Novel G3/DT adjuvant promotes the induction of protective T cells responses after vaccination with a seasonal trivalent inactivated split-virion influenza vaccine

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A R T I C L E   I N F O
Article history:
Received 4 June 2014
Received in revised form 17 July 2014
Accepted 6 August 2014
Available online 17 August 2014

Keywords:
Adjuvant
Split virion vaccine
T cells
Heterosubtypic immunity

A B S T R A C T
Vaccines used against seasonal influenza are poorly effective against influenza A viruses of novel subtypes that may have pandemic potential. Furthermore, pre(pandemic) influenza vaccines are poorly immunogenic, which can be overcome by the use of adjuvants. A limited number of adjuvants has been approved for use in humans, however there is a need for alternative safe and effective adjuvants that can enhance the immunogenicity of influenza vaccines and that promote the induction of broad-protective T cell responses. Here we evaluated a novel nanoparticle, G3, as an adjuvant for a seasonal trivalent inactivated influenza vaccine in a mouse model. The G3 adjuvant was formulated with or without steviol glycosides (DT, for diterpenoid). The use of both formulations enhanced the virus-specific antibody response to all three vaccine strains considerably. The adjuvants were well tolerated without any signs of discomfort. To assess the protective potential of the vaccine-induced immune responses, an antigenically distinct influenza virus strain, A/Puerto Rico/8/34 (A/PR/8/34), was used for challenge infection. The vaccine-induced antibodies did not cross-react with strain A/PR/8/34 in HI and VN assays. However, mice immunized with the G3/DT-adjuvanted vaccine were partially protected against A/PR/8/34 infection, which correlated with the induction of anamnestic virus-specific CD8+ T cell responses that were not observed with the use of G3 without DT. Both formulations induced maturation of human dendritic cells and promoted antigen presentation to a similar extent. In conclusion, G3/DT is a promising adjuvant formulation that not only potentiates the antibody response induced by influenza vaccines, but also induces T cell immunity which could afford broader protection against antigenically distinct influenza viruses.

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1. Introduction
Influenza virus infections cause excess morbidity and mortality in the human population [1]. For the prevention of severe disease and mortality it is recommended to vaccinate high risk groups against influenza annually. Commonly, inactivated trivalent influenza vaccines are used that elicit strain-specific antibodies [2,3]. However, these vaccines fail to afford life-long protection, mainly due to antigenic drift of influenza viruses [4–7]. Influenza vaccine strains need to be updated almost annually, in order to match circulating influenza strains as closely as possible [8]. A mismatch of vaccine strains may lead to reduced effectiveness of influenza vaccines [9,10]. Furthermore, the emergence of an antigenically distinct influenza virus of a novel subtype, may trigger an influenza pandemic to which inactivated seasonal influenza vaccines afford little protection [4]. Ideally, vaccine formulations are used that not only induce neutralizing antibodies to seasonal influenza viruses but also induce more broadly protective immune responses.

Strain-specific virus neutralizing antibodies are mainly directed to the variable globular head region of the viral hemagglutinin
Table 1
Experimental groups and design of the study.

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<th>Experimental group</th>
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(HA) [5,11], although cross-reactive antibodies have been identified [12–16]. In addition, virus-specific CD4+ T cells and CD8+ cytotoxic T lymphocytes (CTLs) contribute to protective immunity. CTLs, which are directed to conserved internal proteins predominantly, contribute to heterosubtypic immunity [17–30]. The main function of CTLs is to recognize and eliminate virus infected cells, thus restricting virus replication and accelerating viral clearance. The existence of cross-reactive CTLs induced by previous infections and their role in protection against heterologous influenza virus strains in humans was demonstrated in several studies [20–23,31–34]. Currently used inactivated influenza vaccines inefficiently elicit cross-reactive CTL responses [35,36], which may be related to their inability to deliver proteins into the cytosol of antigen presenting cells (APCs) for endogenous antigen processing and MHC class I presentation. Therefore, there is considerable interest in adjuvants and antigen delivery systems that not only improve antibody responses of current (inactivated) influenza vaccines, but that also induce cross-reactive CTLs. It has been demonstrated that immune stimulating complex (ISCOM) based vaccines induce strong antibody responses as well as virus-specific CTL responses both in animal models and humans [37–45], whereas current adjuvants, like aluminum salts (Al(OH)3 and AlPO3) and oil-in-water adjuvants (e.g. MF59, AS03 and Freund’s adjuvant) enhance antibody responses, but fail to induce CTL responses [37,46–48]. Most likely, ISCOMs facilitate delivery of viral proteins into the cytosol of APCs where protein degradation and liberation of antigenic peptides by the proteasome takes place [49,50].

In the present study, we evaluated G3, a novel nanoparticle adjuvant as an alternative for ISCOMs. In contrast to ISCOMs, G3 lacks the phospholipid component, is easy to produce, allows incorporating molecules of various kinds, like additional adjuvant components or antigens, and further reduces possible side effects associated with the use of ISCOMs. In this study, the G3 adjuvant was formulated with or without steviol glycosides (deterpenoids (DT); G3/DT), which are known for their ability to promote lymphocyte responses [51]. We evaluated these novel G3 adjuvants for their capacity to enhance the immunogenicity of a commercially available seasonal trivalent inactivated split virion influenza vaccine in a mouse model. It was concluded that G3/DT is a versatile adjuvant system that not only enhances the antibody responses, but that also induces cross-reactive virus-specific CD8+ T cell responses.

2. Materials and methods

2.1. Adjuvants

G3 formulations were prepared with quillaja saponins isolated from the bark of Quillaja saponaria Molina and supplied as QS21 (Desert King CF, USA). In contrast to the preparation of conventional 40 nm ISCOM particles, G3 is produced without phosphatidylycholine, resulting in a smaller spherical particle size of 20 nm (data not shown). The G3/DT adjuvant preparation differs from the G3 preparation by the incorporation of DT. DT was isolated from a water extraction of Rebaudiana Bertoni (Prodalysa, Concón, Chile) from which steviol glycosides were purified by membrane purification carried out with ultrafiltration and nanofiltration membranes and water eluted ion exchange to obtain a purity of >95%. The molar ratio quillaja saponin (a triterpen) and DT was 2:1. The adjuvant dose for immunization of mice was 5 µg G3 or G3/DT based on quillaja saponin content.

2.2. Vaccine preparation

A commercially available seasonal trivalent split virion vaccine of the 2012/2013 influenza season (VAXIGRIP®) (2012/2013, Sanofi Pasteur MSD, Brussels, Belgium) was used in the present study and contained components of the vaccine strains: NYMC X-179A derived from strain A/California/7/2009 (H1N1pdm09), IRV-165 derived from strain A/Victoria/361/2011 (H3N2) and NYMC BX-39 derived from B/Hubei-Wujiang/158/2009, a B/Wisconsin/1/2010 like virus. The vaccine dose used in mice contained 5 µg hemagglutinin of each vaccine strain.

2.3. Influenza virus

Influenza viruses A/PR8/34 (H1N1), X181 (derived from A/California/7/2009), IRV-165 and NYMC BX-39 were propagated in embryonated chicken eggs as described previously [28]. Infectious virus titers were determined in Madin-Darby Canine Kidney (MDCK) cells as described previously [52].

2.4. Immunization and inoculation of mice

Specified pathogen free, 6–8 weeks old female C57BL/6j mice were purchased from Charles River (Sulzfeld, Germany). Mice received two subcutaneous (s.c.) immunizations in the dorsal neck region in a total volume of 200 µl with an interval of four weeks. Mice (n = 7–14 per group) received 5 µg HA of each of the vaccine strains in the trivalent split virion vaccine with 5 µg G3/DT (group 1), G3 (group 2) or without adjuvant (group 3). Control mice received phosphate buffered saline (PBS) with 5 µg G3/DT (group 4), G3 (group 5) or without adjuvant (groups 6 and 7). Four weeks after the second immunization mice were inoculated intranasally (i.n.) with a lethal dose (5 × 104 TCID50) of influenza virus A/PR/8/34 in a volume of 50 µl (groups 1–6). Group 7 received 50 µl of PBS i.n. (Table 1). Clinical signs, including weight loss, were monitored after vaccination and during the infection. Mice were euthanized on day 4 (n = 8 for groups 1–6, n = 4 for group 7) and day 6 post inoculation (p.i.) (n = 6 for groups 1–6, n = 3 for group 7) or when they displayed weight loss of >25% (humane endpoint, mice were scored as dead). Mice were bled via orbital puncture and lungs and spleens were resected. Blood was collected via submandibular bleed just before the first and second vaccination and before challenge infection. All biotechnical procedures were performed under anesthesia with 4% isoflurane in O2. Animals were housed in individually ventilated cages (IVC-units) and had access to food and water ad libitum. An
observed significant immunization Wujiang/158/2009 in 2.6.

2.5. Geometric mean serum antibodies titers after immunization. Serum antibody titers against vaccine strains A/California/7/2009 (A), A/Victoria/361/2011 (B) and B/Hubei-Wujiang/158/2009 (C) and the A/PR/8/34 challenge strain (D) were determined before immunization (T=0), 28 days after the first immunization and 28 days after the second immunization (day of challenge with A/PR/8/34) and day of euthanasia (4 and 6 days after challenge) by HI assay. (*) and (**) indicates that the difference in antibody titers in mice immunized with the split virion vaccine adjuvanted with G3 or G3/DT and split virion only was statistically significant (p<0.05 and p<0.01, respectively). The only significant difference between the split virion immunized mice with the G3 adjuvant versus the split virion immunized mice adjuvanted with the G3/DT adjuvant was observed in the A/Victoria/361/2011 strain at day 62 (*, p<0.05).

Fig. 1. Geometric mean serum antibodies titers after immunization. Serum antibody titers against vaccine strains A/California/7/2009 (A), A/Victoria/361/2011 (B) and B/Hubei-Wujiang/158/2009 (C) and the A/PR/8/34 challenge strain (D) were determined before immunization (T=0), 28 days after the first immunization and 28 days after the second immunization (day of challenge with A/PR/8/34) and day of euthanasia (4 and 6 days after challenge) by HI assay. (*) and (**) indicates that the difference in antibody titers in mice immunized with the split virion vaccine adjuvanted with G3 or G3/DT and split virion only was statistically significant (p<0.05 and p<0.01, respectively). The only significant difference between the split virion immunized mice with the G3 adjuvant versus the split virion immunized mice adjuvanted with the G3/DT adjuvant was observed in the A/Victoria/361/2011 strain at day 62 (*, p<0.05).

2.5. Serology

Serum samples were obtained before and twenty-eight days after the first and twenty-eight days after the second vaccination as well as on day 4 or 6 p.i. and were stored at −20 °C until use. Sera were tested for the presence of antibodies to either influenza virus A/PR/8/34, IVR-165, X181 or BX-39 using a hemagglutination inhibition (HI) assay as described previously [53]. Post infection ferret sera, raised against the influenza viruses mentioned above were used as positive controls. In addition sera were tested for the presence of virus-neutralizing antibodies specific for A/PR/8/34 using a micro virus-neutralization (VN) assay as described previously [54].

2.6. Lung virus titers

Lungs were snap frozen on dry ice with ethanol and stored at −80 °C. Lungs were homogenized using a FastPrep-24® (MP Biomedicals, Eindhoven, The Netherlands) in transport medium (MEM with Hanks’ balanced salt solution (Lonza, Basel, Switzerland) containing 0.5% lactalbumin enzymatic hydrolysat (Sigma-Aldrich, Zwijndrecht, The Netherlands), 200 U/ml penicillin (Lonza), 200 μg/ml streptomycin (Lonza), 250 μg/ml gentamycin (Life technologies, Bleiswijk, The Netherlands), 100 U/ml polymyxin B sulfate (Sigma-Aldrich), 50 U/ml nystatin (Sigma-Aldrich) and 10% glycerol (Sigma-Aldrich)) and centrifuged briefly. Quadruplicate ten-fold serial dilutions of the lung samples were used to determine the virus titers in a confluent layer of MDCK cells as described previously [52].

2.7. Detection of virus-specific CD8+ T cells by dextramer (Dm)-staining

Single-cell splenocyte suspensions were prepared as described previously [28]. Splenocytes were washed with 5% FBS in PBS and stained for flow cytometry with fluorochrome-labeled monoclonal antibodies (mAbs) specific for selected cell differentiation markers: CD3e-PerCP, CD8b.2-FITC (BD Pharmingen, Breda, The Netherlands) and H-2Db dextramers with the NP366–374 epitope (ASNENMETM) (APC-labeled) or the PA224–233 epitope (SLSLENFRAYV) (PE-labeled) (Immudex, Copenhagen, Denmark) and LIVE/DEAD aqua Fixable Dead Cell stain (L/D) (Invitrogen, Breda, The Netherlands). After extensive washing cells were analyzed using a FACSCanlott flowcytometer and FACS Diva software (BD Biosciences, Breda, The Netherlands)

2.8. Peptides and intracellular interferon gamma (IFN-γ) staining

Splenocytes were cultured in the absence or presence of 5 μM peptide NP366–374 (ASNENMETM) or PA224–233 (SLSLENFRAYV)
virion as different group previously maturation o/n 2.9. Healthy CD8b.2-FITC, (immunograde Netherlands) and were subsequently stained with CD3e-PerCP, CD8b.2-FITC, L/D and IFN-γ-PacificBlue (Biolegend, London, United Kingdom) as described previously [55]. Cells were analyzed using a FACSCantoll flowcytometer and FACS Diva software.

2.9. Human monocyte-derived DCs collection and in vitro maturation

Peripheral blood mononuclear cells (PBMCs) from three healthy blood donors (18 to 64 years of age) (Sanquin Bloodbank, Rotterdam, The Netherlands) were isolated and cultured for 6 days to obtain immature human DCs as described previously [56]. Subsequently these cells were stimulated o/n at 37°C with PBS (negative control), G3 [20 μg/ml], G3/DT [20 μg/ml] or LPS [1 μg/ml] (positive control) (Sigma-Aldrich). After stimulation, cells were stained for CD80-FITC, CD83-APC and CD86-PE (BD Pharmingen) or CD11c-APC, HLA-DR-PerCP (BD Biosciences) and β2-microglobulin-PE (BD Pharmingen) and expression was determined by flow cytometry using a FACSCantoll flowcytometer and Flowjo software (Flowjo, Ashland, USA). Experiments were performed in quadruplicate. Culture supernatants from the above mentioned DC-stimulations were used to assess the concentrations of TNF-α, IFN-γ, IL12p70, IL1-β, IL-6, IL-4 and IL-10 via enzyme-linked immunosorbent assay (ELISA) using Ready-Set-Go ELISA-kits (eBioscience, Vienna, Austria).

2.10. Antigen presentation by human monocyte-derived DCs and BLCLs

Influenza virus-specific CD8+ T cell clones directed against the HLA-A*0201 restricted M158–66 G1GFVFTL epitope and the

Virus titers in the lungs after A/PR/8/34 virus inoculation. Mean and range of lung virus titers of each group at day 4 and 6 post inoculation (p.i.) were determined. The different groups are indicated underneath the figure. Dotted line (- - -) indicates the detection limit. Statistically significant differences are indicated (**: p < 0.01).
HLA-B*2705 restricted NP$_{174-184}$ RRSGAAGAVK epitope were generated as described previously [57].

Immature human DCs of an HLA-typed healthy blood donor were obtained as described above. HLA-typed immature human DCs or B lymphoblastoid cell lines (BLCLs) were incubated o/n at 37 °C with vaccine [40 μl/ml] only or in combination with G3 [20 μg/ml] or G3/DT [20 μg/ml]. Negative controls were incubated with PBS, G3 or G3/DT only and positive controls were incubated with 100 μM peptide (GILGFVFTL or RRSGAAGAVK) (Eurogentec) or infected with A/PR/8/34 (MOI 3). The DCs and BLCLs were used as target cells for the stimulation of influenza virus-specific CD8$^+$ T cell clones. IFN-γ responses of in vitro stimulated CD8$^+$ T cell clones were determined by ELISpot assay as well as intracellular staining (ICS) for IFN-γ.

The IFN-γ ELISpot assay was performed according to manufacturer’s instructions (Mabtech Nacka Strand, Sweden) with an effector-to-target (E:T) ratio of 1:3 in duplicate as described previously [23].

For the ICS, cells of the respective CD8$^+$ T cell clones were stimulated with targets cells (E:T of 1:5) in duplicate for 6hr at 37 °C in the presence of Golgistop. Cells were stored o/n at 4 °C and subsequently stained with fluorochrome-labeled mAbs CD3-PerCP (BD Bioscience), CD8-FITC (Dako, Glostrup, Denmark) and L/D in the presence of Golgistop, fixed and permeabilized using Cytofix and Cytoperm and stained with IFN-γ-PE (BD Pharmingen). Cells were analyzed with a FACSCan flowcytometer and FACS Diva software.

2.11. Statistical analysis

Data for weight loss, lung virus titers, dextramer staining and IFN-γ staining between pairs of groups were analyzed using the Mann–Whitney U test. Time to death were compared using the Kaplan–Meier curves and the log-rank test. Differences were considered significant at $p < 0.05$.

3. Results

3.1. G3 and G3/DT enhance the vaccine-induced antibody response

Upon vaccination none of the mice displayed any signs of discomfort. Limited weight loss (<2%) was observed during two days post vaccination in some mice randomly distributed over the experimental groups, including those that received PBS only (data not shown).

All mice that received the trivalent inactivated influenza vaccine, with or without adjuvant developed HI antibody titers against the three vaccine strains (A/California/7/2009, A/Victoria/361/2011 and B/Hubei-Wujiang/158/2009) after the first vaccination (Fig. 1A–C). However, for all vaccine strains the geometric mean titers (GMTs) of mice that received the vaccine with adjuvant G3/DT or G3 (groups 1 and 2, respectively) were significantly higher than those of mice that received the unadjuvanted vaccine (group 3). Four weeks after the second vaccination the GMTs against the vaccine strains increased in the mice of group 3. Although the GMTs of groups 1 and 2 that received the G3/DT and the G3 adjuvanted vaccine, respectively, did not increase after the second vaccination, they remained significantly higher than the GMTs of group 3 (no adjuvant) ($p < 0.01$). No differences in GMTs were detected between groups 1 and 2, except for antibody titers against A/Victoria/361/2011 on day 56, which were higher in mice of group 2 ($p < 0.05$). No HI antibodies were detected in any of the control mice (groups 4–7). In none of the mice, vaccine-induced antibodies were detected that cross-reacted with the influenza virus strain A/PR/8/34 that was used for challenge infection of the mice (Fig. 1D). This was confirmed with a VN assay (data not shown).

3.2. G3/DT improves the protective efficacy of the split virion vaccine against an antigenically distinct influenza virus

Four weeks after the second vaccination mice were inoculated with a lethal dose of influenza A/PR/8/34, except for control group 7. All groups displayed similar weight loss, starting at day 2 p.i. until day 4 p.i. (Fig. 2A). From day 5 p.i. onward, mice vaccinated with the G3/DT-adjuvanted vaccine (group 1) started to gain weight, while mice of the other groups continued to lose weight up to day 6 p.i. From day 5 onward, the body weight of mice from group 1 remained significantly higher compared to that of mice from group 2 and 3 that received the G3-adjuvanted and unadjuvanted vaccine, respectively ($p < 0.01$).

All mice from group 1 survived until day 6 p.i. while only 20% and 0% of the mice survived of group 2 and group 3, respectively ($p < 0.01$) (Fig. 2B).

3.3. G3/DT adjuvanted vaccine-induced immunity restricts virus replication

Lung virus titers were assessed on day 4 and 6 p.i. (Fig. 3). On day 4 p.i. mice of group 1 (10$^{6.4}$ TCID$_{50}$) and 2 (10$^{6.2}$ TCID$_{50}$) (G3/DT or G3-adjuvanted vaccine, respectively) had significantly lower virus titers compared to their PBS control groups 4 (10$^{7.5}$ TCID$_{50}$; $p < 0.01$)
and 5 (10^{7.1} TCID_{50}; p < 0.01), while no statistically significant difference was detected between the unadjuvanted group 3 (10^{6.9} TCID_{50}) and the PBS control group 6 (10^{7.4} TCID_{50}). No statistically significant difference was detected between groups 1 and 2. Group 2 (G3 adjuvanted vaccine) had a significantly lower mean titer than group 3 (unadjuvanted vaccine) (p < 0.01). The most remarkable difference in lung virus titers was observed on day 6 p.i.: The mean virus titer of group 1 (G3/DT adjuvanted vaccine) was 10^{1.8} TCID_{50}, which was significantly lower than that of control group 4 (10^{3.3} TCID_{50}), but also than that of group 2 (10^{5.3} TCID_{50}) and 3 (10^{6.6} TCID_{50}) (p < 0.01). A smaller but significant reduction in lung virus titer was also observed between group 2 and group 3 (p < 0.01).

### 3.4. Detection of virus-specific CD8+ T cells by Dm-staining in the G3/DT adjuvanted vaccine group only

Since HI antibodies against A/PR/8/34 were undetectable in all mice at day of inoculation (Fig. 1D), we wished to investigate whether virus-specific CD8+ T cells had contributed to the protection observed in mice that received the G3/DT-adjuvanted vaccine. Dextramers-staining was used to measure the frequency of CD8+ T cells specific for the NP_{366-374} and the PA_{224-233} epitope on day 6 p.i. (Fig. 4). Both epitopes are present in the A/PR8/34 backbone of the influenza A vaccine strains and the A/PR8/34 virus used for challenge infection. The mean frequencies of NP_{366-374} and PA_{224-233} specific CD8+ T cells were significantly higher in mice of group 1 that received the G3/DT adjuvanted vaccine (7.5% and 3.0%, respectively) than in mice of group 4 (G3/DT adjuvant only) (1.2% and 2.1%, respectively; p < 0.01). These frequencies also exceeded those observed in mice of group 2 (G3 adjuvanted vaccine) (NP_{366-374} 1.7% and PA_{224-233} 2.2%; p < 0.05) and those observed in group 3 (unadjuvanted vaccine) (NP_{366-374} 1.4% and PA_{224-233} 2.3%; p < 0.01). A small but significant difference (p < 0.05) was observed for NP_{366-374} between group 3 and group 6 (no vaccination) (1.0%).

#### 3.5. Detection of virus-specific CD8+ T cells by intracellular IFN-γ staining in the G3/DT adjuvanted vaccine group only

To determine the frequency of NP_{366-371} and PA_{224-233} specific CD8+ T cells, intracellular IFN-γ staining was performed. To this end, splenocytes obtained on day 6 p.i. were stimulated with synthetic peptides NP_{366-371} and PA_{224-233} and the percentage of IFN-γ positive cells was measured (Fig. 5). The highest response was observed for the NP_{366-371} peptide in group 1 (G3/DT adjuvanted vaccine) (22.2%; p < 0.01). In none of the other groups a NP_{366-371} peptide-specific CD8+ T cell response could be detected. In none of the groups a significant CD8+ T cells response to the PA_{224-233} epitope was observed.

#### 3.6. Both G3 and G3/DT have a similar effects on maturation of human monocyte-derived DCs

In vivo results clearly showed that both G3 and G3/DT promoted antibody responses after vaccination with inactivated split virion vaccine, but only G3/DT was able to induce CD8+ T cell responses. Since the mechanism underlying this discrepancy is unknown we wished to address this in vitro. Since ISCOM adjuvants are known to have an effect on maturation and the cytokine response of APCs [58,59] we determined the effect of G3 and G3/DT on the maturation of immature human DCs (Fig. 6A). Both G3 and G3/DT enhanced the levels of CD83, a DC maturation marker, in vitro. Both adjuvants also equally enhanced the expression of HLA class I and II molecules on the DCs surface. Neither G3 or G3/DT affected the expression of T cell co-stimulatory markers CD80 and CD86 on the surface of human DCs. In addition cytokine production by DCs stimulated with G3 or G3/DT was similar (data not shown).

#### 3.7. Both G3 and G3/DT are able to enhance antigen presentation

Next, we tested if G3/DT could improve antigen presentation and activation of CD8+ T cells in vitro. To this end, in vitro...
Fig. 6. Effect of G3 and G3/DT on the maturation of human DCs and antigen presentation. (A) Maturation of human HLA-un-typed DCs. DCs were unstimulated (gray curve) or treated with G3 (−), G3/DT (− −) and LPS (− − −) and the surface expression of CD11c, CD83, CD80, CD86, HLA class I and II molecules was monitored. Results representative for three human subjects are shown. Numbers in the upper right corner of each histogram indicate how many of the three subjects display this particular result. In case of CD86 two out of the three subjects showed no difference in CD86 expression when DCs were stimulated with G3 or G3/DT whereas in one subject CD86 increased after G3 and G3/DT stimulation. Experiments were performed in quadruplicate.

Antigen presentation by human HLA-typed DCs (B, C and E) and BLCLs (D and F) after stimulation with split virion vaccine adjuvanted with G3, G3/DT or without adjuvant and their ability to stimulate CD8^+^ T cell clones directed against the HLA-A^*^0201 restricted M158–66 GILGFVFTL epitope (B) or the HLA-B^*^2705 restricted NP174–184 RRSGAAGAVK epitope (C–F) was determined in an IFN-γ ELSpot assay (B–D) and by ICS of IFN-γ (E and F). Experiments were performed in duplicate.
immature HLA-types human DCs and HLA-types BLCLs were incubated with split virion vaccine with or without G3 or G3/DT and subsequently their ability to activate CD8+ T cells clones specific for influenza M158-66 or NP174-184 epitopes was determined. As shown by ELISpot (Fig. 6B–D) and ICS (Fig. 6E and F) G3 and G3/DT promoted the activation of the influenza virus-specific T cell clones in the presence of the split virion vaccine that in the absence of adjuvant failed to activate virus-specific CD8+ T cells efficiently.

4. Discussion

In the present study, it was demonstrated that the G3/DT adjuvant improved the antibody response to a trivalent inactivated split virion vaccine in mice and promoted virus-specific CD8+ T cell responses. Furthermore, G3/DT improved the protective efficacy of the vaccine against a lethal infection with the antigenically distinct influenza virus A/PR/8/34. Since the unadjuvanted vaccine and the vaccine adjuvanted with G3 only failed to induce protective immunity, the addition of DT was pivotal for the vaccine-induced protection. However, both the G3 and G3/DT adjuvant promoted the induction of vaccine-induced antibody responses. These antibodies did not cross-react with the challenge virus strain A/PR/8/34 in HI and VN assays, which explains the failure to induce sterile immunity and the inability to protect mice from weight loss after infection with A/PR/8/34. However, mice that received the G3/DT-adjuvanted vaccine started to gain weight and displayed 100% survival. The mean lung virus titer of mice that received the G3/DT-adjuvanted vaccine was significantly reduced on day 6 p.i. compared to the other groups. This accelerated clearance of infection in the G3/DT-adjuvanted vaccine group might be attributed to the presence of cross-reactive T cell immunity. Indeed the kinetics of virus replication resembled that of mice in which cross-protective T cell responses were induced by primary influenza virus infection [28,29]. The induction of influenza A virus-specific CD8+ T lymphocytes in mice that received the G3/DT-adjuvanted vaccine was confirmed and was not detectable in mice of any of the other groups. The mechanism underlying the induction of virus-specific CD8+ T cell responses by the addition of G3/DT remains unclear. Human instead of mouse DCs were used to unravel the underlying mechanism because the use of human DCs not only allowed studying DC activation, but also antigen processing and presentation using influenza virus-specific T cell clones. Furthermore, it was shown previously that CD80 and CD86 expression in human and mouse DCs upon stimulation with an adjuvant, resembled each other [56]. Both G3 and G3/DT promoted the activation of virus-specific T cells in vitro. Most likely, both adjuvant preparations are able to introduce viral proteins into the cytosol of APCs, induce maturation of DCs and upregulate MHC class I and II expression. The addition of DT in combination with the vaccine dose used in vivo may have tipped the balance in favor of inducing influenza virus-specific T cell immunity, which may have not been reflected properly in the in vitro experiments.

Although inactivated trivalent influenza vaccines are safe and able to elicit sufficient virus strain-neutralizing antibody responses, they fail to induce broadly protective T lymphocyte responses efficiently [2,35,36,60,61]. Their use even may prevent induction of cross-protective T cell immunity in naive subjects otherwise induced by natural influenza virus infections [62]. Therefore, currently used inactivated influenza vaccines could benefit from the use of adjuvants, like G3/DT, in order to induce more broadly protective immune responses.

It should be realized that the influenza A virus vaccine strains share their back-bone genes with those of the challenge virus A/PR/8/34, which contributes to cross-reactivity of the T cell response and a favorable outcome of the challenge infection. However, T cell responses to the epitopes NP366–371 and PA224–233 also contribute to heterosubtypic immunity as was shown previously [27,29]. Furthermore, it is likely that CTL responses against other conserved epitopes were elicited after vaccination with the G3/DT-adjuvanted vaccine, which are likely to have contributed to protective immunity.

Further research is needed to establish the exact mechanism that enables G3/DT but not G3 to induce virus-specific T cell responses in vivo and to confirm that G3/DT-adjuvanted split virion influenza vaccines also elicit cross-reactive T cell responses in humans.

Since mice that received the G3-adjuvanted vaccine without the DT component were not protected, although these mice developed similar strong antibody responses as mice in the G3/DT vaccine group, it is unlikely that antibodies to HA, NA or any other viral proteins contributed to the protection observed in the latter group. Although it has been shown that antibodies to A/H1N1 viruses that circulated before 1957 can cross-react with H1N1pdm09 viruses and vice versa [63–65], we were unable to detect vaccine (containing the H1N1pdm09 component)-induced antibodies that cross-reacted with the A/PR/8/34 challenge virus by HI or VN assays. Both the G3 and G3/DT adjuvant greatly enhanced the antibody response to all vaccine strains after a single immunization. Since a subsequent immunization did not have a pronounced booster effect, a single dose of the adjuvanted vaccine may be sufficient to induce protective antibody responses, provided that the vaccine is matching the strain causing the infection antigenically. This is especially relevant in case of an emerging pandemic, when the timely availability of sufficient vaccine doses is desired [66–70]. Furthermore, it has been demonstrated that more than one immunization with possibly a high dose of a pandemic vaccine is required for the induction of protective immunity [67,69,70]. The G3 and G3/DT adjuvant may allow substantial antigen dose sparing. In the present study a dose of 5 μg HA was sufficient to induce potent antibody responses.

In conclusion, G3/DT is a promising adjuvant formulation that not only potentiates the antibody response induced by influenza vaccines, but that also induces T cell immunity which could afford broader protection against antigenically distinct influenza viruses. These properties are not only desirable for protection against matching and antigenically mismatched seasonal influenza viruses, they may also contribute to protective immunity against influenza A viruses of alternative subtypes, which continue to pose a pandemic threat [71–74]. Further (clinical) evaluation of G3 and G3/DT-adjuvanted influenza vaccines seems therefore warranted.

Conflict of interest statement

BM is scientific officer of MoreinX, ADMEO and GFR are employed partially by Viroclinics Biosciences BV. The other authors do not declare conflict of interest.

Acknowledgements

The authors wish to thank Marine Hillaire, Lidewij Wiersma, Peter van Run, Gerrie de Mutsert and Theo Bestebroer for excellent technical advice and assistance.

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et al. (2014) / Vaccine 32 (2014) 5614–5623

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