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A B S T R A C T

The widely used influenza subunit vaccine would benefit from increased protection rates in vulnerable populations. Skin immunization by microneedle (MN) patch can increase vaccine immunogenicity, as well as increase vaccine persistence relative to groups given influenza vaccine alone and led to rapid lung viral clearance following lethal infection with homologous virus in the mouse model. Here we demonstrate that successful incorporation of the thermolabile cytokine GM-CSF into MN resulted in improved vaccine-induced protective immunity holding promise as a novel approach to improved influenza vaccination. To our knowledge, this is the first successful incorporation of a cytokine adjuvant into dissolvable MNs, thus advancing and diversifying the rapidly developing field of MN vaccination technology.

1. Introduction

Influenza A virus is a common respiratory pathogen that causes seasonal outbreaks, epidemics and occasional pandemics. Although influenza illness mostly presents with benign symptoms in healthy populations and resolves within 7–10 days, it can become life-threatening in elderly adults, pregnant women, infants and people with chronic conditions [1–3]. Annual infection rates for seasonal influenza are 5–10% for adults and 20–30% for children [4]. Of the many influenza subtypes, H1N1 has the highest prevalence in laboratory-confirmed influenza-like illness (ILI), while H3N2 disproportionately affects individuals ≥65-years of age causing higher morbidity and mortality rates [5].

Influenza vaccination is the most effective public health strategy for reducing influenza mortality rate and economic burden of treatment and hospitalization costs and lost productivity and wages [6,7]. However, during the 2015–2016 flu season influenza vaccination demonstrated modest protection against ILI, with an overall effectiveness of 47% [8]. In addition to the need for increased immunogenicity, current influenza vaccination approaches face obstacles such as limited duration of immunity and lack of protection in high risk groups, such as young children and the elderly, as well as low coverage and participation in vaccination [9–11].

Skin vaccination by microneedle (MN) patch has the potential to overcome the aforementioned hurdles observed in vaccination strategies by generating superior immune responses to vaccine antigen and increasing access to vaccination through improved vaccination logistics and greater patient acceptance [12–15]. MN vaccine patches have specifically been shown to enhance influenza vaccination, including increased immunogenicity [12,13,16], improved acceptability [17,18] and long-lasting stability outside the cold-chain [19]. Additionally, a recent phase I clinical trial showed that influenza vaccination by MN patch administered by study personnel or self-administered by study participants was well tolerated, strongly immunogenic and...
vaccines, BALB/c mice. The LD50 was determined using the Reed-Muench formula [37] and the viral titers were determined by plaque assay [38].

Granulocyte-macrophage colony stimulating factor (GM-CSF) is a monomeric 24 kDa cytokine that promotes the maturation of granulocytes and macrophages from bone marrow progenitor cells [24] and is secreted by a wide array of immune cells, keratinocytes, eosinophils, neutrophils, and endothelial cells [25]. GM-CSF is particularly active in the skin, recruiting epidermal dendritic cells (DC) such as Langerhans cells into draining lymph nodes and enhancing activation and antigen presentation [26,27]. These properties have made GM-CSF an effective therapy to treat opportunistic infections in cancer and AIDS patients for the past 20 years [28,29] and a molecule of interest for adjuvant purposes in therapeutic vaccinations against skin cancers and autoimmune skin disorders [30,31]. However, despite the potential for successful use as an adjuvant and demonstrated safety in cancer clinical trials [32–34], development of GM-CSF as an adjuvant in vaccines against infectious microorganisms has been limited by variability in effectiveness [35].

Due to the immunomodulatory effects and activation of skin immune cells by GM-CSF, we hypothesized that incorporation of this cytokine in MN patches containing influenza subunit vaccine will increase immunogenicity compared to vaccine alone. This is further motivated by prior findings that GM-CSF was identified as a potential adjuvant from a panel of cytokines and chemokines that were upregulated in the skin following insertion of influenza vaccine-coated MN arrays [36]. We tested our hypothesis in the BALB/c mouse model using both intradermal (ID) injection and MN patch delivery. We elaborate on previous work showing the efficacy of MN patches as an improved vaccination technology and develop a pipeline for testing novel adjuvants to enhance the potency of this emerging technology.

2. Materials and methods

2.1. Cells and virus stocks

Madin-Darby canine kidney (MDCK) cells (ATCC, Manassas, VA) were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) (Mediatech, Herndon, VA) containing 10% fetal bovine serum (Thermo, Rockford, IL). Influenza virus stocks A/Brisbane/59/2007 (H1N1), A/California/07/2009 (H1N1), and A/Udorn/307/1972 (H3N2) were propagated in MDCK cells. Egg-grown subunit monovalent influenza vaccines, A/Brisbane/59/2007, A/California/07/2009, A/Christchurch/16/2010 and A/Victoria/210/2009 (A/Perth/16/2009-like) were generously provided by Seqirus (Maidenhead, UK). Vaccine processing for MN patch fabrication (reconstitution of lyophilized preparation and concentration), determination of protein concentration and assay of hemagglutinin (HA) content were carried out as previously described [13]. The following in-house MDCK-grown viruses were used for our studies: H1N1 A/California/07/2009, A/California/10/1978, A/FM/1/1947; and H3N2 A/Texas/50/2012, A/Victoria/210/2009, and A/Aichi/2/1968. Mouse adapted A/California/07/2009 virus 2 months post vaccination.

The optimal GM-CSF concentration for microneedle (MN) vaccination was determined by an initial screen of GM-CSF doses (5, 20, 100 ng) administered ID with 1 μg HA of H3N2 (A/Perth/16/2009-like) subunit vaccine and was compared to 1 μg HA and 5 μg of the same vaccine without adjuvant. Sera was collected 90 days post vaccination (d.p.v.), and mice were infected IN with mouse adapted A/Udorn/307/1972 (H3N2) at a dose of 4xLD50 (35 plaque forming units (p.f.u.)) 4 months post vaccination.

The best route of adjuvanted vaccine delivery was selected by comparing magnitude and breadth of immune responses induced by MN, ID or IM immunization of mice with A/California/07/2009 subunit vaccine. Skin surfaces for MN patch vaccination were prepared as previously described [39]. Mice were anesthetized using xylazine/ketamine cocktail and MN patches were applied with direct pressure for 1 min and left in place for 20 min. Unused and used patches were analyzed by ELISA for vaccine and adjuvant content to determine delivery efficiency. Sera was collected 28, 90, and 120 d.p.v. Mice were infected with 25xLD50 (1500 p.f.u.) mouse-adapted A/California/07/2009 virus 4 months post vaccination.

Cellular immune responses in MN vaccinated cohorts of mice with blank patches or patches containing monovalent subunit vaccine (A/Christchurch/16/2010, a A/California/07/2009-like strain), at 1 μg HA, 3 μg HA, or 1 μg HA mixed with 100 ng recombinant murine GM-CSF. Mice were challenged with 10xLD50 (600 p.f.u.) homologous mouse-adapted A/California/07/2009 virus one month post vaccination. In survival studies, animals were monitored daily for morbidity (body weight loss, hunched posture, ruffled hair and decreased mobility) and mortality for 2 weeks. Mice that lost 25% of their weight were euthanized according to IACUC guidelines. An independent cohort of mice from each group was euthanized at 4 days post-infection to harvest lungs, spleens and lymph nodes.

2.2. Animals

Eight-week-old female BALB/c mice (Charles River Laboratories, Wilmington, MA) were bred and housed in a biosafety level 1 facility at Emory University’s Division of Animal Resources and viral infection experiments were performed on animals housed in a biosafety level 2 facility at Emory University’s Division of Animal Resources. All experiments were conducted in accordance with protocols approved by Emory University’s Institutional Animal Care and Use Committee (IACUC) in accordance with guidelines with the United States Federal Animal Welfare Act (PL 89-544) and subsequent amendments.

Vaccines and adjuvant were prepared in sterile Ca2+ and Mg2+ deficient phosphate-buffered saline (PBS). Mice were intradermally (ID) vaccinated with a tuberculin syringe on the caudal site of dorsum with 1 or 3 μg HA H1N1 A/California/07/2009 subunit vaccine alone or 1 μg HA mixed with 100 ng recombinant murine GM-CSF (PeproTech, Rocky Hill, NJ). Mice were infected intranasally (IN) under isoflurane anesthesia with 25xLD50 (1500 p.f.u.) mouse-adapted A/California/07/2009 virus 2 months post vaccination.

2.3. Immunizations and infections

MN patches were fabricated in a two-step process with polydimethylsiloxane (PDMS) molds as previously described [13]. To measure stability of GM-CSF in the presence of excipients a second-cast solution was prepared containing recombinant GM-CSF (PeproTech, Rocky Hill, NJ) in 3% w/v polyvinyl alcohol (PVA, Millipore, Bellerica, MA) in combination with one or more of the following: 0.1% w/v bovine serum albumin (BSA, Sigma), 10% w/v trehalose (Sigma), 1% w/v carbamylmethyl cellulose (CMC, Spectrum, New Brunswick, NJ). A second-cast solution was prepared containing 18% w/v PVA and 18% w/v sucrose (Sigma). MN patches were prepared by sequentially casting
the first-cast solution to fill the mold cavities and the second-cast solution to cover the mold surface (corresponding to the MN patch backing). The MN patches were removed from the molds and attached to adhesive paper discs. Patches were inspected via light microscopy for integrity and uniformity. Blank placebo patches were made identically, except no antigen was in the first-cast solution. Influenza HA concentration was measured by a modified SRID assay [13,40].

To measure stability of GM-CSF in the presence of influenza vaccine, the first-cast solution was prepared containing A/Brisbane/59/07 and murine recombinant GM-CSF in 3% w/v poly-vinyl alcohol (PVA; Sigma-Aldrich, St Louis, MO) or in combination with 10% w/v trehalose (Sigma) and 3% PVA. Based on pilot delivery efficiency data after microneedle insertion [13], we encapsulated 20 ng GM-CSF per patch for a final working dose of 10 ng and 7 μg HA for a working concentration of 5 μg.

2.5. Bioactivity assays for GM-CSF

Bioactivity of GM-CSF was tested with proliferation assays using 2 cell types: murine bone marrow cells harvested from naïve female mice and TF-1 human erythroleukemia cell line (ATCC® CRL-2003, Manassas, VA). TF-1 cell growth and survival is dependent on GM-CSF or IL-3 [41]. Cells were treated with either solubilized or dried and reconstituted microneedle coating solutions mixed with GM-CSF. Bone marrow was collected from the femurs of two naïve mice, centrifuged at 1000 RPM for 7 min, and incubated in RBC Lysing Buffer Hybri-Max (Sigma-Aldrich, St. Louis, MO) for 10 min. Cells were washed and re-suspended in RPMI with 10% FCS and 1% Penicillin-Streptomycin RPMI (Corning Cellgro, Thermo Fisher Scientific, Waltham, MA). Bioactivity was tested in bone marrow cells using: (i) first-cast solution (with 10% trehalose) without GM-CSF, (ii) GM-CSF suspended in RPMI-10 (200 μl of 200 ng/μl) (iii) first-cast solution mixed with 1:1 with GM-CSF (to reach a final concentration of 100 μg), with GM-CSF and (iv) first-cast solution with GM-CSF dried in microcentrifuge tubes and re-suspended in 200 μl RPMI-10. After 2 days, cells were imaged via Axio Scope software (Carl Zeiss Microscopy, LLC, Thornwood, NY). Cell activation and proliferation was visualized with light microscopy throughout the incubation period. Every 2 days the cells were supplemented with RPMI-10. Following a 4-day incubation, we determined viable cells in proliferation with the colorimetric Promega Cell Titer 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI). An additional liquid vaccine formulation control was included to ensure that the fabrication process did not cause a loss in activity.

Bioactivity of GM-CSF (10 ng delivery concentration) encapsulated in MN patches was tested in TF-1 cells with formulations used in the mold: 1) 0.1% w/v albumin 2) 0.1% albumin and 10% w/v trehalose 3) 1% CMC w/v and 10% trehalose or 4) 1% w/v gelatin for 4 days. Three patches from each group were re-suspended in 200 μl of RPMI-10 to overlay the cells. An additional liquid formulation control for each group was included to ensure that the fabrication process did not cause a loss in activity. As a second liquid control, we air-dried the same first-cast solution with GM-CSF dried in microcentrifuge tubes and re-suspended in 200 μl RPMI-10. After 2 days, cells were imaged via Axio Scope software (Carl Zeiss Microscopy, LLC, Thornwood, NY). Cell activation and proliferation was visualized with light microscopy throughout the incubation period. Every 2 days the cells were supplemented with RPMI-10. Following a 4-day incubation, we determined viable cells in proliferation with the colorimetric Promega Cell Titer 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI). An additional liquid vaccine formulation control was included to ensure that the fabrication process did not cause a loss in activity.

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2.6. Characterization and quantitation of humoral immune responses

Serum samples were collected at 14, 28, 56 or 60, 90 and 120 (d.p.v.). Antibody titers were quantified with ELISA as described previously with bovineinflated anti-IgG, IgG1, IgG2 antibodies (Southern Biotech, Birmingham, AL) against 100 ng/well monovalent H1N1 A/California/07/2009 subunit vaccine, A/Christchurch/16/2010 subunit vaccine, or recombinant HA A/California/07/2009 from BEI Resources (Manassas, VA) [42]. Hemagglutination inhibition (HAI) assays were performed according to the WHO laboratory diagnostics manual using washed turkey red blood cells [43]. Virus-neutralizing (VN) antibody titers were determined by microneutralization assay for A/Perth/16/2009 and A/California/07/2009, as described previously [22]. IgG titers against chimeric HA proteins containing exotic HA head subtypes and commonly found seasonal HA stalks were measured to determine the antibody targets from H1N1 vaccinated mice; these chimeric proteins (cH6/1 and cH14/3) were created in a baculovirus system as described previously [44]. Influenza-specific antibody avidity was measured by ELISA in the presence of increasing concentrations (0–2.0 M) of the chaotropic agent guanidine thiocyanate (GTC) (Sigma), as previously described [42]. The avidity index was determined using Prism 7.03 Software (GraphPad, La Jolla, CA) by calculating the molar concentration of the chaotropic agent required to reduce the initial optical density by 50%.

2.7. Antigen-specific cellular activity in spleen, lungs, and bone marrow

Lymphocytes were isolated from spleen and lung tissue 7 and 14 d.p.v. and 4 days post infection (d.p.i.). Bone marrow was isolated from mice 60 days d.p.v. Antibody secreting cells (ASCs) were quantified by overlaying 1 × 10^5 cells/well in ELISPOT plates coated with 200 ng/well H1N1 A/Christchurch/16/2010. ASCs were incubated at 37 °C for 16 h, washed, and then influenza-specific antibodies were detected using isotype-specific, biotinylated murine Ig antibodies. Cytokine secreting cells (CSC) were quantified by overlaying 5 × 10^6 cells/well in ELISPOT plates (EMD Millipore, Burlington, MA) coated with 100 ng/well of capture antibody (BD Biosciences, San Jose, CA). Cells were incubated with 200 ng/well H1N1 A/Christchurch/16/2010 for 48 h at 37 °C. ASC and CSC plates were washed and incubated with 100 ng/well biotinylated detection antibodies (IL-4 and IFN-γ, BD Biosciences; IgA, IgM, and IgG, Southern Biotech, Birmingham, AL) and developed with streptavidin-HRP and diaminobenzidine. Spots were counted via ImmunoSpot Reader 5.0 (Cellular Technology Limited, Shaker Heights, OH), normalized to 1 × 10^6 cells/well and plotted with GraphPad Prism software.

2.8. Flow cytometry identification of activated cellular subsets

Skin draining (inguinal) lymph nodes were collected at 7 d.p.v. to evaluate the presence of activated T follicular helper cells (Thf) and germinal center (GC) B cells by flow cytometry. Single-cell suspensions were incubated with anti-mouse CD16/32 antibody for 10 min on ice. Cells were then incubated with CD3e (145-2C11), CD4 (GK1.5), PD-1 (29F.1A12), CXC85 (L138D7), CD19 (6D5), Fas (Jo2) and GL7 (GL7) on ice for 30 min. Antibodies were purchased from BioLegend (San Diego, CA) and BD Biosciences (San Jose, CA). Cells were washed and fixed in 2% paraformaldehyde. Samples were acquired on an LSR II flow cytometer (BD Biosciences) and data were analyzed with FlowJo (FlowJo LLC, BD, Franklin Lakes, NJ) (Suppl Fig. 1).

2.9. Quantification of cytokines following infection

Lungs were weighed and homogenized in 0.5 ml DMEM and 1 μl Halt Protease Inhibitor. Lysates were diluted in 1× PBS and cytokines (GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-10, TNF-α) were quantified via a BioPlex Pro Mouse Cytokine 8-plex Assay (Bio-Rad, Hercules, CA).
California) at the Emory Vaccine Center Virology Core and normalized for dilution factor and original tissue concentration.

2.10. Measurement of lung viral titers

Viral titers in lung homogenates were quantified via plaque assay in MDCK cells [39]. Viral titers were assessed per gram of tissue.

2.11. Statistics

For ELISA, ELISPOT and cell-based assays, the statistical significance of differences between two groups was calculated by two-tailed unpaired Student’s t-test. Log_{10} converted HAI and VN titers were analyzed with one or two-way ANOVA with Bonferroni post-hoc test. Unless otherwise stated, antibody assays were performed in duplicate. For survival curves, statistics were calculated using a Log-rank (Mantel-Cox) test. Non-linear regression analyses were performed to determine the IC_{50} (95% confidence interval). A p-value < 0.05 was considered significant.

3. Results

3.1. Intradermal vaccination screen identified GM-CSF as a promising adjuvant in microneedle influenza immunization

The efficacy of GM-CSF as vaccine adjuvant for skin immunization was initially carried out with ID injection studies. The dose of 100 ng for GM-CSF was based on published literature [45]. The immune responses elicited to unadjuvanted A/California/07/2009 subunit vaccine at low or high dose (1 μg HA and 3 μg HA respectively) were compared to those induced by adjuvanted low dose vaccine (Fig. 1). Mice that received the adjuvanted vaccine demonstrated 4-fold higher anti-A/California/07/2009 HAI titers (p = 0.07) when compared to mice vaccinated with low dose unadjuvanted vaccine and similar HAI titers compared to the high-dose vaccinated group (p = 0.025) at 8 weeks post-vaccination (Fig. 1A).

Infection of all vaccinated groups, 2 months post-vaccination with 25xLD_{50} homologous virus, conferred complete protection of the GM-CSF/1 μg HA and the unadjuvanted 3 μg HA vaccine groups (p = 0.04 when comparing survival rates to unadjuvanted 1 μg vaccine group) (Fig. 1B) and minimal morbidity with 5% body weight loss by day 6 (Fig. 1C). In contrast, the group immunized with low-dose unadjuvanted vaccine showed 80% mortality and 20% body weight loss by the survivors. We observed that addition of GM-CSF as an adjuvant can boost protective immune responses comparable to a three-fold higher vaccine dose, thereby conserving the amount of vaccine required for protection.

H3N2 influenza A viruses are known to cause significant disease burden worldwide and the H3N2 strains in inactivated trivalent influenza vaccine have lower efficacy than the H1N1 strains. Hence, we assessed GM-CSF’s ability to boost immune responses to a monovalent H3N2 subunit vaccine using a series of GM-CSF concentrations to optimize the adjuvant dose-response curve. Mice were ID vaccinated with 1 μg HA or 5 μg HA H3N2 A/Victoria/210/2009 (A/Perth/16/2009-like) and a range of GM-CSF concentrations (5, 20, 100 ng) (Fig. 2). Administration of 100 ng GM-CSF with 1 μg HA vaccine doubled the VN titers when compared to unadjuvanted low-dose vaccine at 90 d.p.v. (Fig. 2A). Following infection with 4xLD_{50} of A/Udorn/307/1972 4 months post-vaccination, the co-delivery of 100 ng GM-CSF increased survival rates (Fig. 2B) and prevented significant body weight loss of infected survivors (Fig. 2C) relative to mice that received a low-dose vaccine alone. Thus, we demonstrated that the adjuvanted formulation can improve protective immunity in a second clinically significant influenza subtype.

Fig. 1. Adjuvantation with 100 ng GM-CSF in intradermal H1N1 A/California/07/09 subunit vaccination improves antibody responses and enhances protection to lethal virus challenge. BALB/c mice (n = 4) were vaccinated ID with PBS, 1 μg HA, 3 μg HA, or 1 μg HA/100 ng GM-CSF. (A) Serum influenza-specific HAI titers are plotted at 8 weeks post-vaccination. Statistics for panel (A) were performed with Mann-Whitney test. Mice were challenged with 25xLD_{50} homologous virus 3 months post-vaccination and (B) survival and (C) body weight changes were recorded for 13 days. Percent body weight values are expressed as mean ± SEM. Statistics for panel (B) were performed with Mantel-Cox test.
3.2. Effect of MN patch formulation on GM-CSF’s ability to induce proliferation of bone marrow cells

The proliferative capacity of recombinant murine GM-CSF was initially tested in bone marrow cells (Fig. 3A). Cells were stimulated with increasing concentrations of GM-CSF (0.6–400 ng per well) and supernatants were collected at 60 and 120 min of incubation to test for proliferation. We observed a time-dependent and dose-dependent response that was more significant above concentrations of 25 ng of GM-CSF/well. The maximum bone marrow proliferation occurred after 120 min of cellular exposure at 200 ng GM-CSF.

Since MN patch fabrication may affect the bioactivity of GM-CSF, we measured the ability of GM-CSF to induce activation and proliferation of bone marrow cells before and after incorporation and drying in casting solutions used to fabricate MN patches (Fig. 3B). Bone marrow cells in culture were overlaid with casting solution alone (Fig. 3Ba), murine recombinant GM-CSF dissolved in PBS (Fig. 3Bb), GM-CSF dissolved in first-casting solution (Fig. 3Bc) and GM-CSF in first-casting solution, air dried and resuspended in culture medium (Fig. 3Bd). Visualization of cells with light microscopy showed that

Fig. 2. GM-CSF is an effective adjuvant at low doses in H3N2 vaccinations. Mice were vaccinated ID with 1 μg HA H3N2 A/Victoria/210/2009 with a range of GM-CSF concentrations: 5 ng, 20 ng, and 100 ng. A high vaccine dose (5 μg) was used as a control for superlative immune responses. Serum virus-neutralizing (VN) titers were assessed at 90 d.p.v. Vaccinated mice were challenged with 4×LD₅₀ mouse-adapted H3N2 A/Udorn/307/1972 for (C) survival and (D) body weight changes. VN titers were expressed as GMean ± 95% CI. Percent body weight values are expressed as mean ± SEM. Statistics for survival were performed with Mantel-Cox test.

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inclusion of GM-CSF in the first-casting solution in either liquid or dried formulation caused distinct morphological changes in bone marrow cultures similarly to GM-CSF dissolved in PBS, suggesting that the cytokine retained its biological activity. We also found that the same formulations induced bone marrow cell proliferation and increased the overall cell numbers 4-fold compared to the control (Fig. 3C). Thus, GM-CSF remained stable upon casting processes fundamental to MN production and retained the capacity to proliferate bone marrow cells following reconstitution.

3.3. Stability and bioactivity of GM-CSF following MN patch fabrication with excipients

We next analyzed whether GM-CSF (10 ng delivery concentration) would retain bioactivity following incorporation in MN patches using different formulations. The formulations included trehalose CMC, or gelatin, which have previously been used as structural and stabilizing excipients in MN patch fabrications [13,46] and BSA alone or mixed with trehalose. Bioactivity of reconstituted patches was compared to lyophilized GM-CSF dissolved in PBS and from reconstituted dried solution. Bioactivity was determined by overlaying solutions on TF-1 cells and measuring proliferative capacity (Fig. 4A). Among the formulations studied, MN patches containing 10 ng GM-CSF and fabricated using casting solutions comprised of 0.1% albumin and 10% trehalose generated the highest cellular proliferation within the standard curve (0.31–20 ng/ml). Formulations that induced the highest cellular proliferation in Fig. 4A were reformulated with vaccine, excipients, and GM-CSF in order to validate bioactivity following co-incorporation of vaccine and adjuvant (Fig. 4B).

Next, we tested the bioactivity of 10 ng GM-CSF mixed with subunit influenza vaccine (A/Brisbane/59/2007) (5 μg delivery concentration) and encapsulated in MN patches to enhance TF-1 cell proliferation. These patches were dissolved in RPMI-10 and overlaid on TF-1 cells for 3 days. Bioactivity was compared to patches containing GM-CSF alone, GM-CSF formulated with BSA or trehalose and GM-CSF mixed with vaccine and trehalose (Fig. 4B). Cells cultured in the presence of RPMI-alone were the negative control group. Each group of reconstituted patches was compared to the same formulation in solution. Since reconstituted patches induced cell proliferation at similar levels, we concluded that the formulation that stabilized the adjuvants was also suitable for influenza vaccine.

Mechanical stability of MN patches formulated for GM-CSF delivery was also studied. MN patches containing a red dye were fabricated using a casting solution comprising 0.1% albumin and 10% trehalose and applied to skin. Before application, the red dye was localized mostly in the MNs and not in the patch backing (Fig. 4Ca). After application to the skin, the MNs (and red dye) dissolved in the skin (Fig. 4Cb). Altogether, these results demonstrate that MN patches were fabricated with GM-CSF that retained bioactivity and could be used to puncture and dissolve in skin.

3.4. Humoral and protective immune responses following delivery of recombinant murine GM-CSF-adjuvanted H1N1 vaccine

We previously demonstrated that MN patch delivery of antigen in skin can augment immune responses to influenza vaccination. The goal of this study was to determine if incorporation of GM-CSF into MN patches could induce superior and dose-sparing immune responses...
compared to conventional vaccination (IM or ID) without adjuvant. Mice were vaccinated either cutaneously (MN patches or ID injection) or systemically (IM injection) with the same vaccine (H1N1 A/California/07/2009) whereas the GM-CSF dose was 100 ng per patch and 200 ng for the IM and ID routes as determined in the in vitro bioactivity studies (Fig. 3).

While MN patch vaccination generated superior vaccine-specific IgG responses when compared to IM and ID routes at 28, 90 and 120 d.p.v., we observed no differences in IgG titers among low- or high-dose un-adjuvanted vaccine or in the presence of GM-CSF (Fig. 5A). In contrast, the immunopotentiating effects of GM-CSF were demonstrated in antibody cross-reactivity of MN patch vaccinated mice against a heterologous (homosubtypic) seasonal influenza strain H1N1 A/New Caledonia/20/1999 (Fig. 5B) and the heterosubtypic H3N2 A/Brisbane/10/2007 strain (Fig. 5C). The adjuvanted vaccine induced equivalent antigen-specific IgG serum titers to high vaccine doses at 120 d.p.v. Notably, vaccination in the presence of 2-fold higher dose GM-CSF generated comparable responses across ID and IM groups at 75% lower titers than those achieved with MN patch vaccination.

These data indicate that MN patch vaccination induced robust long-lived antibody responses, and that co-administration with GM-CSF further potentiated heterologous immune responses after MN patch vaccination, but not after ID or IM vaccination. Thus, the combination of MN patch vaccination and GM-CSF adjuvantation may play an important role on increasing breadth of immunity.

While all vaccinated groups showed protection (higher than 80%) (Fig. 6A, C, E) following infection with homologous virus 4 months post-vaccination, only the MN group demonstrated least body weight losses (Fig. 6B) compared to the ID group (Fig. 6D) or IM group (Fig. 6F). Contrary to the initial ID vaccination data (Fig. 1) where administration of GM-CSF with low dose of vaccine improved protection compared to vaccine alone, in this experiment all vaccinated groups irrespectively of route of administration survived lethal infection. These findings are likely due to antibody maturation, considering that the infection study in this experiment took place 4 months post vaccination compared to 2 months in the initial ID vaccination study.

3.5. Longevity of antibody responses to MN vaccination with subunit influenza vaccine and GM-CSF

To assess the potential of GM-CSF to enhance long-term humoral
and cellular immune memory, we fabricated MN patches containing low dose, high dose or low dose H1N1 vaccine (A/Christchurch/16/2010, A/California/07/2009-like) with GM-CSF in an experimental design similar to the initial screening experiments in which we demonstrated superior immune responses after MN patch vaccination compared to the needle and syringe approach (Fig. 7A).

We found that GM-CSF-adjuvanted patches increased by 2-fold A/California/07/2009-specific HAI titers up to 12 weeks post-vaccination (Fig. 7B) and enhanced IgG binding avidity to subunit vaccine (Fig. 7C), showing improved antibody specificity for the influenza antigen.
compared to non-adjuvanted patch vaccination. Addition of GM-CSF to the low-dose vaccine formulation augmented more than two-fold the levels of anti-A/Christchurch/16/2010 IgG antibodies when compared to the same dose of vaccine alone (p = 0.004 at 10 weeks and p = 0.001 for 12 weeks post-vaccination) (Fig. 7D). When comparing the IgG1 titers between the same groups, we found a 2-fold difference by week 10 post-vaccination (p = 0.05), which was further increased by week 12 (p = 0.003). Notably, the adjuvanted formulation also induced 4-fold higher IgG1 responses than the unadjuvanted high-dose vaccine by week 12 (p = 0.003) (Fig. 7E). Although we did not see similar statistically significant differences in IgG2a titers among vaccinated groups due to a higher spread of responses, the antibody levels followed the same trend as in IgG1 subclass (Fig. 7F). Thus, the inclusion of GM-CSF in MN patches increased the production, specificity and duration of antibodies in response to influenza vaccination.

Given the increase in long-term antibody expression for GM-CSF-adjuvanted MN patches, we also investigated whether mice vaccinated against influenza virus with GM-CSF had increased systemic B cell activation and memory responses. Indeed, co-administration of vaccine with GM-CSF doubled the number of influenza-specific IgM-secreting cells (Fig. 8A) and tripled the number of influenza-specific IgG-secreting cells (Fig. 8B) relative to non-adjuvanted low dose vaccine at 14 d.p.v. Most importantly, GM-CSF increased the number of vaccine-specific IgG-secreting cells in the bone marrow 60 d.p.v by 50% compared to all non-adjuvanted groups (Fig. 8C–D). These results, combined with serological analysis, demonstrate that GM-CSF improved the
development of humoral immunity through increased antibody avidity and IgG subtype expression, systemic IgM and IgG cell activation, and maintenance of vaccine-specific IgG-secreting cells in the bone marrow.

### 3.6. T cell activation in the inguinal lymph nodes (ILN) and spleen

Protective immune responses to influenza virus infection require both antibody responses to neutralize free virus and facilitate its opsonization and virus-specific cellular responses to kill infected cells and coordinate immune responses [47]. Healthy adults expressing higher numbers of vaccine-specific CD8+ IFN-γ-secreting cells were less likely to have enhanced viral pathogenesis during the 2009 H1N1 pandemic [48]. Thus, we investigated whether GM-CSF-adjuvanted vaccination could boost the number of activated T cells in draining inguinal lymph nodes (ILN) and the numbers of systemic vaccine-specific cytokine secreting cells. GM-CSF adjuvant-containing patches tripled the frequency of activated follicular CD8+ T cells (CD8+ CXCR5+ PD-1+) as early as day 7 (Fig. 9A–E). In contrast, GM-CSF did not increase the frequency of CD4+ T follicular helper cells (CD4+, CXCR5+, PD-1+) (Suppl. Fig. 2A). These findings suggest that GM-CSF adjuvant improves protective immunity following vaccination by enhancing activation of virus-specific CD8+ cytotoxic lymphocytes (CTLs).

To determine whether inclusion of GM-CSF in MN patches leads to the increased generation of vaccine-specific T cells, we enumerated vaccine-specific IFN-γ and IL-4 producing lymphocytes in ILN at 7 and 14 d.p.v. By day 14, the GM-CSF/low-dose vaccine group induced a 3-fold increase in the number of IFN-γ secreting lymphocytes when compared to placebo (p = 0.02), low dose (p > 0.05) and high dose vaccine groups (p = 0.03) (Fig. 9F). Mice receiving adjuvant-adjuvanted vaccine showed a 50% enhancement in the number of IL-4 secreting lymphocytes (Suppl. Fig. 2B). Thus, in addition to previous results demonstrating the increase of vaccine-specific antibodies, the use of GM-CSF
as an adjuvant in MN patches also exerted a positive effect on CTL activation and vaccine-specific IFN-γ and IL-4 secreting cell numbers, providing a range of cellular and humoral immune responses for an effective response to influenza viral infection.

3.7. Antibody response to H1N1 and H3N2 viruses

Effective influenza vaccination must demonstrate long-lasting and broadly neutralizing immune responses against commonly circulating influenza virus strains in order to reduce disease burden [49]. Breadth of immunity adds value to current seasonal influenza vaccines and it is the goal of successful universal vaccine candidates [50]. In order to measure the range of influenza-specific antibody responses elicited in animals that received the vaccine alone or in combination with GM-CSF, serum collected 3 months post-vaccination was tested for binding to various HAs within the same subtype (H1N1, group 1) or different subtype (H3N2, group 2).

With the exception of significantly higher antibody IgG titers to a homologous-like virus (anti-A/California/07/09) (Fig. 10A), the GM-CSF adjuvanted vaccine generated comparable IgG titers to unadjuvanted vaccine against homosubtypic strains (Suppl. Fig. 3A, B). Interestingly, the adjuvanted vaccine induced 3-fold increase of IgG antibodies against 3 circulating H3N2 strains; A/Texas/50/2012 (Fig. 10B), A/Victoria/210/2009 (Suppl. Fig. 3C), A/Aichi/2/1969 (Suppl. Fig. 3D) relative to non-adjuvanted vaccine. Using recombinant baculovirus proteins containing rare HA heads (H6 and H14) and common seasonal stalks (H1 and H3), it was possible to determine whether the inclusion of GM-CSF as an adjuvant increased the number of antibodies able to bind to H1 and H3 stocks. While the combination of GM-CSF with low vaccine dose did not increase the antibody response to H1 stalks compared to vaccine alone (Fig. 10C), it significantly increased the titer of antibodies binding to H3 stalks (Fig. 10D). These data suggest that the mechanism by which GM-CSF adjuvants increase antibody binding to H3N2 viruses is likely related to enhanced reactivity with H3 stalk rather than the H3 head or receptor binding domain. Thus, co-administration of GM-CSF with a licensed influenza vaccine encapsulated in MN patches generated antibodies with significant cross-reactivity, demonstrating the potential of this adjuvant to expand immune protection across various influenza subtypes.

3.8. Viral growth and immune responses to lethal challenge following MN patch vaccination with GM-CSF adjuvant

To determine the effect of skin vaccination with GM-CSF on protective immunity and identify the implicated mechanisms, mice were infected 30 days post-vaccination with 10xLD50 A/California/07/2009. Survival and weights changes were monitored for 14 days post-infection. A second infected cohort of mice was sacrificed at 4 days post-infection to determine viral titers and cytokine profiles in the lungs and assess vaccine effectiveness in accelerating viral clearance and reducing virus-induced inflammation. Although all vaccinated groups maintained their weight and survived lethal infection (data not shown), immunization with adjuvanted vaccine induced a 2.7-log reduction in viral titers when compared to mice vaccinated with placebo MN patches (p = 0.02), whereas immunization with high-dose vaccine was 42% less effective (2.5-log reduction in viral titers) (p = 0.02). Mice vaccinated with low-dose vaccine in the absence of GM-CSF had one-log decrease in titers compared to placebo infected mice (p = 0.03) (Fig. 11A).

Lung lysates taken from vaccinated mice 4 days after lethal infection and tested for eight major Th1, Th2 and inflammatory cytokines showed significant expression of IL-4, IL-2 and GM-CSF. These cytokines were significantly elevated following lethal infection of mice that received adjuvanted vaccine when compared to the other vaccinated groups (Fig. 11B–D). Early expression of GM-CSF in the lungs has been shown to promote alveolar macrophage activation and recruitment in order to reduce viral replication [51,52]. These findings may explain
efficient viral clearance seen shortly after infection (Fig. 11A). In contrast, IL-2 and IL-4 play important roles in reducing CTL-mediated immunopathogenesis [53] and promoting tissue repair through activation of innate lymphoid cells, respectively [54]. These studies point to a potential role of GM-CSF in influenza vaccination by MN patch to elicit a unique signature of cytokines that promote elimination of virus from the respiratory compartment while reducing byproduct inflammation and tissue damage due to an overactive immune system.

4. Discussion

Skin immunization is an innovative route of vaccination which takes advantage of a potent network of antigen-presenting cells (APCs) and other innate immune cells in skin that can interact with naive T and B cells proximal to the site of vaccination in draining lymph nodes [55–58]. We have previously reported the importance of langerin+ dendritic cells (DCs) in modulating adaptive immune responses following cutaneous MN vaccination [59]. GM-CSF can recruit and activate APCs [60] and is a potent inducer of NF-κB expression and nitric acid synthase in epithelial DCs [61]. CD103+ langerin+ DCs have shown GM-CSF dependent activation of follicular CD4+ T helper cells that subsequently led to the expression of IFN-γ and IL-17 [62]. Thus, the presence of GM-CSF as an adjuvant is critical for enhancing the potency of vaccines with limited immunogenicity, such as the licensed

Fig. 9. MN patch vaccination with GM-CSF increased CD8+ T cell responses in inguinal lymph nodes (ILN) and vaccine-specific IFN-γ responses in the spleen. I nguinal lymph nodes and spleens were harvested from mice 7 and 14 d.p.v. and measured for activation and vaccine-specific cytokine secretion. CD8+ follicular helper cells (CD8+CDXCR5+PD-1+) were determined for each sample, and representative images for (A) placebo, (B) 1 μg HA, (C) 1 μg HA, and (D) 1 μg HA are shown. (E) Frequency of CD8+ follicular helper cells in ILN were quantified in FlowJo and plotted in GraphPad Prism 7. (F) Vaccine-specific IFN-γ were isolated from the spleen at day 7 and enumerated in ELISPOT plates following stimulation with A/Christchurch/16/2010 subunit vaccine. Values are expressed as mean ± SEM and statistics were performed with Mann-Whitney test.
subunit influenza vaccine, by promoting antigen capture and DC and T cell activation and regulation [63,64].

In this study, we investigated the role of GM-CSF to increase the efficiency of skin dendritic cells APCs and the recruitment and priming of antibody-secreting B cells and CD8+ follicular T cells in primary and secondary lymphoid tissues. Here we demonstrate stable co-incorporation of GM-CSF with influenza vaccine in MN patches that can be used to enhance the vaccine-specific humoral and cellular immune responses. We identified that ID administration of GM-CSF with H1N1 or H3N2 vaccines, improved antibody responses and protection against viral challenge [65,66]. We then identified the formulations for efficient vaccine and adjuvant co-encapsulation within MN patches and delivery in the skin. We also identified adjuvant concentrations required for cellular activation, cytokine stabilization and bioreactivity.

Importantly, we demonstrated the superiority of MN patch vaccination over ID and IM vaccination in enhancement of vaccine-specific antibody responses. Mice cutaneously immunized with H1N1 influenza vaccine and GM-CSF as adjuvant via ID and MN routes showed increased protection with lower morbidity and mortality rates following lethal infection, a phenomenon not seen in IM vaccination. These findings suggest that GM-CSF has unique properties as an adjuvant within the skin that can be exploited when developing MN patch delivery technologies.

Examination of the immune response to GM-CSF as an adjuvant showed increased activation of antigen-specific IFN-γ secreting cells following vaccination, which may explain the persistence of IgG2a antibodies 4 months post-vaccination [67]. Following lethal infection, mice vaccinated against H1N1 virus in the presence of GM-CSF effectively controlled viral growth. Importantly, GM-CSF-adjuvanted H1N1 vaccination improved serum cross-reactivity with a homosubtypic (H1N1) and heterosubtypic (H3N2) viral strains. The broadly cross-reactive antibodies elicited in the presence of adjuvants were directed against the hemagglutinin stalk region. Ultimately, influenza immunization with a trivalent or quadrivalent vaccine co-encapsulated with appropriate adjuvants such as GM-CSF in a MN patch could significantly reduce disease burden due to strain mismatch or drift within an influenza season.

The role of GM-CSF in driving more potent humoral or cellular immune responses has been debated. Previous DNA vaccination studies in rhesus macaques showed that influenza HA responses were improved by the inclusion of recombinant rhGM-CSF [68]. Intramuscular vaccination with GM-CSF encoded in a viral vector increased the frequency of APCs but decreased the antigen-specific CD8+ T cell responses in the draining lymph node [69]. In our studies, MN patch vaccination with GM-CSF increased the number of CD8+ follicular T cells in ILN, highlighting the usefulness of administering this cytokine as an adjuvant during vaccination. Our findings are in alignment with data reported by Chou et al. on co-delivery of GM-CSF with hepatitis B subunit vaccine to skin in a biodegradable hydrogel [70]. The authors demonstrated that presence of GM-CSF increased antibody response to the vaccine and vaccine-specific T cell proliferation in addition to improved activation of APCs in the draining lymph node.

**Fig. 10.** GM-CSF adjuvanted MN patch immunization improved cross-reactive neutralization responses between homologous and heterologous viral strains. Serum was collected from mice 90 days post-A/Christchurch/16/2010 vaccination with dissolving MN. IgG reactivity with monovalent subunit vaccines for (A) H1N1 A/California/07/2009 and (B) H3N2 A/Texas/50/2012 was measured with ELISA. IgG reactivity with chimeric HA proteins was measured with ELISA against (C) H1 stalks (cH6/1 Cal/09) and (D) H3 stalks (cH14/3 HK). Statistics performed in Mann-Whitney test for comparison of 1 μg HA/GM-CSF vs. 1 μg HA groups.
GIFT fusokines, engineered to have peptides from multiple cytokines, have been generated to combine the APC stimulation capabilities of GM-CSF with T cell differentiation signaler IL-4 [71]. These fusokines have shown promise in prophylactic HIV vaccination studies in guinea pigs when anchored via glycolipids to virus-like particles (VLPs) [72]. These reports and our present findings suggest that the potency of GM-CSF as an adjuvant is dependent on administration route and formulation. To our knowledge, this is the first example of the use of MN patches to deliver a bioreactive cytokine as an adjuvant that can augment effective systemic and mucosal immune responses elicited to influenza vaccine.

5. Conclusions

In this study, we repurposed a cytokine already approved for clinical use for stimulating immune cell repopulation following chemotherapy for use as an adjuvant for skin immunization [73,74]. Our preclinical studies in mice showed enhanced immunogenicity of influenza vaccine with recombinant murine GM-CSF administered with a MN patch, proposing a novel use for this cytokine as an active molecular adjuvant. Additionally, our process provides a pipeline to examine other active recombinant molecules as adjuvants and to optimize combined adjuvant doses for maximized vaccine efficacy and mucosal immunity. Lastly, these data demonstrate the usefulness of GM-CSF as an adjuvant in a single context; however, an effective adjuvant strategy may likely employ a combination of cytokines, TLR ligands or STING agonists [75,76].

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Competing financial interests

M.R.P. is an inventor of patents licensed to companies developing microneedle-based products, is a paid advisor to companies developing microneedle-based products and is a founder/shareholder of companies developing microneedle-based products (e.g., Micron Biomedical). This potential conflict of interest has been disclosed and is managed by Georgia Tech and Emory University.


Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jconrel.2018.02.033.

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