines against H5N1 influenza. *Lancet*. **357**:1937–1943 (2001).
57. I. Stephenson, K. G. Nicholson, A. Colegate, A. Podda, J. Wood, E. Ypma, and M. Zambon. Boosting immunity to influenza H5N1 with MF59-adjuvanted H5N3 A/Duck/Singapore/97 vaccine in a primed human population. *Vaccine*. **21**:1687–1693 (2003).
58. I. Stephenson, K. G. Nicholson, R. Bugarini, A. Podda, J. Wood, M. Zambon, and J. M. Katz. Cross reactivity to highly pathogenic avian influenza H5N1 viruses following vaccination with non-adjuvanted and MF-59-adjuvanted influenza A/Duck/Singapore/97 (H5N3) vaccine: a potential priming strategy. *J. Infect. Dis.* **191**:4962–4970 (2005).
59. WHO. *2nd WHO meeting on evaluation of pandemic influenza prototype vaccines in clinical trials*. WHO, Geneva, 2006.
60. R. L. Atmar, W. A. Keitel, S. M. Patel, J. M. Katz, D. She, H. El Sahly, J. Pompey, T. R. Cate, and R. B. Couch. Safety and immunogenicity of nonadjuvanted and MF59-adjuvanted influenza A/H9N2 vaccine preparations. *Clin. Infect. Dis.* **43**:1135–1142 (2006).
61. D. T. O'Hagan, M. Singh, J. Kazzaz, M. Ugozzoli, M. Briones, J. Donnelly, and G. Ott. Synergistic adjuvant activity of immunostimulatory DNA and oil/water emulsions for immunization with HIV p55 gag antigen. *Vaccine*. **20**:3389–3398 (2002).
62. T. C. Heineman, M. L. Clements-Mann, G. A. Poland, R. M. Jacobson, A. E. Izu, D. Sakamoto, J. Eiden, G. A. Van Nest, and H. H. Hsu. A randomized, controlled study in adults of the immunogenicity of a novel hepatitis B vaccine containing MF59 adjuvant. *Vaccine*. **17**:2769–2778 (1999).
63. A. G. Langenberg, R. L. Burke, S. F. Adair, R. Sekulovich, M. Tigges, C. L. Dekker, and L. Corey. A recombinant glycoprotein vaccine for herpes simplex virus type 2: safety and immunogenicity [corrected]. *Ann. Intern. Med.* **122**:889–898 (1995).
64. L. Corey, A. G. Langenberg, R. Ashley, R. E. Sekulovich, A. E. Izu, J. M. Douglas Jr., H. H. Handsfield, T. Warren, L. Marr, S. Tyring, R. DiCarlo, A. A. Adimora, P. Leone, C. L. Dekker, R. L. Burke, W. P. Leong, and S. E. Straus. Recombinant glycoprotein vaccine for the prevention of genital HSV-2 infection: two randomized controlled trials. Chiron HSV Vaccine Study Group. *JAMA*. **282**:331–340 (1999).
65. W. Borkowsky, D. Wara, T. Fenton, J. McNamara, M. Kang, L. Mofenson, E. McFarland, C. Cunningham, A. M. Duliege, D. Francis, Y. Bryson, S. Burchett, S. A. Spector, L. M. Frenkel, S. Starr, R. Van Dyke, and E. Jimenez. Lymphoproliferative responses to recombinant HIV-1 envelope antigens in neonates and infants receiving gp120 vaccines. AIDS Clinical Trial Group 230 Collaborators. *J. Infect. Dis.* **181**:890–896 (2000).
66. C. K. Cunningham, D. W. Wara, M. Kang, T. Fenton, E. Hawkins, J. McNamara, L. Mofenson, A. M. Duliege, D. Francis, E. J. McFarland, W. Borkowsky, and Pediatric AIDS Clinical Trials Group 230 Collaborators. Safety of 2 recombinant human immunodeficiency virus type 1 (HIV-1) envelope vaccines in neonates born to HIV-1-infected women. *Clin. Infect. Dis.* **32**:801–807 (2001).