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*J Immunol* 2005; 175:3882-3891; doi: 10.4049/jimmunol.175.6.3882

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Activation of Innate Immunity, Inflammation, and Potentiation of DNA Vaccination through Mammalian Expression of the TLR5 Agonist Flagellin

Steven E. Applequist,² Erik Rollman, ‡ Mark D. Wareing, * Martin Lidén, § Björn Rozell, † Jorma Hinkula, ‡ and Hans-Gustaf Ljunggren *

Improving DNA vaccination remains a fundamental goal in vaccine research. Theoretically, this could be achieved by molecules encoded by DNA capable of activating TLRs to mimic inflammatory responses generated by infection. Therefore, we constructed an expression vector that allows mammalian cells to express the TLR5 agonist flagellin (FliC) at the cell surface. In vitro, cell lines expressing FliC stimulated production of proinflammatory cytokines and the up-regulation of costimulatory molecules on monocytes. Mice given the FliC expression vector intradermally exhibited site-specific inflammation and, in combination with vectors expressing Ags, developed dramatic increases in Ag-specific IgG as well as IgA. Surprisingly, mice also developed strong Ag-specific MHC class I-restricted cellular immunity. To determine whether vaccination using FliC vectors could elicit protective immunity to an infectious agent, mice were given dermal injections of FliC expression vector together with a vector encoding the influenza A virus nucleoprotein. This vaccination strategy elicited protective immunity to lethal influenza A virus infection. These results demonstrate that expression of DNA-encoded TLR agonists by mammalian cells greatly enhance and broaden immune responses, imposing new possibilities on DNA vaccination to infectious agents and cancer. The Journal of Immunology, 2005, 175: 3882–3891.

Delivery of naked DNA encoding Ags is able to induce adaptive immune responses (1). This vaccination method has great potential in its ability to induce focused immune responses to defined Ags of different infectious diseases and tumors. It also has benefits in its ease of preparation and stability (2). Furthermore, DNA vaccination avoids the need for in vitro growth of virulent microorganisms, purification and modifications of protein/peptide preparations, a continuous “cold chain” (2), and circumvents the impact of pre-existing immunity to the carrier organism on vaccine efficacy. However, DNA vaccination has thus far been met with limited success likely due, at least in part, to the lack of strong elicited immune responses and in specific situations undesired polarized immune responses (primarily only Ab or CTL responses) (2, 3).

For DNA vaccinations to have broad applications in both humans and animals, new approaches to delivery, adjuvant formulation, and the discovery of new Ags are needed. Eliciting stronger immune responses as well as inducing both humoral and cellular immunity are critically dependent on the formulation of new adjuvants. Additionally, if successfully developed, needle-free DNA vaccination methods would also reduce the transmission of infectious diseases from person to person through needle reuse, which is a serious public health problem in developing countries (4).

Activation of the innate immune system through TLRs is an effective way to prime the immune system to elicit strong adaptive immune responses. Once activated, TLR-expressing cells can activate multiple arms of the immune system, including antimicrobial effector molecules, type I and type II IFNs, cytokines, chemokines, costimulatory molecules, and effective T and B cell priming by APCs (5).

The observation that flagellin, a TLR5 agonist (6), is a polypeptide has opened up the possibility that eukaryotic cells may be able to produce this molecule. Phase 1 flagellin from Salmonella (called FliC) is a monomeric subunit protein, which polymerizes to form bacterial flagella. It has been extensively studied, and the regions and residues of flagellin that are required for TLR5 interaction have been defined (7, 8). FliC activates proinflammatory cytokine production and polymorphonuclear granulocyte recruitment in lung (9) and intestinal epithelia (10, 11). It activates mouse macrophages to produce inflammatory mediators (12), human monocytes to produce TNF-α (13), as well as induce human monocyte-derived dendritic cells (DCs) (3) to mature and up-regulate costimulatory molecules (14) and produce IFN-γ, IL-10, IL-6, TNF-α, and IL-12p70 but low IL-5 and IL-13 (15). This suggests that FliC can induce Th1-like responses. However, in certain situations, the induction of Th2-like responses have also been observed (16, 17). Taken together, these observations demonstrate that FliC induces inflammatory immune responses typical of TLR activation.

With these properties of FliC in mind, we hypothesized that DNA-encoded TLR agonists with potent immune stimulatory capacity could function as novel molecular adjuvants in conjunction with DNA vaccination. We demonstrate in the present study, using

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Received for publication January 14, 2005. Accepted for publication June 23, 2005.

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1 This work was supported by the Swedish Foundation for Strategic Research, the Swedish Cancer Society, the Swedish Research Council, and the Wallenberg Consortium North.

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3 Abbreviations used in this paper: DC, dendritic cell; g.g., gene-gun; HA, hemagglutinin; NP, nuclear protein; ORF, open reading frame; Endo H, endoglycosidase H.
flagellin as a model DNA-encoded TLR agonist, the activation of innate immunity, inflammation, and potentiation of DNA vaccination through mammalian expression of flagellin.

Materials and Methods

Cell culture and cell lines

Cell lines were grown in RPMI 1640 medium (293FT) or DMEM (HeLa) (Invitrogen Life Technologies) with the addition of 10% heat-inactivated FCS (Integro), 2 mM L-glutamine (Invitrogen Life Technologies), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen Life Technologies), 50 μM 2-ME (Sigma-Aldrich), and 100 mM HEPES (Invitrogen Life Technologies). 293FT was obtained from Invitrogen Life Technologies and HeLa from American Type Culture Collection.

Cloning of fliC and expression vector assembly

A Salmonella enterica serovar Typhimurium (strain ATCC no. 14028) culture was used to clone fliC (phase-1 flagellin, serotype H 1) into a vector. Fifty micro liters of a liquid culture grown in Luria Broth were mixed with 50 μl of 10 mM Tris (pH 8)–1 mM EDTA, heated to 95°C for 15 min, and centrifuged at high speed. Two microliters of the supernatant were subjected to PCR using 1.5 mM dNTPs (Invitrogen Life Technologies), 2 μM MgCl2, 1 × PCR buffer (Invitrogen Life Technologies), 200 μM of Taq DNA polymerase (Invitrogen Life Technologies), and 20 μM concentrations of each primer in a total volume of 50 μl. The fliC primer pairs used were as follows: forward primer (fliC 5'-BglII), 5'-GGAAAGATCTAGGCACAGAGTCTATAAAACACGATTTGAGAGAGGAC-3' and reverse primer (fliC 3'-SmaI), 5'-TCTCAGCTGCTTGAAGAGAGGACC-3'. Amplified products were captured using pCR2.1 (Invitrogen Life Technologies), and plasmids containing an insert of the appropriate length were sequenced. The fliC ORF was identified by GenBank accession no. D13689. The fliC ORF was removed with BglII, SmaI digestion, and cloned into the mammalian surface display plasmid pDisplay (Invitrogen Life Technologies). The resulting plasmid was subjected to site directed mutagenesis (QuickChange as described by the manufacturer (Stratagene)) to eliminate the naturally occurring stop codon (at 1706–1708), as well as modify residues between the stop codon and the mutagenic thrombocytes and resuspended in RPMI 1640 medium supplemented with 2 mM L-glutamine. A total of 5 × 105 PBMCs/ml/well was plated in a 24-well plate (BD Falcon) and incubated for 2 h at 37°C, 5% CO2. Nonadherent cells were removed by gentle pipetting, and 1 ml of RPMI 1640 medium containing 5% FCS, 100 mM HEPES, 2 mM L-glutamine, penicillin/streptomycin, 50 μM 2 ME was added to the remaining cells and incubated overnight. Transfected, adherent 293FT, or HeLa cells were harvested by gentle pipetting, stained with trypan blue, and counted. Alternatively, adherence-enriched PBMCs (monocytes) were activated with LPS (Sigma-Aldrich), rFliC polypeptide (Alexis Biochemicals), or mixed with either 5 × 105 transfected 293FT or transfected HeLa cells and allowed to incubate for 18 h. Total cells were then stained and subjected to flow cytometric analysis.

Flow cytometric analysis

pFliC-Tm expressed at the cell surface of 293FT and HeLa cells was detected using HA1.1 (at 1/100) followed by FITC-rat anti-mouse IgG1 (at 1/100; BD Pharmingen) or rabbit anti-FliC (at 1/100; Statens Serum Institu) followed by FITC-swine anti-rabbit Ig (at 1/100; DakoCytomation). Briefly, cells were resuspended, stained 30 min on ice, and washed. Cells were stained with secondary Abs if necessary. Cells were kept on ice until analysis using a four-color FACScan (BD Biosciences). Data were processed using CellQuest (BD Biosciences). Monocytes were stained with FITC-mouse IgG1, anti-human CD80 (at 1/100); PE-mouse IgG1, anti-CD25 (at 1/100); and PerCP-mouse IgG2a anti-HLA-DR (at 1/100; all BD Pharmingen) for 30 min on ice and washed. Monocytes CD80 and CD25 levels studied were gated on HLA-DR+ populations.

Skin injection experiments and histology

C57BL/6J mice (aged 8–12 wk) from Charles River Laboratories were housed under standard specific-pathogen-free conditions (Swedish Institute for Infectious Disease Control). All procedures were reviewed, approved, and performed under both institutional and national guidelines. Mice were vaccinated using the Helios gene-gun (g.g.) system as described by the manufacturer (Bio-Rad). Briefly, 0.5 mg of gold particles was coated with 0.5 μg of each plasmid DNA and used to coat the delivery tube. Endotoxin/μg DNA were all ≤5.5 × 10−7 EU/μg DNA. Endotoxin units were determined using the Limulus amebocyte lysate kit (BioWhittaker). The binding of DNA to gold beads was controlled for by eluting plasmids from bead-coated delivery tubes followed by transformation, isolation, and DNA restriction enzyme analysis (data not shown). The abdominal skin of mice was shaved, and the spacer of the g.g. held directly against the abdon- men and discharged at a helium pressure of 500 psi for OVA and nuclear

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Table I. Summary of amino acid changes made to eliminate ASN-linked glycosylation signal sequences

<table>
<thead>
<tr>
<th>Predicted Asn-Linked Glycosylation Site</th>
<th>Predicted Asn-Linked Glycosylation Sequence</th>
<th>Amino Acid Change</th>
<th>Organism and GenBank Accession No. on Which the Sequence Change Is Based</th>
</tr>
</thead>
<tbody>
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<td>19* NKSQ</td>
<td>19 N/V</td>
<td>Helicobacter felis no. Y11602</td>
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</tr>
<tr>
<td>101 NSTN</td>
<td>101 N/T</td>
<td>Escherichia coli no. AF169323</td>
<td></td>
</tr>
<tr>
<td>200 NSTF</td>
<td>200 N/D</td>
<td>Salmonella choleraesuis no. AF159459</td>
<td></td>
</tr>
<tr>
<td>346 NTTK</td>
<td>346 N/D</td>
<td>Salmonella typhimurium no. M11332</td>
<td></td>
</tr>
<tr>
<td>446 NLSI</td>
<td>446 T/N</td>
<td>Salmonella enterica no. U06206</td>
<td></td>
</tr>
<tr>
<td>465 NMSR</td>
<td>468 R/K</td>
<td>Salmonella enterica no. U06205</td>
<td></td>
</tr>
</tbody>
</table>

* Amino acid position.
protein (NP) experiments as indicated in their respective figures. For histopathological studies, n = 2/group. Mice were photographed, then skin complete biopsies with abdominal wall from the site of injection was taken. Samples were preserved in neutral-buffered 4% formalin followed by 70% EtOH. Samples were trimmed to include regions adjacent to the injection site, embedded in paraffin, sectioned, and stained with H&E. Multiple, consecutive sections were taken from the region encompassing the central injection site (as defined by the presence of gold beads) as well as adjacent un.injected tissue. Samples were analyzed by light microscopy and photographed at ×10, ×20, and ×40 magnifications.

ELISA
ELISAs were conducted on cell culture supernatants and mouse sera. To test for cytokines, supernatants were collected from monocyte cultures after stimulation and frozen at −20°C. Samples were tested in duplicate for the presence of TNF-α (Quantikine; R&D Systems). Mouse anti-OVA Abs was detected as follows. Ninety-six-well ELISA plates (Costar assay plate; Costar) were coated with 10 μg/ml purified OVA (Sigma-Aldrich) in PBS overnight at 4°C. Plates were washed (PBS/0.1% Tween 20) and blocked with PBS/1% FCS for 1 h at room temperature. Serum samples were diluted 1/2 beginning at 1/1000 for all IgG tests and 1/10 for IgA tests in PBS/1% FCS and added to OVA-coated plates in duplicate followed by incubation overnight at 4°C. All dilutions were titrated to extinction. Wells were washed, and either HRP goat anti-mouse IgG (Fc) (at 1:5000; Pierce), HRP donkey anti-mouse IgG1 (at 1:3000; Caltag Laboratories), HRP-rabbit anti-mouse IgG2a (at 1:2000; Caltag Laboratories), or HRP goat anti-mouse IgG2c (at 1:4000; Southern Biotechnology Associates), or HRP goat anti-mouse IgA (at 1:1000; Sigma-Aldrich) was added to the wells and incubated at room temperature for 2 h. Wells were washed, and 100 μl of Enhanced K-Blue Tetrabenzidine Substrate (Neogen) were added. Plates with identical secondary detections were incubated for identical times, and substrate reactions were stopped by the addition of 1 M HCl. Plates were analyzed using a Labsystems Genesis ELISA plate reader (LabSystems).

T cell analysis
For analysis of responses to OVA, fresh mouse PBMCs were pooled from mice of each group and analyzed 21 days after primary immunization and 31 days after boost one. OVA responses were also studied from individual splenocyte preparations at the final time point. Analysis of response to NP was done 28 days after boosting. ELISPOT analysis was performed as previously described (18) using a commercial IFN-γ, IL-4, and IL-2 ELISPOT kit (Mabtech). Ag restimulation was performed using the Ags described below. Briefly, PBMCs or splenocytes were purified using Ficoll gradient (Amersham Biosciences) duplicates (PBMCs) or triplicates (splenocytes) of 200,000 cells/well into 96-well ELISPOT plates (Millipore MAIPN4510). In vitro restimulation was performed using whole OVA (5 μM; Sigma-Aldrich), the H-2Kb OVA-derived peptide SIINFEKL (5 μM; Thermo Hybaid), the HIV-1 envelope protein gp160 (1 μg/well; Protein Sciences), the H-2Kb immunodominant LCMV peptide gp33 (KAVYNAYATM) (5 μM; Thermo Hybaid), the Influenza A/Puerto Rico/8/34 (H1N1) H-2Kb-based NP366–374 peptide ASNENMETH (5 μM; Thermo Hybaid), or the I-A2-based NP576–583 peptide ARSALILRGSVAHK (5 μM; Thermo Hybaid). Cell reactivity was confirmed by incubation with Con A. Spot-forming cells were quantified after 36 h incubation and counted by an AID ELISPOT reader (Autoimmun Diagnostika). Statistical analyses were conducted using the Student t test.

Influenza A infections titers, viral infection, and sampling
Influenza virus strain A/Puerto Rico/8/34 (A/PR/8/34 (H1N1)) was grown in Madin Darby canine kidney cells. Infectious titers were determined by plating 10% (v/v) cell homogenate dilutions on Madin Darby canine kidney cells (19). C57BL/6J mice (aged 14–20 wk at time of vaccination) were infected intranasally with 2500 PFU/mouse suspended in 30 μl of PBS under heavy anesthesia (i.p. injection with Avertin (2,2,2-tribromoethanol) (n = 20/group)). After infection, mice were monitored daily for weight loss and survival. Inflammatory cells infiltrating the airways were studied by harvesting cells using bronchoalveolar lavage via the trachea by performing three lavages with 1 ml of PBS. Viable leukocyte counts were determined by trypan blue exclusion. Cytosin preparations were performed as previously described (20) using cell suspensions containing 1.5 × 106 cells/ml in PBS, 2% FCS. Cytocentrifuge preparations were fixed for differential staining using May–Grunwald/Giemsa staining.

Results
Human expression of FltC
To express FltC on the surface of mammalian cells, vectors were constructed containing the fltC gene from S. typhimurium in the mammalian expression vector pDisplay (pDisp/fltC-Tm). The complete ORF was transferred to pcDNA3.1/Zeo (+) for use in additional experiments. 293FT cells were transfected with the pcDNA3.1/fltC-Tm (pfltC-Tm) expression vector and analyzed by Western blotting. A representation of the primary structure of the expected preprocessed polypeptide (~61 kDa) and expression of the mature polypeptide is shown in Fig. 1. A and B, respectively. Proteins of identical molecular mass were detected using both anti-HA tag (Fig. 1B) and anti-FltC Abs (Fig. 1C) but with larger than expected size at ~77 kDa and a more disperse band at ~83 kDa. Flagellin isolated from S. typhimurium is not glycosylated. However, multiple eukaryotic N-linked glycosylation sites were identified in the coding sequence of fltC. Thus, it was possible that the larger molecular mass products of FltC-Tm produced by 293FT cells were due to N-linked glycosylation. To address this, whole cytoplasmic cell lysates of pfltC-Tm-transfected 293FT cells were treated with Endo H. Upon Endo H treatment, the ~77
kDa FliC-Tm migrated at the expected size of ~61 kDa, while the migration properties of the larger ~83 kDa polypeptide did not change (Fig. 1D).

To prevent N-linked glycosylation, the coding sequence of pfliC-Tm was changed by site-directed mutagenesis to eliminate predicted N-linked glycosylation sites (called pfliC-Tm(-gly)). Changes were chosen by identifying alternative amino acid residues present at similar locations within flagellin molecules of other bacteria (Table I). Polypeptides produced by 293FT cells transfected with pfliC-Tm(-gly) were of ~66/69 kDa, respectively (Fig. 1D).

Cell surface expression of FliC

To determine whether cells transfected with the expression constructs expressed FliC-Tm at their surface, cells were stained with anti-FliC and anti-HA Abs followed by FACS analysis. 293FT cell cultures transfected with either pfliC-Tm- or pfliC-Tm(-gly) contained cells detectable with an anti-HA epitope Ab (Fig. 2) but not with an isotype-control Ab (data not shown). Mock-transfected cells (data not shown) or cells transfected with an empty vector (Fig. 2) gave background staining. Cells transfected with pfliC-Tm- or pfliC-Tm(-gly) also expressed proteins detectable by anti-FliC Abs (Fig. 2). Similar or greater percentages of cells staining positive for FliC-Tm or FliC-Tm(-gly) were detected using anti-HA and anti-FliC Abs (data not shown).

Activation of human monocytes by cells expressing FliC-Tm

Adherence-enriched human PBMCs (monocytes) produce inflammatory factors in response to recombinant S. typhimurium flagellin (13). To assess whether human cells expressing FliC-Tm on their surface can activate human monocytes, we incubated pfliC-Tm- or pfliC-Tm(-gly)-transfected 293FT cells with resting monocytes. Cultures of 293FT cells expressing FliC-Tm or FliC-Tm(-gly) were analyzed. Total cultures of transfected cells were washed with PBS, then mixed with monocytes, incubated for 18 h, and analyzed for TNF-α production and changes in surface expression of CD80 and CD25. Cultures of 293FT cells expressing FliC-Tm or FliC-Tm(-gly) induced monocytes up-regulation of CD80 and CD25 compared with controls (Fig. 3A). Changes were also seen after treatment with LPS or rFliC polypeptide (Fig. 3C). FliC-Tm- or FliC-Tm(-gly)-expressing cells also induced production of TNF-α (Fig. 3D). NF-κB activation in response to Salmonella-derived FliC (inducible of TLR activation) has been reported to occur in 293 but not in HeLa cells (21), raising the possibility that transfection of 293FT cells with FliC-Tm-expressing constructs leads to the production of undefined factors able to activate monocytes. To test this hypothesis, experiments were also performed using HeLa cells transfected
with pFlC-Tm or pFlC-Tm(-gly). These cells, mixed with monocytes, were also able to induce monocyte activation similar to 293FT-expressing FlC-Tm or FlC-Tm(-gly) but not cells transfected with the empty vector (Fig. 3, B and D). Both 293FT and HeLa cells were negative for staining by anti-CD80, anti-CD25, and anti-HLA-DR (data not shown).

Flagellin-expressing vectors induce local inflammation

To determine whether FlC-Tm-expressing vectors are capable of inducing an inflammatory response in vivo, we used the g.g. method to inject pFlC-Tm or pFlC-Tm(-gly) plasmids into mice. Gold beads were coated with pOVA together with pcDNA3.1/Zeo(+) (vector) or in combination with pFlC-Tm or pFlC-Tm(-gly). Mice were immunized, and the site of injection was photographed immediately after sacrifice at the indicated days (Fig. 4). Gross morphology of the injection sites revealed clear differences (Fig. 4A). Mice injected with pOVA+ vector showed a slight local reaction 2 days postinjection, characterized by a yellowish-brown tinge likely due to deposition of the gold particles. In contrast, mice injected with pOVA+pFlC-Tm or pOVA+pFlC-Tm(-gly) developed local tissue reactions characterized by swelling and central ulceration of the injection site. Seven days postinjection, the skin was grossly normal in all groups of mice.

In all samples, the distribution of gold particles was found in the epidermis and dermis (Fig. 4, B–D). On days 1 (data not shown) and 2 postinjection (Fig. 4, B–D), mice given pOVA+ vector developed epidermal hyperplasia, subcorneal pustule formation, increased infiltration of neutrophilic granulocytes, and an inflammatory reaction extending to, but not involving, the hypodermal fat. This inflammation resolved by day 3 (data not shown) while the superficial necrotic epidermal layers detached from the site of injection. In contrast, injection of pOVA with either of the FlC-Tm-expressing plasmids led to a more rapid and severe inflammatory reaction involving also the hypodermis, extending to and involving the superficial part of the panniculus muscle. On days 1 (data not shown) and 2 (Fig. 4, B–D), epidermal necrosis was observed in the injection site and was densely infiltrated by granulocytes. In the dermis and hypodermis, the inflammatory reaction led to the development of a panniculitis, with dense infiltrates of neutrophilic granulocytes. By day 3 (data not shown), the acute inflammatory reaction in

**FIGURE 4.** FlC-Tm expression vectors induce acute, local inflammation. Gross morphology of the site of injection and histological analysis of the site after H&E staining are shown at days 0, 2, and 7 after one injection with the indicated DNA (0.5 μg of each plasmid). A, Observations of the skin at and immediately adjacent to the site of injection. B, Magnifications of sections focusing on changes in the upper dermis and epithelial layers. C, Magnifications of identical sections focusing on changes in the upper dermis and epithelial layers. D, Smaller cropped sections from magnifications representing shaded areas from identical sections in B. Analyzed areas adjacent to sites of injection revealed no differences from normal skin (data not shown).
Flagellin-expressing vectors potentiate DNA vaccination

To determine whether FliC-Tm-expressing vectors could enhance adaptive immune responses to DNA-encoded soluble Ag (OVA) mice were immunized with pOVA+vector, pOVA+pflIC-Tm, or pOVA+pflIC-Tm(-gly) by g.g., according to the schedule illustrated in Fig. 5A. Blood was taken at the indicated days, and serum was tested for the presence of anti-OVA Abs. Anti-OVA IgG responses were undetectable at day 21 in all groups (data not shown). After one boost, increases in anti-OVA total IgG responses in pOVA+pflIC-Tm and pOVA+pflIC-Tm(-gly) vaccinated mice were seen but not in mice given pOVA+vector (Fig. 5B). After a second boost, higher anti-OVA total IgG titers were observed (Fig. 5C), including increases in anti-OVA IgG-isotypes IgG1 (Fig. 5D), IgG2b (Fig. 5E), and IgG2c (Fig. 5F), as well as IgA (Fig. 5G). IgG2c was analyzed instead of IgG2a due to the fact that C57BL/6 mice do not have the IgG2a gene (22). Corresponding Ab responses were slight or undetectable in mice receiving pOVA+vector alone. Mice receiving pflIC-Tm or pflIC-Tm(-gly) alone do not produce anti-OVA Abs (data not shown).

Lymphocytes were tested for the presence of Ag-specific T cells in peripheral blood at days 21 and 61 and in the spleens of mice at day 74 by ELISPOT. Analysis of PBMCs at day 21 failed to detect Ag-specific T cell responses in all groups (data not shown). However, analysis of PBMCs at day 61 revealed the presence of IFN-γ-producing T cells responding to the H-2Kβ-restricted OVA peptide SIINFEKL (residues 257–264) in mice that had received pOVA+pflIC-Tm or pOVA+pflIC-Tm(-gly) but not in mice that had received pOVA+Vector (Fig. 5H). Spleens from mice at day 74 were tested for the presence of IFN-γ- and IL-4-producing T cells able to respond to SIINFEKL as well as whole OVA. Levels of IFN-γ-producing cells detected in response to SIINFEKL and whole OVA were significantly higher in mice vaccinated with pOVA+pflIC-Tm or pOVA+pflIC-Tm(-gly) than in mice receiving pOVA+vector alone (Fig. 5I). Significant increases in the numbers of IL-4-producing cells in response to whole OVA but not SIINFEKL peptide were also seen (Fig. 5J). T cells from all day 74 (n = 5–6/group). H. IFN-γ ELISPOT analysis of pooled peripheral blood T cell responses to SIINFEKL peptide at day 61 (n = 6/group). IFN-γ (I) and IL-4 (J) ELISPOT analysis of splenic T cell responses to SIINFEKL and whole OVA polypeptide at day 74 (n = 5–6/group). The sampling day is indicated in each panel. The concentration of OVA-specific Abs in serum samples are expressed as the reciprocal of the last dilution of samples giving an OD equal to, or higher than, the mean + 3 SDs (IgG) or 2 SDs (IgA) (the determined cutoff value for the assay) of the values of preimmunization serum samples. Absorbance values equal to or above the cutoff value were considered positive. IgA responses seen are from whole sera. ELISPOT data is expressed as the calculated geometric mean of the Ag-stimulated cells minus unstimulated cells. The cutoff for a given Ag was calculated as the group geometric mean of naive animals + 2 SDs. The error bars represent 95% confidence intervals calculated from the geometric mean titers or groupwise geometric mean SFC. * and **, Significant difference of the response relative to pOVA immunizations without FliC-Tm-expressing vectors defined as p < 0.05 and p < 0.01, respectively.
Flagellin-expressing vectors protect against infectious disease

To determine whether vaccination using a FliC-Tm-expressing plasmid elicits protective immunity to an infectious agent, we challenged mice with influenza A virus after DNA vaccination. Mice were vaccinated with a plasmid vector encoding the intracellular influenza A Ag nucleoprotein (called pNP) shown to elicit protective immunity against influenza A/PR/8/34 (H1N1) viral challenge after i.m. DNA vaccination but not by g.g. vaccination (23–25). Vaccinations were conducted using pNP by i.m. delivery or pNP+vector, pNP+pFliC-Tm, or pFliC-Tm+vector by g.g. delivery as indicated in Fig. 6A. Groups of mice immunized i.m. with pNP or by g.g. with pNP+pFliC-Tm plasmid had significant increases in cells producing IFN-γ in response to NP class I- and class II-specific peptides relative to mice vaccinated with pNP+vector by g.g. (Fig. 6B). Mice not given pNP did not have IFN-γ responses to these (Fig. 6B) or control peptides (data not shown). The results observed in Fig. 6B were also confirmed by intracellular cytokine staining (data not shown). Six days after infection, mice immunized i.m. with pNP or by g.g. with pNP+pFliC-Tm had significant decreases in the numbers of lung infiltrating leukocytes (Fig. 6C), monocyte/macrophages (Fig. 6D), and neutrophils (Fig. 6F) relative to mice given pNP+vector or pFliC-Tm+vector by g.g. as well as naive mice. When parameters of health were studied, mice vaccinated by g.g. with pNP+pFliC-Tm were protected significantly against weight loss (Fig. 6G) and mortality (Fig. 6H) compared with mice given g.g. vaccination with pNP+vector or with pFliC-Tm+vector. Control experiments confirmed previous observations that i.m. delivery of pNP confers protection against weight loss and mortality (26, 27) (Fig. 6, G and H).

**FIGURE 6.** FliC-Tm expression vectors used in DNA vaccination elicit protective immunity. A, Immunization, sampling, and infection timeline. B, ELISPOT analysis of splenic T cell responses to class I and class II NP peptides after vaccination. Error bars represent 95% confidence intervals. *, A significant increase in the numbers of IFN-γ producing cells relative to the pNP+vector group vaccinated by g.g. (p < 0.01) (n = 4/group). C–F, Total numbers of lung infiltrating cells and subtypes from bronchoalveolar lavage samples determined at day 6 after influenza A infection (n = 4/group): total leukocytes (C), monocytes and macrophages (D), lymphocytes (E), and neutrophils (F). Data are presented as the mean percentage of total cells ± SE. *, Significant decreases in the numbers of indicated cell type relative to the pNP+vector group vaccinated by g.g. (p < 0.05). G, Loss of total body weight over time after infection. Average group body weight at day of infection was taken as 100%. Individual total body weights were measured daily for 21 days postinfection and presented as means ± SE. n = 10–12/group at the beginning of the experiment. ***, Significant difference in weight of pNP+vector group vaccinated by g.g. relative to all other groups and is defined as p < 0.001 and is dependent on the sample size due to survival. Mice losing >30% of their total body weight were removed from the study for ethical reasons, euthanized, and counted as deceased. Statistical analysis could not be performed after day 8 due to insufficient sample size in the pNP+vector group vaccinated by g.g. group. H, Survival rate of immunized mice infected with influenza plotted as a function of time and means. Comparison of the survival rate after immunization with the indicated plasmids by either i.m. or g.g. route (n = 10–12/group). End points were monitored daily for 21 days postinfection. ***, Significant difference in survival of pNP+pFliC-Tm mice vaccinated by g.g. and pNP mice vaccinated by i.m. injection relative to all other groups (p < 0.001) (two-tailed Fisher’s exact test).
**Discussion**

Based on the hypothesis that DNA-encoded TLR agonists would improve DNA vaccination, we constructed an eukaryotic expression vector able to express the TLR5 agonist flagellin. The expression of the FliC-Tm polypeptide by human cells led to the production of molecules of identical molecular mass that were detected by both anti-HA and anti-FliC Abs, suggesting that they are the same molecule. However, the two polypeptides were larger than expected, but only the $\approx 83$ kDa polypeptide was found to be Endo H resistant, indicating that it had advanced glycosylation modifications indicative of proteins, which have left the endoplasmic reticulum and have been transported toward the surface. Disruption of the potential N-linked glycosylation sites led to the production of FliC-Tm polypeptides ($\approx 66/69$ kDa) that migrated similarly to the unmodified native molecule. One of which still contained residual Endo H resistance ($\approx 69$ kDa). However, regardless of glycosylation, both versions of FliC-Tm appeared to be expressed on the surface of transfected cells to a similar degree. In addition to these FliC-Tm modifications, we observed a low degree of surface expression of FliC-Tm and FliC-Tm(-gly) in Fig. 2. This could be due to a variety of factors related to the difficulty of expressing a bacterial protein on the surface of eukaryotic cells and/or the stability of molecules once they reach the cell surface. Taken together, these results highlight possible difficulties in the eukaryotic production of desired polypeptides from microorganisms. It remains to be seen if posttranslational modifications and expression levels of other TLR polypeptide agonists produced in eukaryotic systems affects their ability to activate innate immunity.

Human monocytes incubated with cells expressing FliC-Tm responded by producing inflammatory cytokines, up-regulating costimulatory molecules and cytokine receptors, which are all hallmarks of monocyte encounter with a pathogen-associated molecular pattern molecules such as LPS. Both reduced glycosylated and glycosylated versions of FliC-Tm effectively activated monocytes showing that this modification did not affect its ability to induce activation. It also suggests that the residues glycosylated in the native version of FliC-Tm do not affect TLR5 interactions in any major way.

To test the ability of FliC-Tm-expressing vectors to effect immune responses in vivo, we injected mice with a vector-expressing OVA with and without FliC-Tm-expressing vectors. We used the g.g. vaccination method for a number of different reasons. First, its demonstrated reproducibility in inducing immune responses after delivery of plasmid vectors to skin (28), a target tissue that serves as a major immunological site. Second, this method directly targets the skin where TLR5 transcripts (29, 30), protein (31), and flagellin-sensing Langerhans cells have been observed (32). However, TLR5 transcripts are not present in muscle (33). Finally, local inflammatory responses at the site of injection could be easily assessed.

When pOVA was injected with the pFLific-Tm or pFLific-Tm(-gly) expression vectors, clear differences in the severity of inflammatory responses were seen compared with those induced by pOVA+vector. More extensive analysis of the injection sites revealed striking differences in the appearance of ulceration and the degree of subepithelial inflammation (panniculitis, hyperemia). However, the inflammatory responses were localized and transient. These inflammatory responses appear to be unique to the use of FliC-Tm as similar responses have not been observed or reported with the use of any other genetic encoded adjuvants such as IL-2, IL-12, or GM-CSF (our unpublished observations) (34). In the context of translation to human systems, it should be noted that human skin is generally thicker than mouse skin (1–2 mm vs 0.4–0.5 mm, respectively) and may effect the degree of inflammation after a g.g. challenge. Indeed, preliminary g.g. injections on the outer shoulder and upper back of nonhuman primates with 2 μg of pFliC-Tm (500 psi) resulted in only a general redness and slight swelling at days 2–3 relative to control vectors, not the severe inflammation seen in mice (data not shown). However, careful dose-response studies will need to be done to determine the amounts needed to reduce inflammation but retain adjuvanticity in both animals and humans.

Gene-gun vaccination of naive mice with plasmids encoding soluble Ags (such as pOVA) results primarily in a Th2-polarized response dominated by Ab production (35–37). However, analysis of the immune responses induced by codelivery of FliC-Tm-expressing vectors and pOVA revealed several interesting observations. After only one boost, increases in anti-OVA total IgG responses were seen in pOVA+pFlic-Tm- and pOVA+pFlic-Tm(-gly)-vaccinated mice. Yet, anti-OVA IgG was still undetectable in mice given a boost of pOVA+vector (0.5 μg) delivered by a single injection. Indeed, a second boost of pOVA+vector induced anti-OVA IgG responses. The 3-log higher titers of anti-OVA IgG and increases in all IgG isotypes when pFlic-Tm plasmids were included indicate that Flic-Tm-expressing vectors act as potent adjuvants. The appearance of IgA in the sera is also interesting, suggesting that Flic-Tm-expressing plasmids could be a useful addition to DNA vaccinations with the goal of eliciting mucosal Ab defenses.

Thus far, successful generation of CD8$^+$ responses in mice to DNA-encoded soluble OVA requires needle-dependent i.m. injection or intradermal injection at the base of the tail of large amounts of DNA (50–100 μg) (38–40). To our knowledge there is no published work demonstrating the efficient generation of OVA-specific CD8$^+$ T cells subsequent to g.g. vaccination with unmodified OVA. Surprisingly, when FliC-Tm-expressing plasmids were included in g.g. vaccinations, we were able to induce class I MHC-restricted immune responses to soluble OVA, which has previously been seen to elicit only Ab responses (37, 41, 42). It has been suggested that levels of secreted OVA may be too low to load the MHC class I presentation pathway and elicit CD8$^+$ CTL responses after g.g. vaccination (37). However, in the present study, we have vaccinated with less plasmid and fewer injections compared with other g.g. studies but were able to clearly elicit Ag-specific MHC class I-dependent T cell responses when Flic-Tm-expressing vectors were used. SIINFEKL peptide (class I-restricted Ag)-stimulated T cell responses revealed high numbers of cells that produced IFN-γ but not IL-4. However, IL-4 production was seen, albeit at lower frequency, in response to whole OVA polypeptide but not SIINFEKL peptide, indicating that Flic-Tm-expressing vectors are also able to boost multiple types of T cell immunity. IL-2 production by T cells detected by ELISPOT was also seen in splenocytes responding to whole OVA but not SIINFEKL in mice receiving pOVA+pFlic-Tm or pOVA+pFlic-Tm(-gly) but not pOVA+vector (data not shown).

It appears as if the reduced-glycosylated form of FliC-Tm is able to induce higher numbers of CTLs than glycosylated FliC-Tm. Apparently at the expense of certain Ab responses such as IgA, and IgG2c production. It is unclear how “self”-glycosylation of a foreign protein, much less a TLR agonist, can effect the outcome of immune responses to an independent Ag. However, if these trends hold up under further scrutiny using various doses of pFliC-Tm and pFliC-Tm(-gly) and various Ags, it may be advantageous to use one form compared with the other to tailor the desired immune response.

Our results using mammalian expression of protein-based TLR agonists (FliC) indicate that they could have benefits over other TLR-based approaches to improve DNA vaccination. So far, TLR-based strategies have involved the TLR9 activator CpG DNA (bacterial and viral DNA) and the TLR7/8 activators imidazoquinoline compounds/ssRNA (43). However, CpG adjuvant effects with DNA vaccination seem to be limited to needle-dependent delivery.
(35), reduce immune responses when used in trans (44), and dependence on TLR9 has recently even been called into question (45). Imiquimod is effective if delivered dermally (46) or subdermally (47), but it doesn’t eliminate polarized immune responses.

Bacteria expressing flagella containing antigenic inserts have been used in experimental systems as vaccines (48), and recombinant flagellin has also been used as an adjuvant in combination with peptide and protein Ags to induce CD4 T cell responses (49), increased Ab responses (16, 17, 50), or as a fusion protein to elicit cellular immune responses (51). However, currently, the use of recombinant flagellin polypeptides and whole bacteria are less applicable to DNA vaccination. The use of a DNA expression vector that enables mammalian cells to express flagellin has distinct advantages for vaccination efficacy such as ease of preparation, ability to remove contamination with unwanted inflammation promoting molecules, and stability.

To test the ability of FliC-Tm-expressing vectors to elicit protective immunity against an infectious agent, we vaccinated mice with an influenza A NP-Ag expressing plasmid with and without pFliC-Tm. NP is an intracellular Ag and elicits protective immunity after i.m. DNA vaccination, which is conferred by both NP-specific cytotoxic CD8+ T cells (52) and NP-specific effector CD4+ T cells (26, 27, 52) but not Abs (23, 25). Although g.g. vaccination with pNP can elicit detectable CD8+ T cells (41, 53), vaccination is unable to induce protective immune responses to viral challenge (25, 53). It may be that the numbers, type, or location of NP-specific T cells primed by conventional g.g. vaccination are not sufficient to confer protection. In the present study, we also find that g.g. vaccination with pNP+vector elicits slight CD8+ T cell responses; however, CD4+ response were negligible. In contrast, i.m. vaccination with pNP or g.g. vaccination with pNP+pFliC-Tm elicited significant increases in the numbers of IPN-γ-producing CD8+ and CD4+ T cells responding to NP. Interestingly, with our vaccination schedule, g.g. vaccination with pNP+pFliC-Tm appears to give even stronger immune responses to NP than traditional i.m. vaccination. When the lungs of infected mice were studied, we observed significant decreases in the numbers of infiltrating leukocytes, macrophages, and neutrophils in mice from groups having strong anti-NP CD8+ and CD4+ T cell responses. Decreases in neutrophil infiltration after influenza A infection in the lungs of successfully vaccinated animals has also been observed (54). Mice vaccinated i.m. with pNP or g.g. vaccinated with pNP+pFliC-Tm also clearly resisted cachexia-induced weight loss and survive challenge with a lethal dose of virus. Taken together, these results demonstrate that vaccination using Ag-encoding plasmids together with pFliC-Tm can induce immune responses, which strongly correlate with protective immunity to infectious disease.

Gene-gun delivery of DNA-encoded Ags has been shown to induce Th2-polarized immune responses that are dominant to immunostimulatory CpG motifs (35). Therefore, it is unlikely that the 13 CpG motifs found in the FliC-Tm ORF (data not shown), of which only 1 is optimal (55), contribute to the T cell responses observed. It could be that the spectrum of inflammatory factors FliC has been seen to induce in vitro (Th1) are able to “license” local or recruited APCs in vivo to initiate CD8+ T cell responses against secreted OVA (15). Indeed, cross-priming has been observed in other in vivo systems studying the effects of bacterial products (56–58) and viral infection (59). Alternatively, it has been observed that TLR agonists can induce apoptosis (60). Our observations of skin ulceration, epithelial cell layer damage, and the appearance of class I MHC-dependent responses after vaccination suggests that expression of FliC-Tm by TLR5-expressing keratinocytes in the epithelia could be inducing apoptosis and the formation of apoptotic bodies; macromolecular structures known to contribute to cross-presentation of Ags (61). In contrast to our observations, s.c. (16) or i.p. (17) immunization with FliC and Ag polypeptides appears to induce a “Th2-like” immune response. However, class I MHC-restricted immune responses induced by FliC were not studied. Regardless of the molecular mechanisms involved, our results demonstrate that the delivery of FliC-Tm expressing plasmids during DNA vaccination not only strengthens humoral immunity after fewer immunizations but also increases the breadth of the response to induce MHC class I-restricted cellular immunity, thereby eliminating polarized immune responses from DNA vaccination.

It is interesting to speculate exactly how FliC-Tm acts as an adjuvant. As TLR5-deficient mice are not generally available, we vaccinated MyD88-deficient mice to determine whether our observations are generally dependent on the TLR-system. Interestingly, we observed large decreases in Ab production to mice vaccinated with pOVA+vector alone in MyD88-deficient mice compared with C57BL/6 (data not shown).

The applications of expressing biologically active TLR agonists in mammalian cells are broad. Results presented here demonstrate they potentiate immune responses to DNA-encoded Ags and elicit protective immunity to infectious disease. Their use as molecular adjuvants may have significant advantages over the use of vectors containing a single cytokine, costimulatory molecules due to the ability of TLR agonists to induce a variety of immune responses. The use of improved molecular adjuvants applicable to needle-free vaccine delivery brings the promise of an “ideal vaccine” closer to reality (62). However, DNA-encoded TLR agonists are clearly applicable to DNA vaccination using other established DNA delivery vehicles such as virus-like particles or lipids. In addition to vaccination, expression of TLR agonists by mammalian cells may have potential as a new class of gene products able to induce the body’s own immune system to eliminate cells producing the agonist, a thought-provoking application for cancer therapy.

Acknowledgments
We thank X. Hong for technical assistance, M. Rhen, P. Berglund, and members of the H. G. Ljunggren and B. Wahren groups for stimulating discussions, and D. Fuller-Heydenburg and J. Haynes for support with g.g. equipment/methodology.

Disclosures
The authors have no financial conflict of interest.

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