表　インフルエンザワクチンの効果を高める対策と具体例

目　的	対　策	具体例
・高い抗体価を誘導させる	・接種量を増やす	・小児接種量をWHO推奨量に（日本） ・高齢者向け高用量ワクチン（米国）
	・アジュバントを加える	・高齢者向けMF59入りワクチン（EU） ・沈降インフルエンザワクチンH5N1の開発（日本） ・プロトタイプワクチンの開発*（EU）
	・立体構造をもった抗原	・ビロゾーマルワクチンの製造（EU） ・全粒子ワクチンをプロトタイプワクチン（米国，日本）
・変異に対して対応できる抗体の誘導	・IgA抗体の誘導	・経鼻生ワクチンの製造（米国） ・経鼻不活化全粒子ワクチンの開発（日本）
	・立体構造をもった抗原	・全粒子不活化ワクチンの再評価（EU，日本）
・B型への効果を高める	・2系統を入れる	・4価ワクチンの開発（生，不活化とも）

* EUではプロトタイプワクチンとして，AS03またはMF59を含むワクチンを認可している。一方，米国は全粒子ワクチンをプロタイプワクチンとして認可しており，日本も2013年に認可した。
・2011/2012シーズンから日本ではWHO推奨量で接種するようになった。
・B型にはビクトリア系統とヤマガタ系統があり，抗原性は異なっている。B型対策を考慮し，2系統のB型，A/H1N1，A/H3N2を含んだ4価ワクチンが，2013/2014シーズンから米国で使用が開始された。

ンザウイルスの変異の面から，インフルエンザ発症予防のためには，高い血中抗体価が必要である。

Ⅱ．インフルエンザワクチンの効果を高める対策（表）

1．原　則

インフルエンザワクチンの効果を高めるためには，高い抗体価を誘導させることと，変異に対して対応できる抗体を誘導させることが大切である。高い抗体価を誘導させるためには，①1回の接種量を増やす，②アジュバントを加える，③立体構造をもった抗原を接種する，などの対策がある。実際，米国では高齢者向けに通常の4倍量の抗原量を含む高用量ワクチン[4]が，ヨーロッパでは高齢者向けにMF59を含むワクチンが使用されている。また，virosomeにヘマアグルチニン（hemagglutinin：HA）とノイラミニダーゼ（neuraminidase：NA）を付着させたビロゾーマルワクチンが開発されている。

変異に対して対応できる抗体を誘導させる方法としては，粘膜に接種してIgA抗体を誘導させる方法，立体構造をもった抗原を接種する方法，ブースティングにより反応できる抗原の幅を広める方法などがある。粘膜に接種するワクチンとして米国ではインフルエンザ生ワクチンが使用されている。適応は2～49歳である。成人では血中抗体の上昇が悪い点が問題である。わが国では鼻腔接種する全粒子不活化ワクチンの開発が進んでいる。成人に接種すると，粘膜IgA抗体と血中IgG抗体ともに賦活される。なお，ブースティングにより反応する抗原の幅が広がる現象はA/H5N1ワクチンで認められている[2]。

2．日本の対策―乳幼児への接種量増量

日本で臨床上インフルエンザワクチンに関して問題となっている点は，①乳幼児での効果が低いこと，②高齢者での効果が低いこと，③B型に対する効果が低いこと，などである。実際，インフルエンザワクチンの効果をみると，乳幼児の有効率は20～40%，学童では60～70%，成人では80～90%，高齢者では発症予防に対しては50%と，年齢群により異なっている[5]。乳幼児の有効率が低い一つの要因として，2010/2011シーズン以前は，接種量がWHO推奨量よりも少ないことが関与していると考えられていた[3,6]。

2009/2010シーズンおよび2010/2011シーズンに行った臨床研究の成績では，WHO推奨量で接種すると，6か月以上の乳幼児でもヨーロッパ医薬品局のインフルエンザワクチン有効基準を少なくとも1つ以上を満たし[7~9]，しかも，接種量増量によるインフルエンザワクチンの副反応の増

加は認められなかった。この結果を受け，乳幼児や学童でのインフルエンザワクチン接種による抗体価を少しでも高め，発症予防効果を高めるために，2011/2012シーズンから乳幼児や学童のインフルエンザワクチンの接種量が，WHO推奨量に増量となった。なお，インフルエンザワクチン接種量増量による臨床効果に関しては，今後の検討課題である。

3．B型への対策

2010/2011シーズンに行った臨床研究によると，A/H3N2，A/H1N1に対する抗体陽性率は年齢が上がるにつれ上昇したが，B型における抗体陽性率は，3〜6歳までは年齢が上がるにつれ上昇し，7〜13歳未満群では逆に低下していた[8]。B型にはビクトリア系統とヤマガタ系統があり，この2つの系統は抗原性からは別の系統とされている。欧米ではB型2種類を含む4価ワクチンの開発が行われている[9]。

まとめ

ウイルス感染症の発症予防のためには高い抗体価が必要である。高い抗体価を誘導し，効果的なインフルエンザワクチンの効果を期待して，2011/2012シーズンから小児のインフルエンザワクチン接種量が，WHO推奨量に増量された。

Key Points

❶ ウイルス感染症の発症予防のためには，発症予防レベル以上の高い抗体価が必要である。

❷ ワクチン接種により高い抗体価を誘導させる方法として，接種する抗原量を増やす方法がある。

❸ 小児におけるインフルエンザワクチンの予防効果を高めるために，2011/2012シーズンからインフルエンザワクチンの接種量が，WHO推奨量に増量された。

❹ 1回接種量は生後6か月〜3歳未満0.25 mL，3歳以上は0.5 mLである。

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インフルエンザー対象

202. インフルエンザワクチンの接種がもっとも大切な人は，どの年齢層，どのような職業の人ですか

回答・解説　庵原俊昭*

回答要旨

インフルエンザワクチンの接種がすすめられる人は，インフルエンザを発症すると重症化するリスクが高い人（インフルエンザハイリスク者），職業上インフルエンザハイリスク者と接触する機会が多い人（医療従事者，介護施設従事者など），およびインフルエンザハイリスク者の同居者や生活支援者である。

インフルエンザハイリスク者が入所している施設では，面会の機会が多い人にもインフルエンザワクチン接種を推奨すべきである。

解説

インフルエンザワクチンの接種がすすめられる人は，まずインフルエンザを発症したときに重症化するリスクが高い人である。米国がインフルエンザワクチン接種をすすめている人を表に示した[1]。乳幼児は高齢者と同様に発症すると入院率が高いため，6～59か月児にはインフルエンザワクチン接種がすすめられている。なお，6か月未満児のインフルエンザ対策として，妊婦にインフルエンザワクチンを接種し，移行抗体で発症を予防する方法が提唱されている[1,2]。

慢性の肺疾患（喘息を含む），循環器疾患（高血圧を除く），腎疾患，肝疾患，神経疾患，血液疾患，糖尿病を含む代謝疾患もインフルエンザワクチン接種がすすめられる集団である。とくに重症身体障害児（者）を含む慢性神経疾患の人は呼吸機能も低下しているため，とくにハイリスク者である。また，生後6か月～18歳までのアスピリン服用者も，インフルエンザ罹患時にReye症候群を発症するリスクが高いため，発症予防のためのインフルエンザワクチン接種がすすめられている。

表　インフルエンザワクチン接種がすすめられる人

- 6～59か月（4歳）までの小児
- 50歳以上の成人
- 慢性肺疾患（喘息を含む），慢性循環器疾患（高血圧を除く），慢性腎疾患，慢性肝疾患，慢性神経疾患，慢性血液疾患，慢性内分泌疾患（糖尿病を含む）に罹患している小児と成人
- 免疫抑制状態の人
- 妊娠している人またはインフルエンザ流行期間中に妊娠すると予測される人
- 長期間アスピリンを服用している6か月～18歳の小児
- 高齢者施設や障害児（者）施設に入所している人
- 高度の肥満（BMI≧40）の人
- 医療従事者および介護施設従事者
- 5歳未満の子どもや50歳以上の成人・高齢者の家族の人や同居者
- 慢性疾患を基礎にもつなどのインフルエンザに罹ると重症化するリスクが高い人の家族や同居者

（CDC[1]，2010より一部改変）

妊婦がインフルエンザに罹患すると，肺炎の合併リスクが高まるだけではなく，胎児死亡のリスクも増加する[3,4]。妊婦へのインフルエンザワクチン接種によりインフルエンザ罹患のリスクが減少する[4]。なお，妊婦への不活化ワクチン接種は原則第1三半期を避けることがすすめられているが，インフルエンザワクチンに関しては，インフルエンザ発症による母体と胎児への影響を考慮し，いずれの妊娠時期でも接種は可能としている。

次にインフルエンザワクチン接種がすすめられる人は，職業上インフルエンザハイリスク者と接触する機会が多い人である。医師，看護師だけではなく，受付業務に携わるなどの医療機関に働く

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すべての職種の人や，高齢者施設や障害者施設に働く人たちが該当する。インフルエンザワクチンには集団免疫効果があり，施設に働く医療従事者や生活支援者のインフルエンザワクチン接種率が高いほど，入所者のインフルエンザ罹患が減少する[1]。

重症身体障害児（者）などの基礎疾患をもつ人が入所する施設や高齢者施設では，面会者からもインフルエンザウイルスが感染する。このような施設では面会の機会が多い人にもインフルエンザワクチン接種を推奨すべきである。

さらに米国では，日常生活上ハイリスク者と接触する機会が多い人にもインフルエンザワクチン接種をすすめている。具体的には，6か月未満の子どもを含む5歳未満児，50歳以上の人，在宅の障害者などと接触する機会が多い同居家族，保育園や幼稚園の職員，生活支援者などの人たちである。

質問者への回答

インフルエンザワクチンの接種がすすめられる人は，インフルエンザを発症すると重症化するリスクが高い人（インフルエンザハイリスク者），医療従事者や介護施設従事者などの職業上インフルエンザハイリスク者と接触する機会が多い人およびインフルエンザハイリスク者と一緒に住んでいる人や生活支援者です。インフルエンザハイリスク者の施設では，面会者からの感染リスクを軽減させるために，面会の頻度が高い人にもインフルエンザワクチン接種が推奨されます。

Key words：インフルエンザワクチン，インフルエンザハイリスク者，医療従事者，生活支援者，集団免疫効果

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VACCINATION

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インフルエンザ－効果

204. インフルエンザが流行してきたというので心配になり，ワクチンを受けたいと思います。今から受けても効果はありますか

回答・解説　庵原俊昭*

回答要旨

インフルエンザワクチンは不活化ワクチンであるが，少なくとも3種類のインフルエンザウイルス〔A(H1N1)，A(H3N2)，B型〕に対して免疫記憶がある人では，急いで1回接種すれば効果的なブースター効果が期待される。

3種類のインフルエンザウイルスに対して免疫記憶がない人では2回接種が必要なため，効果的な免役が誘導されるまでに発症する危険性はあるが，現在流行している型に対して免疫記憶を有している可能性もあり，急いで1回接種し，間に合えば2週後に2回目を接種する。

解説

インフルエンザに限らず，ワクチン予防可能疾患（vaccine preventable diseases：VPDs）が流行すると急いでワクチン接種を受け，発症を予防しようとする人が増加する。流行時に接種するときは，発症者との接触の心当たりがないものの潜伏期間に接種し，発症が予防されないことも時にはあるが，多くの人がワクチンを受けることで流行を早期に終息させることが可能となる[1]。

流行時にワクチンを接種するときに考慮する因子は，①接種するワクチンが生ワクチンか不活化ワクチンか，②不活化ワクチンの場合接種を受ける人が当該VPDsに対して免疫記憶を有しているか，③ワクチン以外に明らかな接触があった場合の発症予防対策があるか，などである。

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生ワクチンの場合は1回の接種で免疫が誘導されるので，流行時に接種を行えば多くは発症予防が期待される。不活化ワクチンの場合，1期初回を終了し免疫記憶がある人では1回の接種で早期に（接種後3週頃までに）免疫の賦活が起こるため発症予防が期待される。一方，免疫記憶がない場合は，原則4週間隔で少なくとも2回接種し，免疫記憶細胞および免疫実行細胞を誘導する必要があり，少なくとも発症予防のための免疫が誘導されるまでに8週間必要とする。このため，流行時にワクチン接種により発症を予防することは時間的に困難であり，このような場合明らかな接触があったハイリスク者には，抗菌薬や抗ウイルス薬を用いた発症予防対策が行われる。

インフルエンザの場合も同様であり，インフルエンザが流行し始めるとインフルエンザワクチンの接種希望者が増加する。このとき考慮する因子は，①接種希望者が3種類または4種類のインフルエンザウイルス〔A(H1N1)，A(H3N2)，Bビクトリア系，Bヤマガタ系〕に対して免疫記憶を有しているか，②流行しているインフルエンザウイルスの型は何かである。

米国では，どこかのシーズンでインフルエンザワクチンを2回受けていると免疫記憶ができていると判断し，以降は1回の接種でよいとしており，また多くの9歳以上の人は，インフルエンザワクチンを一度も受けていなくても，3種類〔A(H1N1)，A(H3N2)，B型〕のインフルエンザウイルスに対する免疫記憶があるので，以降のインフルエンザワクチン接種は1回でよいとしている[2]。米国の考えに立つならば，上記の条件を満たす人は，インフルエンザ流行時に急いで1回接種すれば発症予防が期待される。

表　インフルエンザワクチン接種後のHI抗体陽転率と保有率（3歳以上13歳未満）

		A(H1N1)			A(H3N2)			B		
		対象	陽性	%	対象	陽性	%	対象	陽性	%
3歳以上6歳未満										
陽転率	1回接種後	72	50	69.4	72	49	68.1	72	40	55.6
	2回接種後	71	57	80.3	71	58	81.7	71	48	67.6
保有率	1回接種後	72	54	75.0	72	60	83.3	72	46	63.9
	2回接種後	71	61	85.9	71	64	90.1	71	52	73.2
6歳以上13歳未満										
陽転率	1回接種後	56	42	75.0	56	37	66.1	56	19	33.9
	2回接種後	56	44	78.6	56	41	73.2	56	21	37.5
保有率	1回接種後	56	47	83.9	56	53	94.6	56	34	60.7
	2回接種後	56	48	85.7	56	55	98.2	56	36	64.3

HI抗体40倍以上を抗体陽性とし，抗体陽転率とはHI抗体が40倍以上かつ4倍以上上昇した症例の割合，抗体保有率とはHI抗体が40倍以上の症例の割合である。

（文献3，2011より作表）

一方，わが国では13歳未満は毎年2回の接種をすすめており，インフルエンザワクチンの接種歴にかかわらず，多くの人が3種類のインフルエンザウイルスに対する免疫記憶をもっているのは13歳以上と考えている。この考えに立つならば，流行時であったとしても13歳未満ならば2回の接種が必要である。このような場合，接種間隔を4週間よりも短くすることで発症予防を期待する。なお，13歳未満は最短2週間隔で接種できるので，流行時に接種するときは2週間隔での接種がすすめられる。

2010/2011シーズンに行われた臨床治験のデータからは，A(H1N1)，A(H3N2）に対しては6歳以上ならば多くの子どもは1回の接種で効果的な免疫反応が期待される（表）[3]。B型の流行ならば6歳以上では取り急ぎ1回接種し，間に合えば2週間後に2回目を接種し，発症予防を期待する。なお，学童のインフルエンザワクチンの発症予防効果は60〜70%であるので，ワクチン接種時には発症予防効果の限界を伝えておくことも大切である。

最後にB型に対しては，近年Bビクトリア系とBヤマガタ系は免疫学的に異なるウイルスと考えられるようになっている[4,5]。この結果，欧米ではBビクトリア系とBヤマガタ系を同時に含む4価ワクチンの開発が行われている。米国医薬食品局（FDA）は，2012年12月にグラクソスミスクライン社のインフルエンザ4価ワクチンを承認した。適応は3歳以上の小児と成人である。

質問者への回答

インフルエンザワクチンは不活化ワクチンであり，少なくとも3種類のインフルエンザウイルス〔A(H1N1)，A(H3N2)，B型〕に対して免疫記憶がある人は，急いで1回接種すれば効果的なブースター効果が期待されます。一方，3種類のインフルエンザウイルスに対して免疫記憶がない人では2回接種が必要なため，効果的な免役が誘導されるまでに発症する危険性はあります。しかし，現在流行している型に対して免疫記憶を有している可能性もありますので，急いでまず1回接種し，間に合えば2週後に2回目を接種する方法をすすめます。なお，インフルエンザワクチンの効果には限界がありますので，インフルエンザワクチンを受けていたとしても，発症した場合は抗インフルエンザ薬を服用してください。

Key words：インフルエンザ，インフルエンザワクチン，緊急接種，免疫記憶，接種回数

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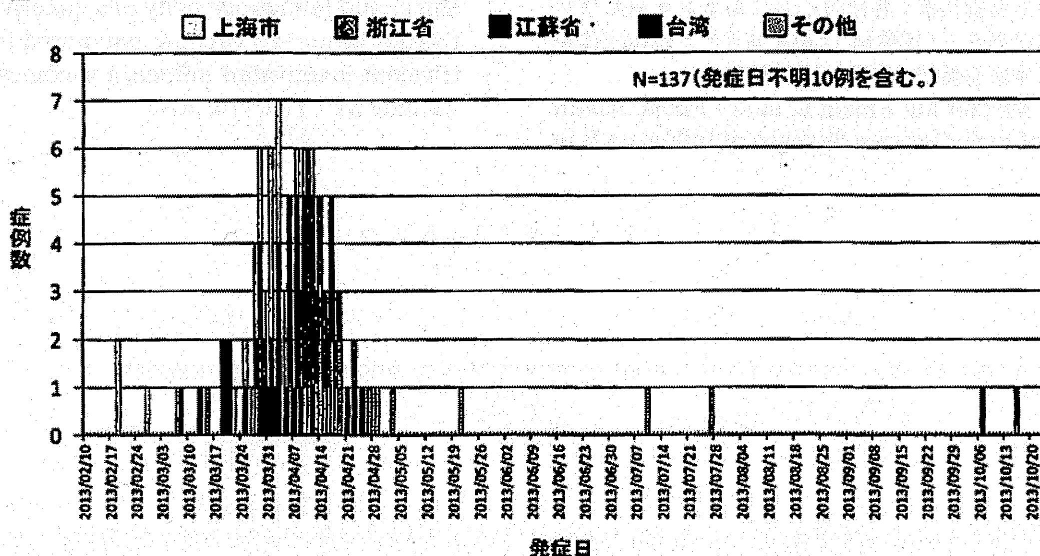
○ VACCINATION ○

図. 鳥インフルエンザA(H7N9)確定例 流行曲線 (2013年10月24日現在)



因なのかは不明である。

当該ウイルスは少なくとも3種類の異なる鳥インフルエンザウイルスの遺伝子交雑体であると考えられる。ヒト分離ウイルス15株は遺伝子系統樹解析の結果から互いに非常に類似していた。しかし，そのうちの1株（A/Shanghai/1/2013）は，塩基配列上では他の14株とは区別され，共通の祖先から分岐した別系統の近縁ウイルスが同時期に伝播していたことが示された。

上海市，江蘇省，浙江省のハト，ニワトリおよび環境からの分離ウイルス7株の遺伝子系統樹解析の結果からは，上記ヒト分離ウイルスのうちの上記14株と類似性が高く，同系統のウイルスと考えられる。しかし，鳥とヒトのウイルス株の間には明らかに異なる塩基配列もあり，今回報告された鳥分離ウイルスが今回報告された患者に直接に感染したものであるとは考えにくい。

ヒト分離ウイルス15株のすべてのHA遺伝子は，ヒト型のレセプターへの結合能を上昇させる変異を有しており，このことはin vitroのレセプター結合実験でも確認された。しかし，これら分離株は，トリ型レセプターへの結合能も併せて保持しているため，まだ継続的にヒト-ヒト間で感染伝播するまでにはヒト型に馴化していないと判断される。しかし，追加の変異によってその能力を獲得する可能性があるので，パンデミックを起こす可能性については，H5N1鳥インフルエンザウイルスよりも高いと推定される。

PB2遺伝子を解析したヒト分離ウイルス11株のすべてに，RNAポリメラーゼの至適温度を鳥の体温（41℃）から哺乳類の上気道温度（34℃）に低下させる変異が観察された。

ヒト分離ウイルス15株および鳥，環境からの分離ウイルス7株，合計22株の遺伝子解析の結果からは，鳥に対して高病原性を示す遺伝的マーカーの変異はみられず，ニワトリやウズラなど家禽への感染実験でも低病原性であることが確認された。またブタへの感染実験においても不顕性感染であることが確認され，この系統のウイルスがこれらの哺乳動物の間で症状を示さずに伝播され，ヒトへの感染源になる可能性が示唆された。

NA遺伝子の塩基配列からは，ヒト分離株のうちの1株A/Shanghai/1/2013が，抗インフルエンザ薬のオセルタミビル，ペラミビルおよびザナミビルに対する耐性変異（R292K）をもつことが指摘されていたが，詳細な遺伝子解析やクローニング実験から耐性株と感受性野生株との混合ウイルスであることが確認された。台湾のヒト分離ウイルスも耐性変異株と感受性野生株の混合ウイルスで，オセルタミビルに感受性が低下していた。

直近で浙江省から2例の患者報告があったことから，冬季にかけてH7N9ウイルスの流行が再び活発になる可能性も否定できず，引き続き中国における患者発生状況および国内への患者の流入の可能性を注視する必要がある。

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2013年9月に分離されたA(H1N1)pdm09ウイルスの性状 ― 三重県

近年，国内でのA(H1N1)pdm09ウイルスの分離・検出状況は2011年には5,284件であったが2012年は39件に減少した。その後2013年には127件（2013年9月18日現在）のウイルスが検出されており，やや増加して

表1. 患者の臨床症状

	検体番号	年齢	性別	発症日	採取日	臨床症状	受診時発熱(℃)
1	2013-576	36歳	男	2013/9/4	2013/9/5	関節痛、口内炎	39.7
2	2013-577	11歳	女	2013/9/4	2013/9/5	筋肉痛、気管支炎、嘔気、腹痛	39.4

いる[1]。本県においてもA(H1N1)pdm09ウイルスは2013年1月上旬に2011年3月以来となる2株が分離され，さらに同年5月中旬には2株の計4株が分離された[2]。その後，2013/14シーズンの初期にインドネシアへの渡航歴のある2名の患者からA(H1N1)pdm09ウイルスが分離された。そこで，これらのA(H1N1)pdm09ウイルスの性状について報告する。

2013年9月5日（第36週）にインドネシアから帰国後に発熱等の症状（表1）を呈し三重県A市の医療機関を受診した2名の患者から採取された咽頭ぬぐい液検体を用い，インフルエンザウイルス遺伝子検査（Conventional RT-PCR法，Real-time RT-PCR法，RT-LAMP法）を実施したところ，2件ともにA(H1N1)pdm09ウイルスが検出された。MDCK細胞によるウイルス分離においても，2件とも初代培養で細胞変性が認められた。これらのウイルス培養上清液に対して0.75%モルモット赤血球を用いた赤血球凝集（HA）試験を行ったところ，両株ともHA価は128を示した。そこで国立感染症研究所より2012年に配布された2012/13シーズンインフルエンザウイルス同定キットにて0.75%モルモット赤血球を用いた赤血球凝集抑制（HI）試験による同定試験を行った。これらの2株はA/California/7/2009 (H1N1)pdm09の抗血清に対するHI価は2,560（ホモ価2,560）を示した。なお，A/Victoria/361/2011(H3N2)の抗血清（同2,560），B/Wisconsin/1/2010(山形系統)の抗血清（同1,280），B/Brisbane/60/2008(Victoria系統）の抗血清（同1,280）に対するHI価は10未満であった。

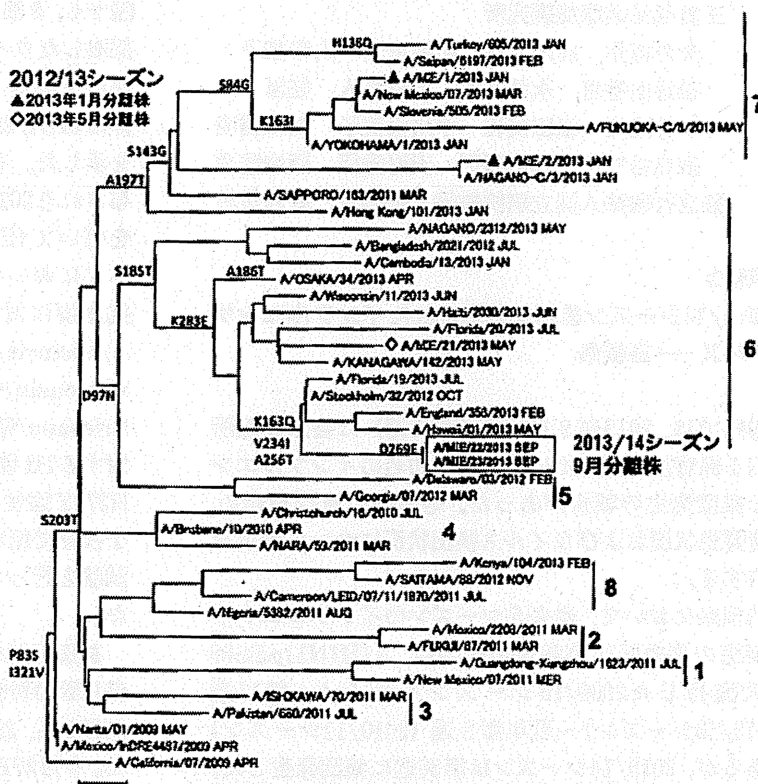

図1. HA遺伝子系統樹A(H1N1)pdm09

HA遺伝子系統樹解析

2013年9月に分離された2株のA(H1N1)pdm09ウイルス〔A/MIE(三重)/22/2013株，A/MIE/23/2013株〕はHA遺伝子系統樹解析により，HAタンパク質にD97N，S185Tのアミノ酸置換を持つクレード6に分類された（図1）。また，2013年1月上旬の2株（A/MIE/1/2013株，A/MIE/2/2013株）は，2010/11シーズンの国内流行株の特徴であるA197Tアミノ酸置換を持つクレード7に属していた。また同年5月中旬の分離株（A/MIE/21/2013株）はクレード6に分類されるが，同クレード内の9月分離株（A/MIE/22/2013株，A/MIE/23/2013株）のHAアミノ酸と比較すると，6カ所（アミノ酸番号：19, 163, 234, 256, 266, 269）が異なっていた。今回の検出事例は帰国後2日で発症していることから，インドネシアからの輸入事例と推測され，今後，インフルエンザウイルス流行期に分離されるA(H1N1)pdm09ウイルスとの抗原性の違いに興味がもたれる。

なお，9月分離株は2株ともオセルタミビル耐性マーカーである，NA遺伝子内のH275Y変異は検出されていない。

感染予防対策のためにも通年における継続的なインフルエンザウイルスの動向監視を行い，A(H1N1)pdm09ウイルスの国内での再流行およびAH3亜型インフルエンザウイルス，B型インフルエンザウイルスの動向に注視する必要があると思われる。

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(三重県感染症情報センター，インフルエンザウイルス分離・検出状況)

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<速報>

2013/14シーズン最初に分離されたインフルエンザウイルス ― 島根県

当県では，2013年9月17日（第38週）に益田保健所管内1保育園（園児108名）から，10名のインフルエンザの集団発生の報告があった。地域内小流行を含め，患者発生状況およびウイルス検出状況について概要を報告する。

島根県において，過去5シーズンのインフルエンザ様疾患の学級閉鎖等報告の初発は，A(H1N1)pdm09が大流行した2009/10シーズンを除くと，第47週(2012/13シーズン)～翌年第2週（2010/11シーズン）であるが，2013/14シーズンは第38週に集団発生した。報告のあった県西部の益田保健所管内インフルエンザ定点（定点数5）患者報告数は，第37週（9月9日）から12件，第38週（9月16日）に10件，第39週（9月23日）には17件であり，益田保健所管内でこの時期10～17件（定点当たり2.0～3.4）と地域的な流行となっていた（図）。また，入院サーベイランスにおいても第38週に3名（80歳，64歳，5歳)，第39週に1名（1歳)，第40週に1名（12歳)，第41週に1名（4歳）のインフルエンザ入院患者報告があった。

この時期に集団発生を探知することは稀であり，今後のインフルエンザの流行を早期発見し，対策を迅速かつ的確に実施するために益田保健所と管内の定点医療機関にインフルエンザ患者検体の提供を依頼し，集団発生のあった保育園の患者1名（5歳）を含む計6名（前述の5歳の他，1歳2名，8歳1名，35歳1名，43歳1名）の患者から咽頭ぬぐい液検体が得られた。

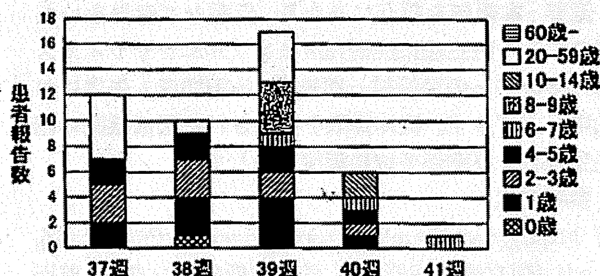

図. 益田保健所管内のインフルエンザ報告患者数

検体からRNAを抽出し，リアルタイムRT-PCR（TaqMan Probe法）によりインフルエンザウイルス遺伝子検査を行い，6検体ともにA(H1N1)pdm09ウイルスが検出された。MDCK細胞によるウイルス分離でも，6検体とも初代培養で細胞変性が認められた。採取したウイルス培養上清に対して0.75％モルモット血球を用いて赤血球凝集（HA）試験を行った。1検体は6HA/25μl，残りの5検体は4HA/25μlのHA価を示した。そこで，国立感染症研究所より2012年に配布された2012/13シーズンインフルエンザ同定キットを用いてHI試験による同定を行ったところ，6株すべてにおいてA/California/7/2009(H1N1)pdm09の抗血清に対するHI価は320（ホモ価640）を示した。A/Victoria/361/2011(H3N2)の抗血清（同5,120)，B/Wisconsin/01/2010(山形系統）の抗血清（同160)，B/Brisbane/60/2008(Victoria系統）の抗血清（同80)に対するHI価は10未満であった。また，A/H1N1pdm09 H275Y耐性株検出法実験プロトコールに基づき，遺伝子検査で用いたRNAからのH275Y耐性マーカー検出試験を行ったが，6株すべて感受性（H275）株であった。

益田保健所のその後の調査では，集団発生のあった保育園の新規患者は9月18日1名，20日1名，21日1名，24日2名，25日1名であり，合計16名の患者報告があった。9月27日以降，10月16日現在までは保育園での新たな患者発生報告はなかった。また，職員からの患者発生報告はなかった。益田保健所管内インフルエンザ定点患者報告数も第40週（9月30日）は6件，第41週（10月7日）は1件と減少し，散発的な発生となっている。保育園の集団発生が探知の端緒であったが，患者は小児のみでなく広範な年齢層で認められた（図)。

例年より早期の集団発生であり，県内の他地域ではほとんど患者報告は認められていないが，県薬事衛生課を通じて各保健所等と情報を共有し，今後のインフルエンザウイルスの動向を注視する必要がある。

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<速報>

渡航歴のない麻疹集団発生からのB3型麻疹ウイルス検出 ― 愛知県

2013年8月23日～9月12日の期間に愛知県内で麻疹と診断された患者のうち，愛知県衛生研究所にて行った麻疹ウイルス遺伝子検査陽性を示した13例について，ウイルス検査の概要を報告する。このうち遺伝子型別のできなかった1例を除く12例の遺伝子型はB3

CrossMark

Nonagonistic Dectin-1 ligand transforms CpG into a multitask nanoparticulate TLR9 agonist

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CpG DNA, a ligand for Toll-like receptor 9 (TLR9), has been one of the most promising immunotherapeutic agents. Although there are several types of potent humanized CpG oligodeoxynucleotide (ODN), developing "all-in-one" CpG ODNs activating both B cells and plasmacytoid dendritic cells forming a stable nanoparticle without aggregation has not been successful. In this study, we generated a novel nanoparticulate K CpG ODN (K3) wrapped by the nonagonistic Dectin-1 ligand schizophyllan (SPG), K3-SPG. In sharp contrast to K3 alone, K3-SPG stimulates human peripheral blood mononuclear cells to produce a large amount of both type I and type II IFN, targeting the same endosome where IFN-inducing D CpG ODN resides without losing its K-type activity. K3-SPG thus became a potent adjuvant for induction of both humoral and cellular immune responses, particularly CTL induction, to coadministered protein antigens without conjugation. Such potent adjuvant activity of K3-SPG is attributed to its nature of being a nanoparticle rather than targeting Dectin-1 by SPG, accumulating and activating antigen-bearing macrophages and dendritic cells in the draining lymph node. K3-SPG acting as an influenza vaccine adjuvant was demonstrated in vivo in both murine and nonhuman primate models. Taken together, K3-SPG may be useful for immunotherapeutic applications that require type I and type II IFN as well as CTL induction.

innate immunity | two-photon microscopy | MARCO | Siglec-1 | β-glucan

CpG oligodeoxynucleotide (CpG ODN) is a short (~20 bases), single-stranded synthetic DNA fragment containing the immunostimulatory CpG motif, a potent agonist for Toll-like receptor 9 (TLR9), which activates dendritic cells (DCs) and B cells to produce type I interferons (IFNs) and inflammatory cytokines (1, 2) and acts as an adjuvant toward both Th1-type humoral and cellular immune responses, including cytotoxic T-lymphocyte (CTL) responses (3, 4). Therefore, CpG ODN has been postulated as a possible immunotherapeutic agent against infectious diseases, cancer, asthma, and pollinosis (2, 5).

There are at least four types of CpG ODN, each of which has a different backbone, sequence, and immunostimulatory properties (6). D-type (also called A) CpG ODNs typically comprise one palindromic CpG motif with a phosphodiester (PO) backbone and phosphorothioate (PS) poly(G) tail, and activates plasmacytoid DCs (pDCs) to produce a large amount of IFN-α but fails to induce pDC maturation and B-cell activation (7, 8). The three other types of ODN consist of a PS backbone. K-type (also called B) CpG ODN contains nonpalindromic multiple CpG motifs, and strongly activates B cells to produce IL-6 and pDCs to maturation but barely produces IFN-α (8, 9). Recently, C and P CpG ODNs have been developed; these contain one and two palindromic CpG sequences, respectively, both of which can activate B cells like K-type and pDC like D-type, although C CpG ODN induces weaker IFN-α production compared with P CpG ODN (10–12).

D and P CpG ODNs have been shown to form higher-order structures, Hoogsteen base pairing to form parallel quadruplex structures called G tetrads, and Watson–Crick base pairing between *cis*- and *trans*-palindromic portions, respectively, that are required for robust IFN-α production by pDCs (12–14). Although such higher-order structures appear necessary for localization to early endosomes and signaling via TLR9, they suffer from product polymorphisms, aggregation, and precipitation, thereby hampering their clinical application (15). Therefore, only K and C CpG ODNs are generally available as immunotherapeutic agents and vaccine adjuvants for human use (16, 17). Although K CpG ODN enhances the immunogenicity of vaccines targeting infectious diseases and cancers in human clinical trials (6, 17), chemical or physical conjugation between antigen and K CpG ODN is necessary for optimal adjuvant effects. These results indicate that these four (K, D, P, and C) types of CpG ODN have advantages and disadvantages; however, the

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Significance

CpG oligodeoxynucleotide (ODN), a Toll-like receptor 9 ligand, is a promising immunotherapeutic agent; however, developing an IFN-inducing CpG ODN forming a stable nanoparticle without aggregation has been unsuccessful. Here we generated a nanoparticulate CpG ODN (K3) wrapped by the nonagonistic Dectin-1 ligand schizophyllan (SPG), K3-SPG. K3-SPG stimulates human peripheral blood mononuclear cells to produce large amounts of both type I and II IFN. K3-SPG thus became a potent adjuvant, especially for cytotoxic T-lymphocyte (CTL) induction to coadministered protein antigens without conjugation, which is attributable to its nanoparticulate nature rather than to targeting Dectin-1. Protective potency of K3-SPG as an influenza vaccine adjuvant was demonstrated in both murine and nonhuman primate models. K3-SPG may be used as an IFN inducer as well as a CTL inducer for immunotherapeutic applications.

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Conflict of interest statement: K.S. holds a patent related to schizophyllan forming a complex with nucleic acids. K.K., T.A., and K.J.I. have filed a patent application related to the content of this manuscript.

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development of an "all-in-one" CpG ODN activating both B cells and pDCs that forms a stable nanoparticle without aggregation has yet to be accomplished. A better strategy, targeting CpG ODN toward antigen-presenting cells (APCs), is desired to improve immunostimulatory specificity and immunotherapeutic efficacy of CpG ODNs.

Schizophyllan (SPG), a soluble β-glucan derived from *Schizophyllum commune*, is a drug that has been approved in Japan as an enhancer of radiotherapy in cervical carcinoma patients for the last three decades (18). It has been shown to form a complex with polydeoxyadenylic acid (dA) as a triple-helical structure (19). Although we previously demonstrated that mouse and humanized CpG ODN with PO poly(dA) at the 5′ end complexed with SPG enhanced cytokine production and acted as an influenza vaccine adjuvant (20, 21), it has been difficult to achieve high yields of the CpG–SPG complex toward its more efficient and cost-effective preclinical as well as clinical development. Recently, when the PS backbone of the dA sequence was linked to CpG ODN, the efficacy of complex formation was elevated by nearly 100% (22). However, a thorough investigation has yet to be conducted to identify the best humanized CpG sequence and optimization of factors to gain all-in-one activities of the four types of CpG ODN.

To do this, we sought to optimize a humanized CpG–SPG complex as a vaccine adjuvant and immunostimulatory agent in humans (in vitro), mice (in vitro and in vivo), and nonhuman primates (in vivo). In this study, we identified a novel K CpG ODN (K3) and SPG complex, namely K3-SPG. It forms a higher-order nanoparticle that can be completely solubilized. We found that this all-in-one K3-SPG displayed a more potent activity than, and different characteristics from, any other type of CpG ODN and previous CpG–SPG complexes.

Results

A Rod-Shaped Nano-Sized Particle of K3-SPG Gains Dual Characteristics of K- and D-Type CpG ODNs. To make a complex between CpG ODNs and schizophyllan (SPG), CpG ODNs need additional sequences of phosphorothioate backbone of 40-mer polydeoxyadenylic acid (dA_{40}) at the 5′ or 3′ end (20, 22). Fig. 1*A* shows methods of CpG ODN and SPG complexation through denaturing–renaturing procedures. In this study, we selected K3 as a K-type CpG ODN. At first, we examined the immunostimulatory impacts of the 5′ and 3′ ends of CpG ODN. 5′-K3-dA_{40}-3′, but not 5′-dA_{40}-K3-3′, complexed with SPG-activated human peripheral blood mononuclear cells (PBMCs) to produce a robust amount of IFN-α (Fig. 1*B* and Fig. S1). K3, K3-dA_{40}, or dA_{40}-K3, which are able to activate human PBMCs to produce other cytokines such as IL-6, failed to produce IFN-α (Fig. 1*B* and Fig. S1). These results indicate that the 5′-CpG sequence (K3-SPG) is more desirable than the 3′-CpG sequence as a novel TLR9 agonist. Although some CpG ODN-induced cytokine production is known to have a dose-dependent correlation, K3-SPG–induced IFN-α production is not. Given that previous reports showed that IFN-α pro-duction by K CpG ODN stimulation has a bell-shaped dose–response correlation (7), altogether these results suggest that K3-SPG still has the character of K CpG ODN.

Qualification and quantitation of K3-SPG were conducted by scanning electron microscopy (SEM) and dynamic light scattering (DLS). K3-SPG had a rod-like structure, consistent with that seen in a previous report (23) (Fig. 1*C*). It appeared to be a soluble monomeric nanoparticle with an average diameter of 30 nm, comparable to SPG itself and smaller than D CpG ODN (D35) (14, 24) (Fig. 1*D*). Given that K3-SPG forms a nanoparticle, we compared the immunostimulatory activities of K3-SPG with D, C, and P CpG ODNs. PBMCs stimulated with K3-SPG produced larger amounts of IFN-α and IFN-γ but at far lower concentrations than those induced by D35 (Fig. 1*E*) and P and C CpG ODNs (Fig. S2). These results suggest that K3-SPG gains the characteristic of D CpG ODN without losing that of the K type, because these IFNs are known to be D type-specific cytokines (7, 8, 25). To understand the dual functions of K and D

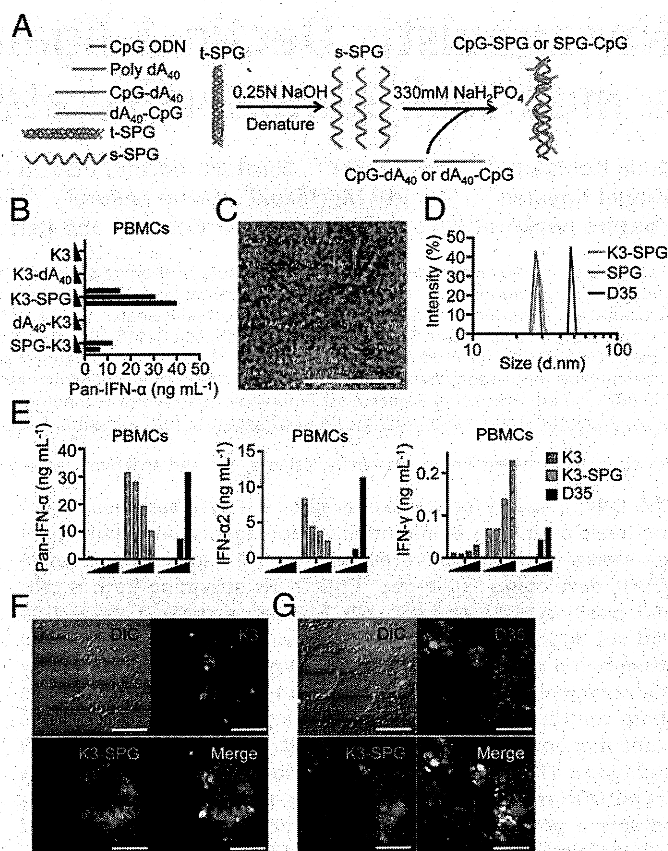

Fig. 1. K (B) CpG ODN and SPG complex forms nanoparticles and gains D (A) CpG ODN characteristics. (*A*) Methods of CpG ODN and SPG complexation. tSPG, triple-stranded SPG; sSPG, single-stranded SPG. (*B*) Production of IFN-α by human PBMCs stimulated with K3, K3-dA_{40}, K3-SPG, dA_{40}-K3, or SPG-K3 (adjusted for K3 ODN concentration at 0.1, 0.3, or 1 μM) for 24 h was measured by ELISA. (*C*) K3-SPG processed for SEM. (Scale bar, 50 μm.) (*D*) Size of K3-SPG, SPG, and D35 was analyzed by DLS. (*E*) Production of type I and II IFNs by PBMCs stimulated with K3, K3-SPG, or D35 for 24 h was measured by ELISA. (*F* and *G*) Mouse BMDMs were stimulated with Alexa 488-K3 (*F*) or Alexa 488-D35 (*G*) and Alexa 647-K3-SPG at 1 μM for 3 h. The cells were incubated with Hoechst 33258, fixed, and analyzed by fluorescence microscopy. DIC, differential interference contrast. (Scale bars, 10 μm.) Data represent one of three independent experiments with similar results.

CpG ODNs, we analyzed the intracellular localization of K3-SPG in bone marrow-derived macrophages (BMDMs). K3-SPG was colocalized with not only the endosomes containing K CpG ODN but also those containing D CpG ODN (Fig. 1 *F* and *G*) such as C CpG ODN (26), suggesting that K3-SPG may transduce endosome-mediated innate immune signaling pathways by K and D CpG ODNs. These results strongly suggest that K3-SPG forms a nanosized higher-order and completely solubilized particle and found that this all-in-one K3-SPG displayed a more potent activity than, and different characteristic from, any other CpG ODNs and previously known CpG–SPG complex.

K3-SPG Is a Prominent Vaccine Adjuvant That Induces Potent CTL Responses to Protein Antigen Without Conjugation. We compared the adjuvant effects of K3, K3-dA_{40}, and K3-SPG in a murine immunization model. When wild-type mice were immunized with LPS-free chicken ovalbumin protein (OVA) alone or OVA with each K3-derived adjuvant, K3-SPG induced significantly higher humoral immune responses (Fig. 2*A*) and stronger T-cell responses than that induced by K3 (Fig. 2*B*). Of note, tetramer assays revealed a significantly greater number of OVA-specific CD8 T cells (Fig. 2*C*). We also observed very strong in vivo CTL activity against

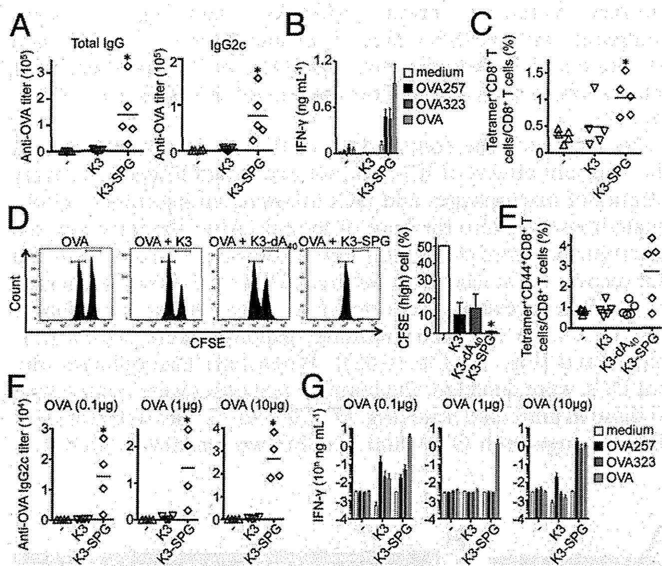

Fig. 2. K3-SPG acts as a potent vaccine adjuvant by simple mixture with antigen. Adjuvant activities of K3-SPG were analyzed. C57BL/6J mice (n = 4 or 5) were immunized s.c. with OVA protein antigen and various adjuvants. OVA-specific serum IgG (*A*), IFN-γ (*B*), and $OVA_{257–264}$-specific tetramer (*C*) were monitored (d17) after immunization (d0 and d10) with OVA (100 μg) with or without K3 (10 μg) or K3-SPG (10 μg). (*D*) In vivo CTL assay 7 d after priming with OVA and various adjuvants as indicated. (*E*) Immunization with $OVA_{257–264}$ peptide (10 μg) with or without adjuvant as indicated. (*F* and *G*) Dose-sparing study; OVA-specific serum IgG and IFN-γ were monitored after immunization as in *A* and *B*. *$P < 0.05$ (Mann–Whitney U test). Data represent one of two or three independent experiments with similar results.

coadministered protein antigens lacking any covalent conjugation (Fig. 2*D*). This strong CTL induction by K3-SPG was reproduced by peptide vaccination (Fig. 2*E*) and was dose-dependent (Fig. S3). The antigen-sparing ability of K3-SPG was so potent that comparable antibody and CD4 T-cell responses were achieved using one-hundredth the amount of OVA antigen (Fig. 2 *F* and *G*). These results clearly indicate that K3-SPG is a more prominent adjuvant than K3 alone.

SPG Is a Soluble Dectin-1 Ligand but Is Not a Dectin-1 Agonist. We examined the role of Dectin-1 in cellular uptake of, and following activation by, SPG and K3-SPG, as Dectin-1 has been shown to be a receptor for β-glucans such as Zymosan (27). Using flow cytometry, we found that HEK293 cells expressing Dectin-1 but not Dectin-2 or a control (vector) increased the uptake of SPG or K3-SPG in vitro regardless of ODN presence (Fig. 3 *A* and *B*). It has recently been reported that the soluble form of β-glucan does not activate Dectin-1 signaling (28). Additionally, Dectin-1 signaling inhibits TLR9-mediated cytokine production through suppressor of cytokine signaling 1 induction (29). Therefore, we examined the agonistic activity of SPG. When splenocytes were stimulated with Zymosan-Depleted but not SPG, dose- and Dectin-1–dependent TNF-α and other cytokine production was observed, whereas cytokine production by Zymosan and Curdlan was Dectin-1–independent (Fig. 3*C* and Fig. S4). Zymosan-Depleted inhibited CpG ODN-induced IFN-α, with this inhibition relieved by Dectin-1 deficiency (Fig. 3*D*). In contrast, SPG did not inhibit CpG ODN-induced IFN-α production (Fig. 3*E*). These results indicate that SPG is a ligand but not an agonist of Dectin-1; therefore, SPG does not interfere with TLR9-mediated IFN-α production.

Adjuvant Effects of K3-SPG Are Dependent on TLR9 and Partially Dependent on Dectin-1. Because K3-SPG is a complex of CpG ODN and β-glucan, we examined the role of TLR9 (1) and Dectin-1 (30) using receptor knockout mice. When splenocytes and Flt3 ligand-induced bone marrow-derived DCs (FL-DCs) from *Tlr9*- and *Dectin-1*–deficient mice were stimulated with K3-SPG, cytokine production was completely dependent on TLR9 but not Dectin-1, excluding IL-12 p40 production (Fig. 4 *A*–*D*). K3-SPG–induced IL-12 p40 production showed two peaks, where the first peak of its production, but not the second peak at a higher dose, was dependent on Dectin-1 (Fig. 4*D*). This result may imply that Dectin-1 expression is involved in IL-12 p40 production at a lower dose of K3-SPG in vitro. Consistent with in vitro results, immunization of *Tlr9*-deficient mice with K3-SPG plus OVA resulted in diminished humoral and T-cell responses (Fig. 4 *E*–*G*). *Dectin-1*–deficient mice showed comparable immune responses with wild-type mice when the mice were immunized with OVA plus 10 μg of K3-SPG (Fig. S5). When *Dectin-1*–deficient mice were immunized with OVA plus 1 μg of K3-SPG, mice exhibited a reduced CD8 T-cell response according to the tetramer assays (Fig. 4*J*), with no significant changes in antibody and cytokine production from T cells (Fig. 4 *H* and *I*). These results suggest that the adjuvant effect of K3-SPG is dependent on TLR9 signaling. Although SPG and K3-SPG do not stimulate Dectin-1 signaling, the effect of K3-SPG is still partially dependent on Dectin-1 in vivo.

MARCO$^+$, but Not Siglec-1$^+$, Macrophages in Draining Lymph Nodes Dominantly Capture K3-SPG with Antigen. Given that K3-SPG provides potent adjuvant effects in vivo through immunization with a simple antigen mixture, we hypothesized that cells that capture both antigen and K3-SPG should play a critical role in mediating adjuvant effects. To examine in vivo distribution of fluorescence-labeled OVA and K3-SPG, we used fluorescence microscopy and two-photon microscopy. After an injection at the

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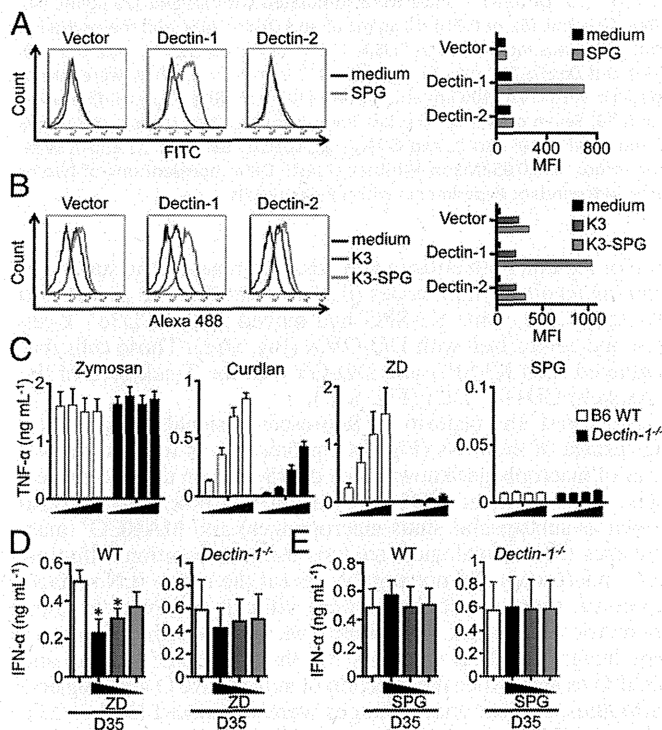

Fig. 3. SPG is a nonagonistic Dectin-1 ligand, but does not interfere with TLR9-mediated IFN-α production. (*A* and *B*) HEK293 cells transiently expressing Dectin-1 or Dectin-2 were treated with SPG-FITC (*A*), Alexa 488-K3, or Alexa 488-K3-SPG (*B*) for 60 min, and then their cellular uptake was monitored by flow cytometry [*Left*, histogram; *Right*, mean fluorescent intensity (MFI)]. Splenocytes from C57BL/6J and *Dectin-1*$^{-/-}$ mice (n = 3) were stimulated with Zymosan, Curdlan, Zymosan-Depleted (ZD), or SPG (3.7–100 μg/mL) (*C*), with D35 (1 μM), or with or without ZD (11.1–100 μg/mL) (*D*) or SPG (*E*) for 24 h and supernatant cytokines were monitored by ELISA. *$P < 0.05$ (t test). Data represent one of three independent experiments with similar results.

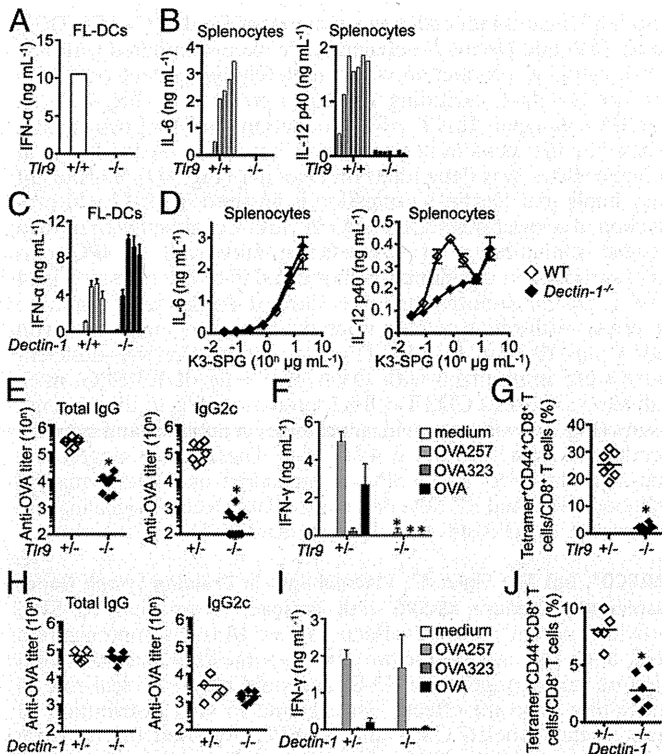

Fig. 4. Adjuvant effects of K3-SPG were completely dependent on TLR9 and partially on Dectin-1. FL-DCs (*A* and *C*) or splenocytes (*B* and *D*) from C57BL/6J, $Tlr9^{-/-}$, or $Dectin\text{-}1^{-/-}$ mice were stimulated with K3-SPG [20 μg/mL (*A*), 0.014–10 μg/mL (*B*), or 0.014–10 μg/mL (*C* and *D*)] for 24 h, and their cytokine production was monitored by ELISA. $Tlr9^{+/-}$ (n = 7) or $Tlr9^{-/-}$ mice (n = 10) (*E*–*G*) and $Dectin\text{-}1^{+/-}$ (n = 5) or $Dectin\text{-}1^{-/-}$ mice (n = 6) (*H*–*J*) were immunized s.c. with OVA (100 μg) and K3-SPG [10 μg (*E*–*G*) or 1 μg (*H*–*J*)] at days 0 and 10. Seven days after the last immunization, OVA-specific serum IgG (*E* and *H*), IFN-γ (*F* and *I*), and $OVA_{257–264}$-specific tetramer (*G* and *J*) were monitored. *$P < 0.05$ (Mann–Whitney *U* test). Data represent one of two or three independent experiments with similar results.

base of the tail, both antigen and adjuvant reached the surface of draining inguinal lymph nodes (iLNs) within 1 h (Fig. 5 *A*, *B*, and *D*). After 24 h, some K3-SPG had moved to the CD3e⁺ T-cell area and colocalized with DQ-OVA (Fig. S6*A*). Those cells that contained both K3-SPG and DQ-OVA in the T-cell area of the iLNs were CD11c⁺ DCs (Fig. S6*B*).

Of interest, the majority of fluorescence signals remained on the surface of the iLNs (Fig. 5*A*), prompting us to focus on two types of macrophages known to be distributed on the LN surface, Siglec-1⁺ (also called CD169 or MOMA-1) macrophages (also known as subcapsular sinus macrophages) and MARCO⁺ macrophages (31). Histological analysis using conventional fluorescence microscopy did not suitably reveal the entire iLN surface; moreover, these macrophages were difficult to isolate for flow cytometric analysis (32, 33). Hence, we used two-photon microscopy imaging analysis to clarify the distribution of antigen and K3-SPG ex vivo. After the injection of anti-MARCO and –Siglec-1 antibodies, specific macrophages were visualized (Movie S1). When the iLN surface was monitored by two-photon microscopy at 1 h postinjection, OVA and K3-SPG were colocalized with MARCO⁺ but not Siglec-1⁺ macrophages (Fig. 5 *B* and *D*, Fig. S7 *A*–*D*, and Movies 2–5). Previous reports suggest that the immune complex and inactivated influenza virus are captured by Siglec-1⁺ macrophages to induce humoral immune responses (34, 35). The distribution pattern perfectly matched that for MARCO⁺ macrophages in the iLNs and did not colocalize with Siglec-1⁺ macrophages, as confirmed by Volocity's colocalization analysis (Perkin Elmer) (Fig. 5 *B*–*E*). In contrast, K3 was more diffusely distributed between MARCO⁺ and Siglec-1⁺ areas compared with K3-SPG (Fig. 5 *D* and *E*, Fig. S7 *C*–*E*, and Movies 6 and 7). Additionally, both *Tlr9*- and *Dectin-1*–deficient mice showed comparable localization of K3-SPG (Fig. S7 *F* and *G*).

To determine the contribution of these macrophages toward the adjuvant effects of K3-SPG, we examined different recovery kinetics of macrophages and DCs following an injection of clodronate liposomes into the base of the tail. After the injection, the macrophages were completely depleted by day 2. These cells did not recover for at least 1 wk, whereas DCs were mostly recovered by day 7, as previously reported (36). When both macrophages and DCs were depleted, immune responses were significantly suppressed [Fig. 5*F*, Clo (−d2)]. When only macrophages, but not DCs, were depleted, the immune responses were comparable to those in untreated mice [Fig. 5*F*, Clo (−d7)]. This would suggest that although both OVA and K3-SPG were mainly captured by

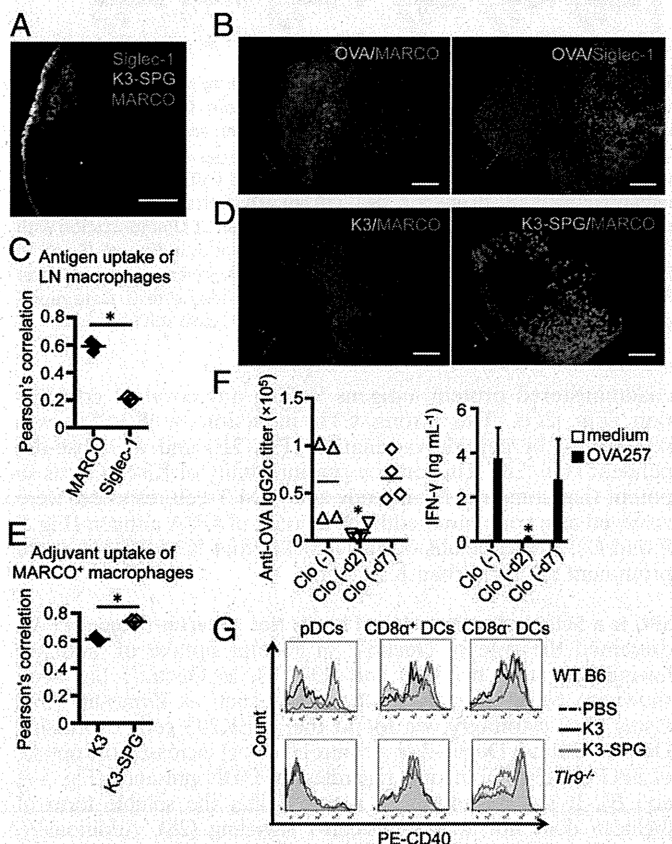

Fig. 5. Role of lymph node macrophages and dendritic cells in uptake and adjuvant effects of K3-SPG. (*A*) Immunohistochemistry of mouse inguinal LNs after Alexa 488-K3-SPG injection. One hour after injection, the LNs were collected and stained with anti–MARCO-phycoerythrin (PE) and anti–Siglec-1-APC antibodies. (*B*–*E*) Two-photon microscopic analysis of LNs. DQ-OVA, Alexa 488-K3, or Alexa 488-K3-SPG was injected as indicated, and anti–MARCO-PE or anti–Siglec-1-PE antibodies were administered. The LNs were collected 1 h later and analyzed by two-photon microscopy. (*C* and *E*) Colocalization of antigen or adjuvant with the stained macrophages was analyzed by Pearson's correlation. (*F*) Clodronate liposomes were injected into C57BL/6J mice either 2 or 7 d before immunization (n = 4). Mice were administered OVA (100 μg) plus K3-SPG (10 μg) at day 0. Eight days after immunization, OVA-specific serum IgG and IFN-γ were monitored. (*G*) C57BL/6J and $Tlr9^{-/-}$ mice were administered s.c. with K3 (10 μg) or K3-SPG (10 μg). At 24 h postadministration, the LNs were collected and the prepared cells were stained and analyzed by flow cytometry. (Scale bars, 100 μm.) *$P < 0.05$ (*t* test or Mann–Whitney *U* test). Data represent one of two or three independent experiments with similar results.

MARCO$^+$ macrophages in the LNs after injection, the macrophages were dispensable to inducing adaptive immune responses. In other words, the adjuvant effect of K3-SPG was largely dependent on the DC population.

K3-SPG Targets and Strongly Activates the Antigen-Bearing DC Population in Vivo. Our findings suggest that although a large portion of nanoparticulate K3-SPG was taken up by MARCO$^+$ macrophages in iLNs after injection, the adjuvant effects appear to be controlled by DCs. We focused on antigen and adjuvant uptake by the DC population in iLNs. At 24 h postinjection, the uptake of antigen and adjuvants by the DC population was analyzed by flow cytometry. The frequency of CpG-positives in three DC subsets (pDCs, CD8α^+ DCs, and CD8α^- DCs) was significantly increased after K3-SPG injection than with K3 (Fig. S8*A*). In contrast, the frequency of OVA-positive DCs was comparable after K3 and K3-SPG injections (Fig. S8*B*). When we focused on both antigen- and adjuvant-positive DCs, there was a substantial increase for K3-SPG over K3 (Fig. S9). Both pDCs and CD8α^+ DCs in iLNs were strongly activated by K3-SPG but not by K3 24 h postinjection, and this was completely dependent on TLR9 (Fig. 5*G*). Our results indicate that pDCs and CD8α^+ DCs preferentially capture nanoparticulate K3-SPG rather than nonparticulate K3 for maturation and to exert adjuvant effects.

K3-SPG Is a Potent Adjuvant for Influenza Vaccine in Murine and Nonhuman Primate Models. Finally, we sought the adjuvant effect of K3-SPG by using more clinically relevant influenza vaccination models in both mice and nonhuman primates. When mice were immunized with ether-treated hemagglutinin antigen-enriched virion-free split vaccine (SV) plus the indicated adjuvant, K3-SPG demonstrated superior adjuvant effects to K3 when antibody responses (Fig. S10*A*) and T-cell responses (Fig. S10*B*) were compared. More importantly, SV plus K3-SPG immunization resulted in a 100-fold greater antibody response, even compared with vaccination using a whole (virion) inactivated vaccine (WIV) (0.2 μg per mouse) (Fig. 6*A*), which contains viral RNA as a built-in adjuvant (21). Interestingly, SV (0.1 μg per mouse) plus K3-SPG strongly induced both CD8 and CD4 T-cell responses (Fig. 6*B*). Mice immunized with SV and K3-SPG exhibited less body weight loss than WIV-immunized mice (Fig. 6*C*). Strikingly, K3-SPG conferred 100% protection against lethal PR8 virus challenge at the dose of which only 10% of WIV-vaccinated mice survived (Fig. 6*D*). These results strongly support the notion that K3-SPG works as a potent adjuvant for protein or protein-based vaccines in a murine model, prompting us to extend this finding to a nonhuman primate model using the cynomolgus monkey (*Macaca fascicularis*). Each group of three cynomolgus monkeys was immunized with SV plus K3 or K3-SPG at days 0 and 14. Serum antibody titers were then monitored for 8 wk. The SV plus K3-SPG induced significantly higher antibody titer at 2 wk postimmunization, and titer levels remained high for at least another 6 wk (Fig. 6*E*). Although antibody titers were reduced at 110 wk after immunization, the K3-SPG group had higher antibody titers than the K3 group (Fig. 6*E*). When PBMCs were stimulated with SV and WIV, IFN-γ was detected from the SV plus K3-SPG–immunized group (Fig. 6*F*). Taken together, these results suggest that K3-SPG is a prominent vaccine adjuvant in a nonhuman primate model.

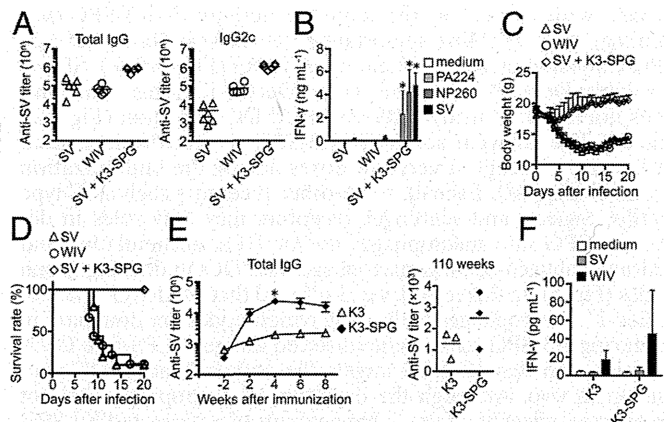

Fig. 6. K3-SPG acts as an influenza vaccine adjuvant in mice and nonhuman primates. (*A–D*) C57BL/6J mice (*n* = 6 or 10) were immunized with SV (0.1 μg), whole inactivated vaccine (WIV) (0.2 μg), or SV (0.1 μg) plus K3-SPG (10 μg) at days 0 and 14. Seven days after the final immunization, SV-specific serum IgG titers (*A*) and IFN-γ (*B*) [specific to SV antigen, $PA_{224-233}$ (PA224) (10 μg/mL) or $NP_{260-283}$ (NP260)] were monitored. (*C* and *D*) Fourteen days after the final immunization, mice were challenged with a 10-LD_{50} dose of influenza virus A/PR/8 (H1N1). Changes in body weights (*C*) and mortality (*D*) were monitored for the next 20 d. (*E* and *F*) Cynomolgus monkeys (*n* = 3) were immunized with SV (5 μg) plus K3 (5 nmol) or SV plus K3-SPG (5 nmol) at days 0 and 14. (*E*) Serum samples were collected at −2, 2, 4, 6, 8, and 110 wk. Antigen-specific serum antibody titers were measured by ELISA. (*F*) PBMCs were prepared from individual cynomolgus monkey blood at 4 wk after the first immunization and restimulated in vitro with medium, SV (10 μg), or WIV for 24 h. Mouse IFN-γ in the supernatants was determined by ELISA. *$P < 0.05$ (*t* test or Mann–Whitney *U* test).

Discussion

The medical need for novel, potent, and safe adjuvants is ever-increasing these days as (*i*) recombinant vaccine antigens such proteins and peptides are short on natural adjuvants, unlike attenuated or inactivated whole microbial antigens, (*ii*) conventional aluminum salts and oil adjuvants are limited or preferred for enhancing humoral immune responses, and (*iii*) new adjuvants that can induce cellular immune responses, including CTLs, are needed, for example for cancer vaccines. The last two decades have resulted in tremendous progress with respect to adjuvant research and development. A hallmark of the new generation of adjuvants is that nucleic acids have been rediscovered to be immunologically active in stimulating specific innate immune receptors of the host, in particular TLRs. CpG DNA, a ligand for TLR9, is one of the most promising immunotherapeutic agents that has been identified.

Although there are several types of potent humanized CpG ODN—K (also called B), D (A), C, and P types—the development of an all-in-one CpG ODN activating both B cells and pDCs to form a stable nanoparticle without aggregation has been less than successful. In this study, we generated a novel K CpG ODN that we designated K3-SPG. Although it had been reported that there are molecular interactions between single-stranded nucleic acids and β-glucan (37) and that murine and humanized CpG ODNs can be wrapped by SPG to increase their original TLR9-agonistic activities (20), our report demonstrates that a rod-shaped nano-sized K3-SPG particle exhibits dual characteristics of K and D CpG ODNs (Fig. 1). K3-SPG is distinct from other previously reported K CpG ODNs, including K3. In turn, K3-SPG becomes a D CpG ODN, stimulating human PBMCs to produce large amounts of both type I and type II IFN, targeting the same endosome where the IFN-inducing D type resides without losing its K-type activity (Fig. 1 *F* and *G*). Another surprising finding is that this K3-SPG forms a rod-like single nanomolecule (Fig. 1 *C* and *D*). This is advantageous over previously demonstrated D or P types, whose ends form higher-order structures that may hamper further development as prodrugs, including good manufacturing practice assignment.

Another prominent feature of this K3-SPG is its potency as an adjuvant for induction of both humoral and cellular immune responses, especially CTL induction, to coadministered protein antigens without conjugation. Such potent adjuvant activity of K3-SPG is attributable to its nanoparticulate nature (Figs. 1 *C* and *D* and 2) rather than targeting Dectin-1 by SPG (Figs. 3 and 4). Initially, we hypothesized that K3-SPG becomes such a potent adjuvant because it targets Dectin-1, because SPG is a β-1,3-glucan, and seems to be a clear Dectin-1 ligand (Fig. 3*A*). Our other results, however, led us to conclude that the role of Dectin-1

in vivo with respect to the adjuvant activity of K3-SPG was minimal (Fig. 4). More importantly, the in vivo activity of K3-SPG was completely dependent upon TLR9 (Fig. 4 *E–G*). SPG is a soluble Dectin-1 ligand but not a Dectin-1 agonist, and thus does not interfere with TLR9-mediated DC activation (Fig. 3 *D* and *E*). The adjuvant activity of K3-SPG is mostly independent of Dectin-1, except at very low doses during the immunization protocol (Fig. 4*J*). Instead, some other receptors such as C-type lectins, Siglecs, and scavenger receptors may play roles in delivering SPG into macrophages and/or DCs, accumulating and activating antigen-bearing macrophages and DCs in draining lymph nodes (Fig. 5). In this regard, we also found that $MARCO^+$, but not $Siglec\text{-}1^+$, macrophages in draining lymph nodes are dominant in capturing K3-SPG, and coadministered antigen (LPS-free OVA protein), and that K3-SPG targets the antigen-bearing DC population in vivo. Although the depletion of macrophages did not ameliorate adjuvant effects, large amounts of antigen and K3-SPG are taken up by the same $MARCO^+$ macrophages, and the two-photon microscopic data suggest that they are activated as they become much bigger than nonstimulated macrophages. Whether this massive accumulation of antigen and adjuvant in $MARCO^+$ macrophages contributes to the following DC activation and adaptive T- and B-cell activation is yet to be elucidated in future work.

The protective potency of K3-SPG as an influenza vaccine adjuvant was demonstrated in vivo in both murine and nonhuman primate models. In the murine model, intradermal immunization with a very low dose of seasonal influenza split vaccine mixed with K3-SPG in solution provoked robust IgG responses and offered better protection than a low but physiological dose of whole inactivated virion vaccination against the heterologous challenge of lethal virus (Fig. 6 *C* and *D*). These data provide better protective potency than our previous results, where we used approximately 10 times higher doses of influenza antigens (21), because many factors for K3-SPG have been improved for its potency: K3–SPG complexation efficiency and optimization of the order between K3 and poly(dA_{40}) (Fig. 1); the immunization route is different as well. The data above prompted us to develop K3-SPG as a potent adjuvant for influenza split vaccine, especially for those urgently needing improvement: seasonal influenza vaccination for the elderly, immunodeficient patients (transplant recipients), and pandemic influenza vaccination.

Taken together, these data suggest that K3-SPG can be used as a potent adjuvant for protein vaccines such as influenza split vaccines, and may be useful for immunotherapeutic applications that require type I and type II IFN as well as CTL induction.

Materials and Methods

All animal studies using mice and monkeys were conducted in accordance with the Institutional Animal Care and Use Committee at the National Institute of Biomedical Innovation. All of the ODNs used in this manuscript were synthesized by GeneDesign. Other details are described in *SI Materials and Methods*.

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Review

Innate Immune Signaling by, Genetic Adjuvants for, DNA Vaccination

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Abstract: DNA vaccines can induce both humoral and cellular immune responses. Although some DNA vaccines are already licensed for infectious diseases in animals, they are not licensed for human use because the risk and benefit of DNA vaccines is still controversial. Indeed, in humans, the immunogenicity of DNA vaccines is lower than that of other traditional vaccines. To develop the use of DNA vaccines in the clinic, various approaches are in progress to enhance or improve the immunogenicity of DNA vaccines. Recent studies have shown that immunogenicity of DNA vaccines are regulated by innate immune responses via plasmid DNA recognition through the STING-TBK1 signaling cascade. Similarly, molecules that act as dsDNA sensors that activate innate immune responses through STING-TBK1 have been identified and used as genetic adjuvants to enhance DNA vaccine immunogenicity in mouse models. However, the mechanisms that induce innate immune responses by DNA vaccines are still unclear. In this review, we will discuss innate immune signaling upon DNA vaccination and genetic adjuvants of innate immune signaling molecules.

Keywords: DNA vaccine; innate immune responses; adjuvant; DNA sensor

1. Introduction of DNA Vaccines

Almost two decades ago, it was reported that plasmid DNA could induce adaptive immune responses against plasmid-encoded antigens [1], indicating it could be used in novel therapeutic applications as a human vaccine for the prevention of various pathogen infections [2], autoimmunity [3], allergy [4], neurological disorders [5], and cancer [6]. In the veterinary field, some DNA vaccines are already licensed for West Nile virus in horse, infectious hematopoietic necrosis virus in salmon, and melanoma in dogs [7]. For human use, DNA vaccines have not been licensed, however, many candidate DNA vaccines are being studied in ongoing clinical trials. The clinical benefits of DNA vaccine are low cost, vaccine stability, high productivity, and easy modification of antigen in comparison with traditional protein vaccines. Conversely, it was reported that the immunogenicity of DNA vaccines was quite low according in clinical trials. Indeed, the immunogenicity of DNA vaccines tended to be weaker than other types of vaccines using live virus, virus vectors, or traditional protein plus adjuvant vaccines. Therefore, the immunogenicity of DNA vaccines was improved by changing promoters, codon usage of antigen sequences, the insertion of genetic adjuvants such as cytokines and innate immune activation molecules, strategies to prime and boost vaccination, and the route of administration [8].

Furthermore, elucidation of the molecular mechanisms of DNA vaccines is also important for developing DNA vaccines for human use. TANK-binding kinase 1 (TBK1), and stimulator of interferon genes (STING), was identified as an essential molecule for the induction of adaptive immune responses by DNA vaccination. In addition, double-stranded DNA (dsDNA) is a critical ligand of the STING-TBK1 signaling cascade [9]. These results indicate that dsDNA-induced innate immune signaling lead to induction of DNA-encoded antigen specific adaptive immune responses, like an adjuvant. However, DNA sensing machinery is still controversial. In this review, we will discuss innate immune signaling of DNA vaccines and genetic adjuvants of innate immune signaling molecules.

In 1990, Wolf *et al.* showed that the intramuscular administration of naked DNA led to the induction of DNA-encoded reporter genes in muscle cells [10]. Subsequently, Ulmer *et al.* demonstrated that the intramuscular administration of plasmid DNA encoding influenza viral protein induced encoded antigen-specific cytotoxic T lymphocyte (CTL) responses, which protected against lethal influenza virus infection [1]. These findings were the first evidence that naked DNA administration alone could induce adaptive immune responses against antigens expressed from plasmid DNA, and suggested that DNA vaccine strategies might be useful for clinical use. Indeed, many researchers evaluated novel DNA vaccines using experimental infectious diseases models [11]. The properties of DNA vaccines represent greater stability, low cost, high productivity, and possibility to improve immunogenicity. In 1998, the first human clinical trial of DNA vaccines against human immunodeficiency virus was reported [12]. This study evaluated the safety and efficacy of DNA vaccines. Importantly, one of the safety concerns for DNA vaccines was the integration of plasmid DNA into the host genome [13]. If integration occurs following DNA vaccination, the integrated-DNA may cause oncogene activation, tumor suppressor gene inactivation, or chromosomal instability. Fortunately, experimental data showed the rate of

plasmid DNA integration was lower than the natural rate of mutation in mammalian genomes [14]. Another safety concern is development of anti-DNA antibodies, associated with autoimmune disorders [15]. Anti-dsDNA antibody was increased in mouse after DNA vaccination [16]. In the clinical trials, anti-DNA antibody did not increase in any study subject [17]. However, the improvement of DNA vaccines to enhance immunogenicity may increase the risk of integration and development of anti-DNA antibody. Therefore, evaluation of safety concerns is essential before clinical trials are initiated. Subsequently, research groups have developed novel DNA vaccines against cancer, influenza virus, human papillomavirus, hepatitis, and malaria. However, the early clinical trials showed disappointing results.

1.1. Mode of Action

Although DNA vaccines can induce both humoral and cellular immune responses against plasmid-encoded antigens, the mode of action of DNA vaccines is still unclear. However, when DNA plasmids are administered to muscle, skin, subcutaneous, or the nasal cavity, it is believed that the DNA plasmid enters cells, translocates to the nucleus, and antigen is expressed by the host cellular machinery. In most cases, myocytes and antigen presenting cells (APCs), such as dendritic cells (DCs) or macrophages, appear to capture plasmid DNA. Subsequently, antigen protein is degraded and presented by major histocompatibility complex (MHC)-I in immune cells. Additionally, expressed-antigens can be secreted from cells by active secretion of the protein or released due to apoptosis of the transfected cell. Secreted antigen proteins are taken up, degraded, and presented by APCs on MHC-I and MHC-II molecules. Finally, APCs recruited to the draining lymph nodes activate naïve B cells, CD4+ and CD8+ T cells. In many cases, secreted antigen proteins could induce both IgG1 and IgG2a/c antibody, and cytosolic protein antigens could induce IgG2a/c antibody.

1.2. Methods of DNA Vaccination

Intramuscular electroporation (imEPT) is one method of DNA vaccine administration, which overcomes limitations such as low transfection efficacy and insufficient recruitment of APCs to the injection site, by inducing transient enhancement of cell membrane permeability. Consequently, the increased uptake of DNA into the host cell and induction of low level of inflammation can enhance the influx of APCs to the injection site [18]. This method induces potent immune responses including CTL responses, and is therefore a convenient method for analyzing the intracellular signaling cascade of DNA vaccines. Indeed, for most cases, the contribution of innate immune activation by DNA vaccination is evaluated using imEPT in mouse models. Gene gun [19], needle-free systems [20], and mucosal delivery [21] are studied as other methods for DNA vaccination; however, these methods have not been examined to elucidate the innate immune signaling of DNA vaccination. It is important whether these vaccination methods activate same innate immune signaling cascade.

2. Innate Immunity and DNA Vaccines

2.1. Immunostimulatory Properties of Double-Stranded DNA

At present, it is known that nucleic acids such as DNA and RNA induce innate immune responses such as type I interferon (IFN) and inflammatory cytokine production. Interestingly, the innate immune

activation of DNA is affected by DNA structure and conformation. In 1963, it was reported that rat liver derived-DNA or RNA stimulation could produce type I IFN from chick cells [22]. In 1984, Bacillus Calmette-Guérin-derived DNA was shown to have strong anti-tumor activity [23]. These findings were the first evidence that both host and bacterial DNA induced innate and adaptive immune responses. Subsequently, bacteria-derived unmethylated CpG DNA and synthetic CpG oligonucleotide (ODN) were shown to be direct stimulators of B cells [24]. Additionally, Toll-like receptor 9 (TLR9) was identified as a receptor for CpG motif DNA that activated innate immune responses in immune cells, such as DCs, B cells, and macrophages [25]. Meanwhile, host DNA-induced innate immune activation was forgotten and ignored. In 1999, the independent effects of unmethylated CpG motifs or specific DNA sequences were shown as at least 25 base pairs of synthetic double-stranded (ds), but not single-stranded (ss) DNA up-regulated the expression of genes related to immune responses [26]. Later, the B-form conformation of dsDNA was shown to be more effective at inducing innate immune responses than the Z-form of dsDNA [27]. Stimulation with synthetic B-form dsDNA, poly (dA-dT) poly (dA-dT), resulted in the induction of type I IFN and IFN-inducible chemokines, whereas stimulation with synthetic Z-form dsDNA, brominated poly (dG-dC) poly (dG-dC) only induced CXCL10 release.

Studies then focused on adaptive immune responses and demonstrated genomic DNA derived from dead cells induced the maturation of APCs and cellular immune responses, especially CTL responses [28]. In addition, traditional aluminum adjuvant induced cell death and host-derived DNA release, which induced antigen specific IgE production [29]. These results indicate that the immunostimulatory effect of self-DNA could cause the induction of innate immune responses and side-effects in the host. Adverse effects of aberrant DNA have been shown in relation to the function of DNase, an enzyme that digests DNA. DNase II-deficient mice failed to digest DNA from engulfed nuclei of erythroblasts in hepatic macrophages and resulted in the robust production of type I IFN and inflammatory cytokines, which caused severe anemia and rheumatoid arthritis (RA)-like symptoms in a TLR9-independent manner [30,31]. DNase I and DNase III knockout mice developed systemic lupus erythematosis-like symptoms and inflammatory myocarditis, respectively [32–34]. The functional mutations of DNase I and DNase III in humans were also shown to cause several autoimmune disorders, such as systemic lupus erythematosis [33,35], Aicardi-Goutieres syndrome [36], familial chilblain lupus [37], or retinal vasculopathy with cerebral leukodystrophy [38]. Thus, DNA-induced immune responses are not only involved in the prevention of microbial infection but also of autoimmune responses. These findings indicate that normal cells are equipped with innate sensing machineries to remove aberrant genomic DNA fragments.

2.2. Cellular Signaling of DNA Vaccines

In general, DNA vaccines derived from bacterial plasmids contain unmethylated CpG motifs recognized by TLR9, which induce innate immune responses [25]. Therefore, many researchers have attempted to clarify whether TLR9-induced innate immune responses are required for immunogenicity of DNA vaccines. Unexpectedly, some reports suggested that TLR9 was not essential for the induction of immune responses of DNA vaccines *in vivo*, although plasmid-induced cytokine production from immune cells was completely dependent on TLR9 *in vitro* [39,40]. Importantly, dsDNA, including plasmid DNA, could activate both immune cells and non-immune cells such as fibroblasts or