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Antigen-Containing Liposomes Engrafted with Flagellin-Related Peptides Are Effective Vaccines That Can Induce Potent Antitumor Immunity and Immunotherapeutic Effect

Abdus Faham and Joseph G. Altin

The bacterial protein flagellin can trigger immune responses to infections by interacting with TLR5 on APCs, and Ag-flagellin fusion proteins can act as effective vaccines. We report that flagellin-related peptides containing a His-tag and sequence related to conserved N-motif (aa 85–111) of FltC flagellin, purportedly involved in the interaction of flagellin with TLR5, can be used to target delivery of liposomal Ag to APCs in vitro and in vivo. When engrafted onto liposomes, two flagellin-related peptides, denoted as 9Flg and 42Flg, promoted strong liposome binding to murine bone marrow-derived dendritic cells and CD11c+ splenocytes, and cell binding correlated with expression of TLR5. Liposomes engrafted with 9Flg or 42Flg induced functional MyD88-dependent maturation of dendritic cells in vivo. The vaccination of mice with 9Flg liposomes containing OVA induced OVA-specific T cell priming, increased the number of Ag-responsive IFN-γ–producing CD8+ T cells, and increased Ag-specific IgG1 and IgG2b in serum. Importantly, the vaccination of C57BL/6 mice with syngeneic B16-OVA–derived plasma membrane vesicles, engrafted with 9Flg or 42Flg, potently inhibited tumor growth/metastasis and induced complete tumor regression in the majority of mice challenged with the syngeneic B16-OVA melanoma, in the lung and s.c. tumor models. Strong antitumor responses were also seen in studies using the s.c. P815 tumor model. Therefore, vaccination with Ag-containing liposomes engrafted with 9Flg or 42Flg is a powerful strategy to exploit the innate and adaptive immune systems for the development of potent vaccines and cancer immunotherapies. *The Journal of Immunology*, 2010, 185: 1744–1754.

TLRs are expressed by APCs, such as monocytes, macrophages, and dendritic cells (DCs), and play a key role in triggering innate immunity and in initiating adaptive immune responses to pathogens (1–3). Several TLRs function as sensors of external stimuli, which can recognize specific microbial products and structures, such as pathogen-associated molecular patterns, which are conserved in pathogens (1–5). One TLR of special interest is TLR5, which recognizes the protein flagellin (6, 7), the major constituent of bacterial flagella involved in motility. TLR5 is a transmembrane protein ∼100 kDa expressed by a variety of cells, including monocytes, DCs, epithelial cells, and mast cells (8–10). Several studies showed that stimulation of TLR5 by monomeric flagellin can activate macrophages, DCs, neutrophils, and intestinal cells to produce inflammatory mediators, such as TNF-α, IL-6, IL-8, and IL-12, to an extent depending on cell type (6, 7, 10–12). The binding of flagellin to TLR5 on APCs in vitro activates the MyD88-dependent intracellular signaling pathway, leading to APC maturation and secretion of proinflammatory cytokines and chemokines (6, 13–15). Interestingly, when administered in vivo, flagellin induces a proinflammatory response similar to that of LPS; however, flagellin is much more potent than LPS, inducing responses at concentrations as low as 1 pg/ml (15, 16).

The fact that flagellin is one of the first Ags to which DCs are exposed during an early stage of bacterial infection suggests that it could play a crucial role in generating protective immunity (17). Consistent with this, a number of studies showed that flagellin has adjuvant-like properties (17, 18). Vaccination with recombinant flagellin–Ag fusions induced potent T and B cell responses, including Ag-specific CD8+ T cell and Ab responses to model Ags (19, 20), as well as to a number of disease-relevant Ags (21–23), including influenza M2e Ag (24), Plasmodium vivax merozoite surface protein-1 (25), and Mycobacterium tuberculosis Ag p27 (26). The responses induced were often much greater than those induced by vaccination of the Ag emulsified in CFA (20–26).

Also, the linking of flagellin to Ag is essential to induce detectable Ab responses, because a simple mixing of the two is not sufficient (20–26). This underscores the notion that TLR activation and Ag delivery to the same APC, or indeed to the same location/compartment at the cell surface or within the cell, may be required to elicit an effective adjuvant response.

The ability of Ag–flagellin fusion proteins to induce potent Ag-specific immunity when used as vaccines suggests that the targeting of Ag to TLR5 is an effective strategy to elicit Ag-specific immunity. However, the production of fusion proteins can be cumbersome and expensive and may lack versatility, especially for the delivery of...
multiple Ags simultaneously. In this study, we explored the potential of using relatively short flagellin-related peptides (pFlgs) to manipulate immunity by targeting Ag-containing liposomes and plasma membrane vesicles to TLR5 on APCs. Using the chelator lipid 3-(nitrotriacetic acid)-diethytriacylamine (NTA3-DTDA) (27, 28), we engaged Ag-containing liposomes and tumor cell-derived plasma membrane vesicles (PMVs) with synthetic His-tagged peptides that contain a sequence derived from flagellin and explored the ability of these to enhance immunity when used as vaccines. The results show that peptides having a sequence related to conserved regions in bacterial flagellin can be used to elicit dramatic Ag-specific and antitumor responses in mouse tumor models.

Materials and Methods

Reagents

The phospholipids 1,2-distearoyl-phosphatidylcholine, 1,2-palmitoyl-oleoyl-sn-glycero-3-phosphatidylcholine (POPC), and cholesterol were obtained from Sigma-Aldrich (Castle Hill, New South Wales, Australia). NTA3-DTDA was produced in the Research School of Chemistry (Australian National University [ANU]), as described previously (27, 29). Distearoyl-phosphatidylethanolamine (polyethylene glycol)-750 was obtained from Avanti Polar Lipids (Alabaster, AL). The fluorescent lipid Oregon Green-488–(1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine) (OG488-DHPE) was purchased from Invitrogen (Eugene, OR). 1,2-palmitoyl-oleoyl-sn-glycero-3-phosphatidylethanolamine was reacted with Alexa Fluor-647 carboxylic acid succinimidyl ester (Invitrogen) and purified to produce Alexa Fluor-647–1,2-palmitoyl-oleoyl-sn-glycero-3-phosphatidylethanolamine (AF647–DOPE), as previously described (30). Paraformaldehyde was from BDH Chemicals (Kilsyth, Victoria, Australia). RPMI 1640 (Invitrogen) medium was obtained from the Media Unit, John Curtin School of Medical Research (ANU).

Cells

Day-6 bone marrow-derived DCs (BMDCs) from C57BL/6 mice were generated as previously described (31, 32). BMDCs were cultured in RPMI 1640 medium containing 10% FCS, 2 mM l-glutamine, 50 μM 2-ME, 0.1 mg/ml penicillin-streptomycin-neomycin (referred to as complete medium), plus 10 ng/ml recombinant murine GM-CSF (PeproTech, Rocky Hill, NJ). Splenic semiadherent cells, hereafter referred to as splenic DCs (SpDCs), were prepared from RBC-depleted splenocytes by the method of plastic adherence, as described (32). B16-OVA melanoma (C57BL/6 (H-2b)) (a gift from Dr. Hilary Warren (The Canberra Hospital, Garran, Australian Capital Territory). HepG2 cells (human hepatocellular carcinoma) were obtained from Prof. Chris Parish (Division of Immunology and Genetics, John Curtin School of Medical Research). All other cell lines were prepared, respectively, by Dr. Mark Hulett and Prof. Ian Young (Division of Molecular Bioscience, John Curtin School of Medical Research). Human embryonic kidney HEK-293 and mouse P815 mammary mastocytoma [DBA/2J (H-2d)] cells were provided by Dr. Hilary Warren (The Canberra Hospital, Garran, Australian Capital Territory). HepG2 cells (human hepatocellular carcinoma) were obtained from Prof. Chris Parish (Division of Immunology and Genetics, John Curtin School of Medical Research). All cell lines were cultured in RPMI 1640 medium with 10% newborn calf serum (plus 0.5 mg/ml genitin for B16-OVA cells), except for CHO cells, which were cultured in F15 medium (Invitrogen) with 10% newborn calf serum.

Peptides

pFlgs for engulfment onto liposomes contained a 12His-tag (to enable engulfment onto liposomes) and a GS repeated spacer of length indicated (to reduce steric hindrance), followed by an amino acid sequence to the indicated region conserved in bacterial FlhC flagellin (Table I). A 12His peptide was used as a nontargeted (control) peptide, because it is known to bind strongly to Ni-NTA-DTDA and to reduce nonspecific binding of Ni-NTA-DTDA liposomes to cells. All peptides were synthesized and HPLC purified at the Biomolecular Resource Facility, John Curtin School of Medical Research, ANU. Stock solutions of the peptides were prepared in distilled water, stored at −20°C, and thawed and vortexed immediately before use.

Preparation of stealth liposomes and plasma membrane vesicles

Stealth liposomes (hereafter referred to as liposomes) were prepared as described (28, 32). Briefly, distearyl-phosphatidylcholine, cholesterol, distearyl-phosphatidylethanolamine (polyethylene glycol)-750, NTA3-DTDA, and tracer lipid OG488-DHPE dissolved in ethanol were mixed (molar ratio 46.5:46.5:5:1:1), dried under a stream of nitrogen gas, and rehydrated in 1 ml PBS (2 mM final lipid) containing Ni2+ (60 μM). Liposomes were produced by sonication (two 25-s bursts at maximum amplitude) in a TOSCO 100W Ultrasonic Disintegrator (Measuring and Scientific, London, U.K.). ZetaSizer measurements indicated that liposomes prepared in this way had an average diameter ∼130 nm and bore a near-neutral surface charge (ζ potential ∼5 mV). Where indicated, the tracer lipid AF647–DOPE was used instead of OG488–DHPE. For immunological experiments, the tracer lipid was omitted, and OVA was encapsulated into the NTA3-DTDA liposomes to give ∼200 μg OVA/mg total lipid, after removal of unencapsulated OVA by gel filtration, as previously described (28, 32). PMVs from cultured cells (B16-OVA, B16, or P815) were prepared as described (32). Cells were washed twice with PBS and suspended in ice-cold PBS (1 × 107 cells/ml) and lyzed by brief sonication (two 15-s bursts) at 4°C. The lysate was centrifuged (1000 × g) in 1 ml Eppendorf tubes at 4°C for 10 min; the supernatant was removed from the pelleted debris and centrifuged (15,000 × g) again for 30 min at 4°C. The supernatant was discarded, and the pellet (enriched in PMVs) was suspended in PBS and resonicated. Typically, 1 × 107 cell-equivalents of PMVs were suspended in 1 ml PBS. Stock PMVs were stored at −20°C and briefly resonicated before use in experiments. PMVs were incorporated with Ni-NTA3-DTDA and tracer lipid (OG488–DHPE) by mixing with Ni-NTA3-DTDA plus POPC in PBS (final concentration 5 × 105 cell-equivalents PMVs/ml containing 12.5 μM Ni-NTA3-DTDA plus 37.5 μM NISO4 and 37.5 μM POPC) and sonicating (two 20-s bursts at 4°C) to promote Ni-NTA3-DTDA incorporation. ZetaSizer measurements indicated that the resulting PMVs had a diameter ∼140 nm and bore a near-neutral surface charge.

PMVs and liposomes were engulfed by mixing with the indicated His-tagged peptide dissolved in H2O with a suspension of NTA3-DTDA–containing liposomes or PMVs and incubating for 30 min at room temperature. The amount of peptide used for engulfment onto liposomes or PMVs was based on a theoretical calculation of the amount of peptide required to be equimolar with anchoring moiety Ni-NTA3-DTDA and an empirical titration to give the optimal binding of the liposomes to mouse BMDCs. Where indicated, the same amount of NTA3-DTDA lipid (∼1.5 μg) and approximately the same amount of peptide (∼3–4.5 μg, depending on the m.w. of the peptide being used) were used for all vaccination conditions for each experiment.

In vitro binding of liposomes to cells and flow cytometry

To assess the binding of pFlg-engrafted liposomes to cells, the cells (BMDCs, SpDCs, and splenocytes, at 1 × 106, 0.5 × 106, and 2 × 105 cells/well, respectively) were suspended in 50% FCS in PBS (50% FCS-PBS) and then added to pre-engrafted liposomes in a 96-well polystyrene V-bottom Serocycler plate (Costar, Cambridge, MA) and incubated for 1 h (at 37°C or on ice, as indicated) with frequent gentle vortexing of the plate (32). Where indicated after this incubation, the cells were washed and blocked for 15 min on ice with mouse IgG before incubating with a fluorochrome-conjugated mAb to the indicated cell surface marker. Cells were then washed thrice in PBS, fixed with 2% paraformaldehyde in PBS, and analyzed for fluorescence by flow cytometry. Cell fluorescence was quantified using a flow cytometer (BD Biosciences LSRII; BD Biosciences, San Jose, CA). Typically, 10,000 or 30,000 cells were counted; dead cells were excluded from the analysis by gating based on the forward scatter versus side scatter. Where indicated, quadrant gates for mAb staining and liposome binding were set to contain >98% of all gated livemock-stained cells (as control) and >98% of all gated live cells stained with 12His liposomes (as control), respectively. For detection of intracellular IFN-γ (see below), ∼200,000 cells were counted for each condition.

Measurement of cell proliferation

Pooled splenocytes from each group of vaccinated mice were incubated with PBS, soluble OVA (100 μg/ml), or pFlg-OVA liposomes for 3 h at 37°C. The cells were washed and incubated in a 96-well flat-bottom plate in complete RPMI 1640 medium supplemented with IL-2 (50 IU/ml) for measurement of cell proliferation using a standard [3H]thymidine incorporation assay, as described (32).
**Intracellular staining for IFN-γ and measurement of OVA-specific Ab production**

Splenocytes (1 × 10⁶ cells) from the spleens of each group of mice were stimulated for 2 h with 1 µg/ml SIINFEKL; a 1:1000 dilution of BD GolgiStop (BD Biosciences) was added, and the cells were incubated for an additional 3 h before staining with anti-CD8-PE (clone 53-6.7; BD Pharmingen, San Diego, CA) and intracellularly with anti–IFN-γ-α/β-Fluor-647 (clone XMG1.2; BD Pharmingen), as described (32). Flow cytometric data were acquired with a BD Biosciences LSR II and analyzed using FlowJo software (Tree Star, Ashland, OR). CD⁸⁺ cells were gated from live cells to give the IFN-γ⁺ CD⁸⁺ cells in a two-color dot plot; for each condition, the background was determined by subtracting the value obtained by adding PBS instead of peptide.

Ab production in response to vaccinations was analyzed in the sera of mice by indirect sandwich ELISA, exactly as described (32). Data are expressed as Ab concentration calculated from standard curves generated with OVA-specific IgG1, IgG2a, and IgG2b mAb standards (BD Pharmingen) using KC4 V3.0 Analysis Software.

**Vaccination of mice, tumor challenge, and measurement of tumor size**

For vaccination, typically 200 µl of the indicated vaccine preparation was administered i.v. to mice by tail vein injection. The preparations of OVA-bearing liposomes contained ~10 µg OVA and 50 µg total lipid; vaccinations with PMVs contained 3 × 10⁶ cell equivalents of the indicated PMV preparation. For the tumor-challenge experiments, mice were first injected i.v. or s.c. with the indicated tumor cells (2-5 × 10⁶ B16-OVA or P815 cells) in RPMI 1640 medium (without serum), and the mice were vaccinated as indicated. Lung metastases of B16-OVA tumor were quantified by sacrificing the mice (at day 21 after challenge), removing the lungs, and counting the number of tumor foci in the lungs visually under a dissecting microscope. The size of s.c. tumors was determined from the 494-aa mature flagellin FliC from Salmonella typhimurium, reveals the existence of at least two regions of high homology, P815 cells) in RPMI 1640 medium (without serum), and the mice were vaccinated as indicated. Lung metastases of B16-OVA tumor were quantified by sacrificing the mice (at day 21 after challenge), removing the lungs, and counting the number of tumor foci in the lungs visually under a dissecting microscope. The size of s.c. tumors was determined from the mean of two perpendicular diameters measured using calipers.

**Statistical analyses**

Data are represented as mean ± SEM. The Student t test was used for statistical analysis, and p < 0.05 was considered statistically significant.

### Results

**Design of pFlgs for targeting liposomes to APCs**

A comparison of the amino acid sequences of flagellins from different strains of bacteria, facilitated by using the National Center for Biotechnology Information’s Conserved Domain Protein Database, reveals the existence of at least two regions of high homology. The representative flagellin sequence used in this study is the 494-aa mature flagellin FltC from Salmonella typhimurium (GenBank accession no. D13689; hereafter referred to as flagellin). The high-homology regions are defined by aa 79–117 (in N region) and aa 420–475 (in C region) (33, 34). The three-dimensional structure, coupled with mutagenesis studies of flagellin, showed that the ability of flagellin to interact with TLR5 resides in the first 117 aa of the N-terminal region, particularly the region defined by aa 79–117, but it may require contributions from C-region aa 408–439 (33–38). Peptides spanning different regions conserved in flagellin and/or that have been implicated in binding to TLR5 can be designed (Table I), produced synthetically, and examined for their ability to interact with APCs.

pFlgs covering aa 79–117 of FltC in the conserved N-region may contain epitopes that can bind/stimulate TLR5 (34–39). Therefore, two peptides overlapping by 4 aa were designed and denoted 41Flg (79–101) and 42Flg (98–117); two peptides denoted 9Flg (85–111) and 45Flg (98–111) were designed to cover, respectively, the central and N- parts of the conserved N-region (Table I). Two other peptides covering the conserved C-region of flagellin were also produced: 10Flg (405–425) and 11Flg (408–438) (Table I). The His-tag for these was designed to be on the C-terminal end of the peptide sequence to avoid possible steric hindrance, as judged by the orientation of this region in the three-dimensional structure of flagellin and the predicted site of interaction with TLR5 (39).

**pFlgs promote binding of liposomes to DCs**

The ability of pFlg to promote binding of liposomes to APCs was explored by incubating (1 h, 37 °C) tracer-containing NTA₂-DTDA liposomes engrafted with 12His peptide (as control) or a pFlg peptide with day-6 BMDCs before washing, staining the cells with APC-conjugated CD11c mAb, and analysis by flow cytometry. The results indicated that BMDCs incubated with 12His liposomes exhibited a mean fluorescence intensity (MFI) ~2-fold above background (data not shown). Preliminary experiments also indicated that 5–15% of all BMDCs bound 9Flg and 42Flg liposomes (data not shown). Additional studies showed that although 1.00 ± 0.05% of BMDCs that bound 12His liposomes were double positives (bind liposomes and were CD11c⁺), 9.6 ± 1.0% and 6.4 ± 0.6% of BMDCs bound 9Flg and 42Flg liposomes, respectively, and were double positives (Fig. 1A). The MFI of the CD11c⁺ BMDCs was increased from a standardized value of 1 (for 12His liposomes) to 9.3 ± 1.0-fold and 4.0 ± 0.5-fold greater for cells incubated with 9Flg and 42Flg liposomes, respectively (Fig. 1A).

Interestingly, a similar pattern of fluorescence was observed in binding assays using SpDCs instead of BMDCs (Fig. 1B). Thus, incubation of SpDCs with liposomes caused the proportion of double-positive SpDCs (CD11c⁺ SpDCs that bound liposomes) to increase from 1% (for 12His liposomes) to 5.6 ± 2.2% and 2.6 ± 0.6% for 9Flg and 42Flg liposomes, respectively; and the MFI of the CD11c⁺ cells increased 6.2 ± 1.4-fold and 3.2 ± 0.2-fold, respectively (Fig. 1B). As shown in Fig. 1C, 9Flg and 42Flg liposomes also bound to CD11b⁺ SpDCs. The proportion of double-positive cells increased from 1% (12His liposomes) to 10 ± 2.2% and 6.6 ± 1.4% for 9Flg and 42Flg liposomes, respectively; the MFI of the CD11b⁺ cells increased 9.5 ± 2.7-fold and 4.1 ± 1.3-fold, respectively (Fig. 1C). Under these conditions, 10Flg and 11Flg liposomes bound weakly and 41Flg and 45Flg liposomes bound very weakly or not at all to BMDCs and SpDCs (Supplemental Fig. 1), indicating that 9Flg and 42Flg are the most effective at promoting binding of liposomes to APCs.

**Table I. Name and amino acid sequence of pFlgs designed for engraftment onto liposomes/PMVs**

<table>
<thead>
<tr>
<th>Peptide Name</th>
<th>Amino Acid Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>9Flg-85-111</td>
<td>HHHHHHHHH--GS-GSGG-INNLNLQRVRLAQVOSanSNSSQDSLDS</td>
</tr>
<tr>
<td>10Flg-405-425</td>
<td>TENPLQKDIALAQVDTLRLSDGAVQVRFNSATG-GSGG-HHNNNHHHHHHHHH</td>
</tr>
<tr>
<td>11Flg-408-438</td>
<td>PLQKIDALAAQVDTLRLSDGLAVQVRFSANIT-GSGG-GHNNNNHHHHHHHHHH</td>
</tr>
<tr>
<td>41Flg-79-101</td>
<td>HHHHHHHH--GS-GSGG-ALNEINNLNLQRVRLAQVOSANS</td>
</tr>
<tr>
<td>42Flg-98-117</td>
<td>HHHHHHHH--GS-GSGG-SANSTNSQSLDSIQAITEQ</td>
</tr>
<tr>
<td>45Flg-98-111</td>
<td>HHHHHHHH--GS-GSGG-GSGG-GSGG-GSGG-QSANSTNSQDSLDS</td>
</tr>
</tbody>
</table>

Each pFlg contains a 12His-tag (to enable engraftment onto NTA₂-DTDA-liposomes), a spacer region (to reduce steric hindrance), and an amino acid sequence (bold text) derived from the indicated conserved region of FltC flagellin.
The cell-binding specificity of pFlg liposomes was examined by incubating the liposomes with splenocytes. Preliminary experiments indicated that ~1% of splenocytes bound 12His liposomes, but 7–10% of splenocytes bound 9Flg and 42Flg liposomes (data not shown). Further studies showed that although 0.50 ± 0.05% of splenocytes were double positives (i.e., bound liposomes and were CD11c<sup>+</sup>) following incubation with 12His liposomes, the proportion of double positives after incubation with 9Flg and 42Flg liposomes increased to 3.0 ± 0.2% and 2.5 ± 0.1%, respectively; the MFI of the CD11c<sup>+</sup> cells increased 9 ± 1-fold and 8.3 ± 1.1-fold, respectively (Fig. 2A). Similarly, the double-positive cells (liposome-positive and CD11b<sup>+</sup>) increased from 0.5% (for control cells) to 2.6 ± 0.5% and 1.3 ± 0.3% for splenocytes incubated with 9Flg and 42Flg liposomes, respectively; the MFI of the CD11b<sup>+</sup> cells increased 3.8 ± 0.4-fold and 3.1 ± 0.7-fold, respectively (Fig. 2A). Also, the proportion of double-positive cells (liposome positive and CD19<sup>+</sup>) increased from 0.5% (for control cells) to 3.6 ± 0.6% and 1.3 ± 0.1% for splenocytes incubated with 9Flg and 42Flg liposomes, respectively; the MFI of the CD19<sup>+</sup> cells increased 4.8 ± 0.5-fold and 2.5 ± 0.1-fold, respectively (Fig. 2A). The binding of liposomes engrafted with 10Flg, 11Flg, 41Flg, and 45Flg to CD11c<sup>+</sup>, CD11b<sup>+</sup>, and CD19<sup>+</sup> splenocytes was generally much lower than for liposomes engrafted with 9Flg and 42Flg (Supplementary Fig. 2). There was no significant binding of 9Flg and 42Flg liposomes to CD3<sup>+</sup> cells (data not shown).

Additional studies, in which 9Flg liposomes were administered i.v. to mice, and the spleens were removed 1 h later for analysis of tracer-containing splenocytes, showed that 9Flg liposomes had bound to 1.7 ± 0.2% of the splenocytes subsequently isolated from the spleen; the liposome-associated fluorescence of the CD11c<sup>+</sup> cells was increased 8.7 ± 1.9-fold (Fig. 2B). Interestingly, the liposome
To confirm that binding of pFlg liposomes to cells occurs through interaction with TLR5, cells were doubly stained with pFlg liposomes and TLR5 mAb. Many of the commercially available TLR5 Abs bind to a cytoplasmic region of TLR5 and, therefore, were unsuitable for correlating the binding of pFlg liposomes with surface TLR5 expression. Intracellular TLR5 mAb staining also cannot be used for the correlation because TLR5 is expressed intracellularly and at the cell surface (40, 41). The TLR5 mAb Clone 19D759.2 (Mouse IgG2a mAb; Imgenex, San Diego, CA) was particularly useful for cell-surface staining of mouse and human TLR5. This mAb stained BMDCs weakly, and it was more effective at staining cells that expressed higher levels of TLR5. Thus, the TLR5 mAb bound HEK-293 cells (widely reported to constitutively express TLR5), and it bound particularly well to the human hepatoma cell line HepG2 (42). Consistent with reports that CHO cells do not express TLR5 (43), we found no significant binding of TLR5 mAb or pFlg liposomes to CHO cells. Experiments in which CHO cells were transfected to express mTLR5 also were carried out, but only very low surface expression of TLR5 expression was achieved, which did not allow any meaningful comparisons to be made with these cells (data not shown). Therefore, we conducted binding experiments with BMDCs and the cell lines HEK-293 and HepG2 that react with the TLR5 mAb.

Preliminary experiments indicated that the binding of liposomes engrafted with the less-active peptides (i.e., 10Flg, 11Flg, 41Flg, and 45Flg) to BMDCs and HEK-293 and HepG2 cells was too low to permit a reliable correlation of the binding of the engrafted liposomes with the binding of the TLR5 mAb (data not shown). Therefore, experiments were performed to correlate the binding of 9Flg liposomes with that of TLR5 mAb to these cells. The two-color dot plots in Fig. 3A show that binding of the 9Flg liposomes correlates well with staining of TLR5 mAb in each of the three cell types. This correlation is strongest for HepG2 cells, which exhibit the strongest binding of TLR5 mAb (Fig. 3A, lower right panel). Other experiments showed a similar binding pattern when using 42Flg liposomes with HepG2 cells, but the level of binding was lower (Fig. 3B). Also, we observed only a small difference in the binding of the TLR5 mAb to cells that had been incubated or not with 9Flg liposomes, indicating no substantial interference between the binding of the 9Flg liposomes and binding of the TLR5 mAb used (data not shown). The mean percentage of double-positive cells \( \text{AF}_{647} \times \text{AF}_{488}^+ \); i.e., cells that bound

![Figure 3](http://www.jimmunol.org/)
The ability of 9Flg liposomes to induce DC maturation was first assessed using BMDCs. Day-6 BMDCs from C57BL/6 mice were incubated with complete medium, containing added PBS, 12His liposomes, 9Flg liposomes, or LPS (10 μg), before washing and culturing the cells for 24 h to assess surface expression of the activation markers MHC class II and CD86, by flow cytometry. The results showed that the incubation with 9Flg liposomes induced a significant (60–80%) increase in the relative expression of MHC class II or CD86, relative to controls treated with PBS or 12His liposomes (Supplemental Fig. 3A). Analogous studies using BMDCs from MyD88−/− mice showed no significant effect of 9Flg liposomes (Supplemental Fig. 3B). LPS, a known potent inducer of DC maturation, induced significant increases in expression of MHC class II and CD86 in these cells (Supplemental Fig. 3).

The effect of 9Flg liposomes on the maturation of DCs in vivo was examined by injecting naive C57BL/6 mice with PBS (control), LPS (10 μg in PBS, as positive control), or liposomes engrafted with peptide 12His or 9Flg and then comparing the level of expression of the activation markers CD86 and MHC class II on CD11c+ splenocytes after 48 h. Splenocytes from mice for each condition were doubly stained with a mAb to CD11c and a mAb to CD86 or MHC class II. As shown in Fig. 4A, the relative expression of CD86 and MHC class II in splenocytes from 12His liposome-injected mice was similar to that seen in the PBS controls (relative expression ∼1). As expected, CD11c+ splenocytes from mice treated with LPS exhibited a significant (∼2-fold) increase in the expression of CD86 and MHC class II (Fig. 4A). Notably, CD11c+ splenocytes from mice injected with 9Flg liposomes exhibited ∼2-fold increase in CD86 and MHC class II expression (Fig. 4A). Interestingly, however, treatment with 9Flg liposomes failed to significantly increase the expression of CD86 and MHC class II in CD11c+ splenocytes from MyD88−/− mice (Fig. 4B).

Stimulation of functional Ag presentation and Ag-specific immunity

Induction of Ag-specific T cell priming. We used liposomes containing the model Ag OVA to test whether 9Flg liposomes can be used to enhance Ag-specific responses. Different groups of five naive C57BL/6 mice were vaccinated on days 0, 7, and 14 with PBS, soluble OVA, or OVA-containing liposomes engrafted with 12His (as control), 9Flg, or 42Flg peptide. At day 21, spleens were isolated, and total splenocytes prepared from each group were pooled and incubated in vitro with soluble OVA to assess cell proliferation by measuring [3H]thymidine incorporation. Control mice, immunized with PBS, OVA alone, or 12His-OVA liposomes exhibited little, if any, proliferative response (Fig. 5A). In contrast, compared with their respective controls, splenocytes from mice vaccinated with 9Flg- and 42Flg-engrafted OVA liposomes exhibited ∼10- and ∼20-fold increases in cell proliferation, respectively (Fig. 5A).

Generation of Ag-responsive CD8+ T cells. The number of IFN-γ–producing CD8+ T cells from splenocytes of mice vaccinated with pFlg-OVA liposomes (as above) was determined by intracellular staining for IFN-γ in response to a 3-h stimulation with 1 mM SIINFEKL peptide in the presence of monensin. Two-color FACS analysis of splenocytes from mice vaccinated with PBS, OVA alone, or 12His-, 10Flg-, 11Flg-, 41Flg-, or 45Flg-OVA liposomes showed no significant IFN-γ production by CD8+ T cells (Fig. 5B). Importantly, splenocytes from mice vaccinated with OVA liposomes engrafted with 9Flg and 42Flg peptide induced a substantially higher proportion (i.e., 1.0 ± 0.1% and 0.7 ± 0.2%, respectively) of SIINFEKL-responsive IFN-γ–producing CD8+ T cells (Fig. 5B), indicating the induction of a modest CD8+ T cell response to the liposome-associated OVA Ag. Interestingly, ∼50% of the SIINFEKL-responsive IFN-γ–producing splenocytes from mice vaccinated with 9Flg liposomes (Fig. 5C) and 42Flg liposomes (data not shown) were CD8−.

Induction of Ag-specific Ab. Mice vaccinated with pFlg-OVA liposomes also were tested for Ab production. Blood samples from the mice vaccinated at day 21 were collected, and sera from the mice in each group were pooled to assess the production of OVA-specific IgG1, IgG2a, and IgG2b by ELISA. No OVA-specific Ab was detected in sera from mice vaccinated with 12His-OVA liposomes (controls), and only low titers of IgG2a (corresponding to IgG2c, in C57BL/6 mice, data not shown) could be detected in sera from mice vaccinated with 9Flg- or 42Flg-OVA liposomes.
Interestingly, however, sera from mice vaccinated with 42Flg-OVA liposomes contained a high titer of IgG1 and a moderately high titer of IgG2b; sera from 9Flg-OVA liposome-vaccinated mice had low/moderate titers of IgG1 and IgG2b (Fig. 5C).

**Effect of pFlg-PMV vaccination on tumor growth in tumor models**

*B16-OVA tumor growth and metastasis.* The potential for pFlg liposomes to be useful as vaccines also can be assessed by their ability to induce antitumor effects. Groups of five C57BL/6 mice were inoculated with B16-OVA cells (2 × 106 cells i.v. at day 0); vaccinated i.v. on days 2, 8, and 14 by PBS or OVA liposomes engrafted with 12His, 9Flg, or 42Flg; and sacrificed at day 21 to assess the response. As shown in Fig. 6A, the lungs of control mice vaccinated with PBS and 12His-OVA liposomes contained an average of ∼120 tumor foci, whereas the lungs of mice vaccinated with OVA liposomes engrafted with 9Flg, 10Flg, 11Flg, and 42Flg contained an average of 1 ± 1, 2 ± 1, 7 ± 5, and 2 ± 1 tumor foci, respectively.

The antitumor effects of vaccinating with liposomal Ag engrafted with pFlg were also explored using tumor cell-derived PMVs that contain an array of Ags associated with the tumor...
and can potentially be used to enhance antitumor immunity (28, 32). We prepared PMVs from cultured B16-OVA cells and modified these by incorporating NTA3-DTDA, followed by engraftment of pFlgs. Binding assays analogous to those in Fig. 1 revealed that the engraftment of pFlgs (particularly 9Flg and 42Flg) also mediated the binding of tracer-containing modified PMVs to BMDCs (Supplemental Fig. 4). In experiments analogous to those above, groups of five mice (C57BL/6) were inoculated with B16-OVA cells (2 × 10^5 cells i.v. at day 0); vaccinated at days 2, 8, and 14 by i.v. injection of PBS or PMVs engrafted with 12His, 9Flg, 10Flg, 11Flg, or 42Flg; and sacrificed at day 21 to assess the response. As shown in Fig. 6B, the lungs of the control mice vaccinated with PBS and 12His PMVs contained an average of ~120 tumor foci. In contrast, the lungs of mice vaccinated with 9Flg PMVs or 42Flg PMVs contained an average of 2 ± 2 and 4 ± 4 tumor foci, respectively, whereas lungs from mice vaccinated with 10Flg PMVs and 11Flg PMVs contained 43 ± 15 and 60 ± 12 tumor foci, respectively (Fig. 6B); representative images of the lungs are shown in Fig. 6C.

**Effect on established B16-OVA tumors.** Experiments also were carried out in mice bearing established s.c. B16-OVA tumors. Mice were injected s.c. on the back with 4 × 10^5 B16-OVA cells on day 0; after 9 d when tumors were palpable (~2 mm in diameter), different groups of five mice were vaccinated i.v. each week for 5 consecutive weeks with PBS or PMVs engrafted with 12His, 9Flg, 10Flg, 11Flg, or 42Flg. Tumor size was monitored every 3 d; the average tumor diameter ± SEM for each group of mice is shown *p* < 0.0001; 9Flg PMV versus 12His PMV.

**FIGURE 6.** Vaccination with 9Flg PMVs inhibits tumor growth and metastasis. C57BL/6 mice were inoculated with 2 × 10^5 B16-OVA cells injected i.v. (day 0). Different groups of five mice were vaccinated at days 2, 8, and 14 with OVA liposomes engrafted with peptide 12His, 9Flg, 10Flg, 11Flg, or 42Flg. A. At day 21, the lungs were removed, and the number of tumor foci was counted. B. Analogous experiments in which C57BL/6 mice were inoculated i.v. with 2 × 10^5 B16-OVA cells and then vaccinated at days 2, 8, and 14 with B16-OVA-derived PMVs (3 × 10^5 cell-equivalents) engrafted with peptide 12His, 9Flg, 10Flg, 11Flg, or 42Flg were also conducted. B. Mean number of tumor foci ± SEM in the lungs for each group of mice at day 21. C. Representative images of the lungs from each group of mice.

**FIGURE 7.** Vaccination with 9Flg PMVs inhibits the growth of established tumors and prolongs survival. A. Subcutaneous B16-OVA tumors were established by s.c. injection of 4 × 10^5 B16-OVA cells in the back of the mice (day 0). At day 9, when tumors were ~2 mm diameter, separate groups of five tumor-bearing mice were vaccinated with B16-OVA-derived PMVs engrafted with 12His, 9Flg, or 42Flg. Tumor size was monitored every 3 d; the average tumor diameter ± SEM for each group of mice is shown *p* < 0.0001; 9Flg PMV versus 12His PMV. B. Subcutaneous P815 tumors were established in syngeneic DBA/2J mice by injecting 4 × 10^5 P815 cells in the back of the mice (day 0). On day 15, when the tumors reached ~5 mm diameter, groups of mice were vaccinated with PBS (six mice) or 12His- or 9Flg-engrafted P815 cell-derived PMVs (seven mice in each group). The vaccinations were administered again every 5 d for a total of three consecutive vaccinations. The plot shows the survival times of the mice in each of the treatment groups.
PBS or 12His, 9Flg, or 42Flg PMVs. As shown in Fig. 7A, control mice vaccinated with PBS and 12His PMVs exhibited a progressive increase in tumor size, and all mice in these groups were sacrificed by day 30 (when tumors reached 15 mm diameter). Surprisingly, however, mice vaccinated with 9Flg PMVs and 42Flg PMVs exhibited marked abrogation of tumor growth and a dramatic regression of their tumors (Fig. 7A). Thus, all of the mice vaccinated with 9Flg PMVs, as well as 4/5 (i.e., 80%) of the 42Flg PMV-vaccinated mice, had no palpable tumor by day 30; furthermore, these mice showed no signs of tumor and were apparently tumor-free 4 mo after tumor inoculation.

**Effect on established P815 tumors.** We also examined the effect of 9Flg PMV vaccination on s.c. P815 tumors. DBA/2J mice were challenged s.c. on the back with $0.5 \times 10^6$ P815 tumor cells (day 0); when tumors reached an average of 5 mm in diameter (day 15), different groups of mice were vaccinated i.v. with syngeneic P815 cell-derived PMVs engrafted with 12His (control) or 9Flg peptide. Vaccinations were repeated every 5 d, and tumor size/survival was monitored. The results show that control mice exhibited rapid and progressive tumor growth, with all mice being euthanized by day 22 because of tumor growth (tumors >15 mm diameter). In contrast, 9Flg PMV-vaccinated mice exhibited a markedly slower rate of tumor growth and prolonged survival: 50% of these mice survived at day 22, with some mice surviving to day 25 (Fig. 7B).

**Discussion**

This study used a novel approach using stealth liposomes engrafted with pFlgs to promote delivery of liposome-encapsulated Ag to APCs in vivo. Previous structure-function studies using truncated and/or mutated forms of flagellin indicate that the proinflammatory properties of flagellin are localized in the conserved N- and C-terminal regions (33–37, 39), with conflicting data claiming an essential role for the central hypervariable domain (36). Although not previously reported, short peptides making up the regions of flagellin implicated in the interaction with TLR5 may also bind TLR5 themselves; however, short peptides generally lack significant secondary/tertiary structure, suggesting that any such interaction is likely to be of low affinity, potentially limiting their use in vaccine development. However, peptides engrafted onto liposomes promote multimeric interactions between the liposome-encapsulated peptide molecules with receptors on the apposing cell (45). This attribute provides a means to reveal and use weakly interacting peptides—an approach exploited in the present work. In this way, we showed that the adjuvant properties of flagellin could be conveniently harnessed to target liposome-associated Ag to APCs, as well as to induce potent Ag-specific and antitumor immunity.

We produced several synthetic His-tagged peptides containing a sequence of 20–30 aa corresponding to different conserved regions in flagellin considered to be important for interaction with TLR5 (Table I). Of the peptides tested, 9Flg and, to a lesser extent, 42Flg promoted substantial binding of NTA 3-DTDA liposomes to CD11c+ BMDCs and SpDCs (Fig. 1, Supplemental Fig. 1). Two-color FACS analysis of cells indicates that 9Flg-liposome binding correlates with TLR5 expression (Fig. 3A), with little or no binding to cells lacking the expression of TLR5. Importantly, 9Flg liposomes bound CD11c+ DCs in vitro and in vivo (Fig. 2A, 2B), and cell-bound 9Flg liposomes were efficiently internalized by cultured DCs (Fig. 3B). This indicates that after interacting with TLR5 on APCs, 9Flg liposomes can be internalized, permitting intracellular Ag processing and presentation.

Recent evidence suggests that a 14-aa α-helical motif within the N-terminal domain (aa 95–108) and a conserved C-terminal motif (around aa 408–438) of flagellin are essential for full activation of TLR5, with the N-region of flagellin (aa 88–97) having been predicted to physically interact with aa 555–559 of TLR5 (33–37, 39). Our finding that liposomes engrafted with 9Flg and 42Flg (which contain aa 85–111 and aa 98–112 of flagellin, respectively) interact with TLR5-expressing cells, but that liposomes engrafted with 10Flg and 11Flg (which contain aa 405–425 and aa 408–438 of flagellin, respectively) exhibit only little binding, underscores the importance of the N-terminal aa 85–111 region and is consistent with the C-terminal region not playing a major role in the binding of flagellin to TLR5 (15, 17, 33, 39).

Although peptides 9Flg (containing aa 85–111 of flagellin) and 45Flg (containing aa 98–111 of flagellin) share considerable overlap, our results show that compared with 45Flg, peptide 9Flg is considerably more potent at promoting the binding of liposomes to TLR5+ cells and at inducing Ag-specific immunity. The reasons for this are unclear. One possible explanation is that 9Flg (and not 45Flg) contains the amino acids crucial for interaction with TLR5. Interestingly, however, a 14-aa region defined by aa 95–108 of flagellin is reported to form a hairpin loop, and deletions or site-specific mutations within this region can apparently abolish the proinflammatory activity of flagellin (35). Because 45Flg contains aa 98–111 of flagellin, but 9Flg also contains amino acids purported to be nonessential for functional TLR5 activation (35), another possible explanation for the activity of 9Flg is that, when anchored onto NTA3-DTDA liposomes, the purported nonessential region (aa 85–95) could contribute to overcoming steric constraints for the interaction between liposome-anchored 9Flg (containing the flagellin hairpin region) and TLR5.

Interestingly, it was reported that asialo-GM1 could play a coreceptor role in the binding of flagellin to TLR5 in lung (36); however, the precise relevance of this for the flagellin–TLR5 interaction remains to be elucidated. The possibility that pFlg liposomes can interact with receptors other than TLR5 on cells also cannot be discounted by the present work. However, our demonstration of a correlation between the binding of pFlg liposomes and TLR5 mAb to cells known to express TLR5 (Fig. 3A) is consistent with 9Flg liposomes interacting with TLR5.

An important finding from the present work is that the interaction of pFlg liposomes with APCs can elicit APC activation/maturation in vitro (Supplemental Fig. 4), as well as in vivo (Fig. 4). The administration of 9Flg liposomes to mice increased the expression of activation markers MHC class II and CD86 on CD11c+ on cells recovered from spleen 24 h later to an extent comparable to that seen with the administration of 10 μg LPS (a TLR4 ligand) (Fig. 4A). Flagellin is known to elicit DC maturation by binding to TLR5 and signaling through the MyD88 signal-transduction pathway, which involves recruitment of the MyD88 adaptor protein. Therefore, it is not surprising that analogous experiments performed using syngeneic MyD88−/− mice failed to show any increase in CD86 and MHC class II expression on CD11c+ splenocytes from mice injected with pFlg liposomes (Fig. 4B). In contrast, splenocytes from MyD88−/− mice injected with LPS still elicited a substantial increase in CD86 and MHC class II expression, presumably because LPS (unlike flagellin) can signal DC maturation through the MyD88- and TRIF-dependent pathways (7, 19). These findings show clearly that vaccination with 9Flg liposomes is effective at inducing MyD88-dependent maturation of CD11c+ DCs in vivo.

The ability to prime Ag-specific T cells and to generate IFN-γ-producing CD8+ T cells is crucial for the induction of an effective CTL response and protective antitumor immunity (23, 47). Further confirmation that pFlg liposomes can be used to elicit Ag-specific responses was provided by our demonstration that vaccination of C57BL/6 mice with 9Flg- and 42Flg-OVA liposome induced Ag-specific T cell priming, as assessed by the ability to induce increased Ag-specific cell proliferation (Fig. 5A), as well as to generate a higher...
Ag-containing liposomes to target Ag-uptake receptors to DCs and induce strong antitumor responses in these different tumor models. Although the effectiveness can depend on the tumor type, our preliminary experiments and Prof. S. Akira and S. Uematsu, Department of Host Defense, Research Institute for Microbial Diseases, Osaka University, Japan, for making the MYD88Δ/Δ mice available for some of the experiments carried out in this study.

Disclosures

J.G.A. has a commercial interest in Lipotek Pty Ltd.

References


