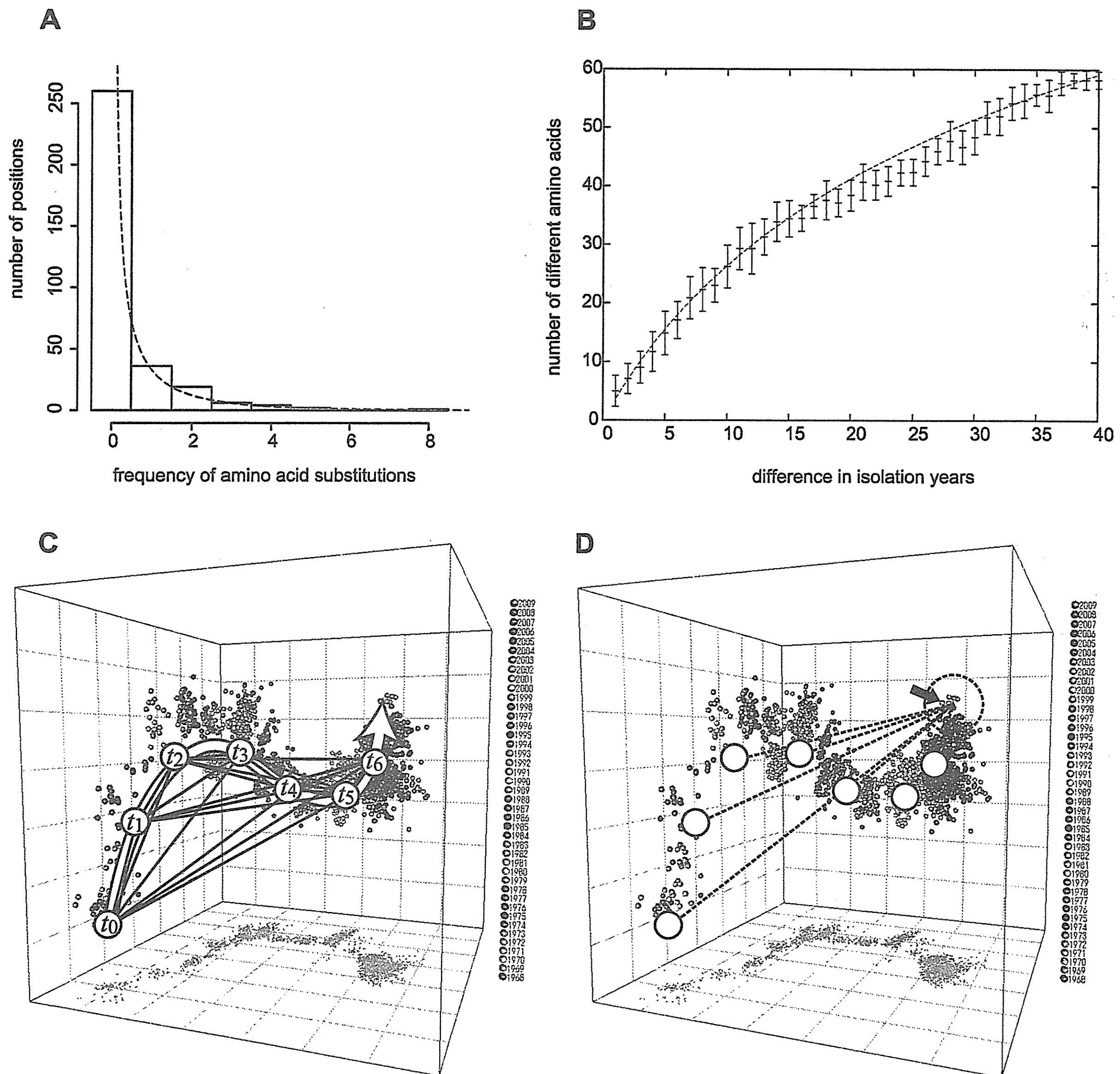

Figure 3. The amino acid substitutions on the gnarled trunk. (A) the distribution of the number of positions that were substituted on the trunk from 1968 to 2009. The gamma distribution that has a mean substitution frequency of 0.418 and a variance of 1.155 is superimposed. (B) The number of different amino acids between the trunk sequences plotted against the difference in their isolation years. The horizontal bars indicate mean values, and vertical lines indicate ±1 standard deviation of the number of different amino acids. A non-linear regression curve using the formula $d = 328 \times (1 - (a/(a + \bar{r}t))^a)$ is superimposed ($a = 0.129$, $\bar{r} = 0.0118$), showing good fit to the actual data ($P < 0.001$). (C) A schematic illustration of trunk viruses and the sequence dissimilarities among them. A trunk virus is a virus located near the main trunk. Circles labelled with $t_0, \ldots, t_6$ are examples of trunk viruses. The sequence dissimilarities among trunk sequences are shown by solid lines. (D) The selection of a Leading Bud. Using the formula of $d = 328 \times (1 - (a/(a + \bar{r}t))^a)$, one may calculate the expected sequence dissimilarities between a future trunk virus and past trunk viruses (dotted lines). The bold arrow indicates a candidate for Leading Buds. For the coming influenza season in 2010, A/Thailand/CU-B110/2009(H3N2) was predicted to be the most likely candidate virus (Table S2).
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was constantly curved in the MDS-constructed 3D space. This unique property of the sequence evolution indicated that the evolution on the trunk favoured multiple substitutions at the same positions on HA molecules. Our study found that the curvature was relatively constant and reasonably explained by assuming that the rate of amino acid substitutions on HA varied from one position to another according to a gamma distribution. The estimated parameters of the gamma distribution allowed us to predict the amino acid substitutions on HA in subsequent years with reasonable accuracy.

The small value of its gamma parameter estimated in this study suggested that most of amino acids on HA remained unchanged,

Table 1. Results of retrospective tests for the prediction of amino acid substitutions.

Test Year	The number of predicted substitutions	The number of actual substitutions	The number of correctly predicted substitutions	Recall	Precision
1998	9	8	8	1.00	0.89
1999	4	3	1	0.33	0.25
2000	4	5	2	0.40	0.50
2001	10	5	5	1.00	0.50
2002	7	3	1	0.33	0.14
2003	13	12	11	0.92	0.85
2004	4	5	3	0.60	0.75
2005	2	0	0	-	0.00
2006	4	2	2	1.00	0.50
2007	5	2	0	0.00	0.00
2008	4	1	0	0.00	0.00
2009	7	1	0	0.00	0.00
overall	73	47	33	0.70	0.45

Recall was defined as the number of correctly predicted substitutions divided by the total number of actual substitutions. Precision was defined as the number of correctly predicted substitutions divided by the total number of predicted amino acid substitutions.
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but amino acid substitutions occurred at a relatively restricted number of positions on the HA. The result was consistent with previous studies identifying several positions that had undergone the positive Darwinian selection, where non-synonymous mutations have been favoured [16,17,28,31,32]. Although the positions that undergo amino acid substitutions could have been moving to different positions over time [33], our analysis indicated that the relative sequence distance between two trunk sequences remained roughly constant with respect to the difference in their isolation years. This stable feature allowed us to predict the relative sequence distance between two viruses located on the trunk, and led us a fully-computerized prediction method.

He and Deem have recently pointed out that an MDS visualization with density estimation allowed us to identify a cluster of 'incipient dominant strains' before it became dominant [15]. They proposed two important criteria for the selection of a new vaccine strain. The first criterion is that a new cluster that does not contain currently circulating strains or vaccine strains is detected. The second criterion is that the current vaccine strain does not provide high protection against strains in the new cluster. The reason why our gamma-distribution-based model achieves high recall and precision can be explained by their two criteria. First of all, the recognition of a Leading Bud conceptually corresponds to the detection of a newly emerging cluster of incipient dominant strains. Since a Leading Bud described in this paper should have a certain amount of different amino acids from dominantly circulating strains, a Leading Bud can be considered as one of the early isolates in a newly emerging cluster. Although we do not consider whether Leading Buds form a cluster or not, this property partially fits their first criterion. Secondly, our gamma distribution-based method recognizes a Leading Bud by finding an HA sequence that has the amino acid substitutions at the same positions as those seen in the past evolution. Since most of past amino acid substitutions are concentrated in the antigenic sites (Table S1), it is highly likely that the Leading Bud having amino acid substitutions at these positions antigenically differ from the dominantly circulating strain and the vaccine strain. This could meet the second criterion.

The overall recall of our prediction method was around 0.70, indicating that the model had a reasonable ability to predict amino acid substitution in the subsequent year for each year. It should be noted that this high recall was achieved by the prediction method that relied only upon the number of different positions in the amino acid sequences and the isolation year of the viruses. The overall precision, on the other hand, was around 0.45. Some of mistaken predictions might be attributable to the delayed

Table 2. Comparison of overall recall and precision with other methods.

Method	Overall Recall	Overall Precision
Select a sequence randomly (n = 100)	0.22±0.012	0.24±0.015
Select the one that has the maximum numbers of substitutions at the 18 codons identified by Bush et al	0.49	0.34
Select the one that has the maximum numbers of substitutions from the current dominant sequence	0.51	0.13
Select the one that has the maximum numbers of substitutions from past dominant sequences	0.55	0.25
Select the one that has the maximum numbers of substitutions from the dominant sequence of two years ago	0.70	0.23
Select the one that has the maximum numbers of substitutions at antigenic domains A–E	0.70	0.32
Select the one that has the minimum errors from the gamma-distribution-based expectation	0.70	0.45

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appearance of amino acid substitutions in the dominant sequences on the trunk. For instance, the predicted substitutions for I144N in 1999, R50G in 2000 and 2001, E83K V202I, W222R G225D in 2001, and V226I and S227P in 2002 did not occur in the next years, but rather 2 or 3 years later (Table S2). For these idle periods, the prediction method could have looked too far ahead, and the Leading Buds might be too early to be used as vaccine strains.

In the retrospective tests, we evaluated the prediction methods by comparing the amino acid sequences of the Leading Buds with the dominant sequences in subsequent years. It was also confirmed that WHO-recommended vaccine strains had amino acid residues that were identical to those predicted by our method (Table S2). The major difference lies in the timing. We accept that an overhasty selection of vaccine strains might lead antigenic mismatch between vaccine and epidemic strains. The prediction of the exact timing of the antigenic change could become a subject of future study. In addition to the timing of antigenic change, a careful investigation on their characteristics such as antigenicity and growth in embryonated hen eggs must be practically important for the vaccine selection.

Vaccine strains must be selected in order to match the antigenicity of viruses that will circulate in the influenza season. The antigenic cartography, which was developed by Smith et.al, enables us to accurately predict antigenic similarity between two virus strains based on a large collection of hemagglutination inhibition (HI) assay data [5]. In the period from 2005 to 2006, for instance, the circulating H3N2 viruses changed from A/California/7/2004-like viruses to A/Wisconsin/67/2005-like viruses [15,34]. The Leading Bud found in 2005 was A/Okinawa/18/2005. The antigenic-cartography-based antigenic distance between A/Okinawa/18/2005 and A/Wisconsin/67/2005 was found to correspond to a twofold difference in HI titers of antisera [34]. Therefore, it is likely that the antigenicity of A/Okinawa/18/2005 could match the epidemic strain in 2006. Although the Leading Buds may not be perfect candidates for the vaccine strains, we believe that our prediction method could provide useful information for the formulation of influenza vaccines.

Retrospective tests for 2006–2007, 2007–2008, and 2008–2009 failed to predict the actual amino acid substitutions. The low recall and precision are likely due to the limited number of amino acid substitutions during these periods. Since antigenic changes of H3N2 viruses occur every 3–5 years in a punctuated manner [5], the conservation of dominant sequences in a few contiguous years is common in the evolution of H3N2 viruses. We have not taken such periodicity into account in our evolutionary model, and our method could not predict the exact timing when a dominant strain is replaced by another strain. This result highlighted the need to develop a method that can predict the exact timing of the antigenic change of the virus. However, the HA of the dominant H3N2 virus after the September in 2009 possessed 3 of 6 amino acid substitutions that were predicted by our method using the sequence data before August 2009 (Table S2). A/Perth/16/2009, a similar strain to our Leading Bud (A/Thailand/CU-B110/2009), was recommended as a vaccine strain for H3N2 viruses by WHO on Dec 1st in 2010.

Further understanding of the gnarled trunk might be achieved by combined efforts with experimental studies. Future research direction include the association of the gnarled trunk evolution with the prediction of antigenic evolution [5,35,36], the effect of mutations upon biological activity of the protein [37,38], and the effect of cross immunity to previously circulating dominant strains [21,22]. Finally, the 3D visualization technique we present here enables us to represent the direction of sequence evolution as well as sequence phylogeny, providing additional information that is not obtained via traditional phylogenetic analysis.

Materials and Methods

Sequence Data

Nucleotide sequences for HA genes of H3N2 influenza A viruses isolated from humans during the period from 1968 to 2009 were downloaded from the Influenza Virus Resource at the National Center for Biotechnology Information (NCBI) [39] on Feb 23 in 2010. The isolation date of the latest sequence was July 11th 2009. After eliminating sequences that contained ambiguous nucleotide codes, 6,806 amino acid sequences of the HA1 domain were determined by translating the nucleotide sequences using the standard genetic code. All the amino acid sequences were 328 amino acids long. Nucleotide sequences that gave an identical amino acid sequence were grouped together, and the nucleotide sequence having the fewest mutations from the pandemic strain in 1968 was used as a representative. By removing all identical amino acid sequences except one, 2,640 unique amino acid sequences were obtained.

MDS analysis

For every pair of the 2,640 amino acid sequences of the HA1 domain, the sequence dissimilarity, which is the total number of positions where the two sequences possess different amino acids, was calculated. The resulting 3,483,480 pair-wise dissimilarities were stored in a dissimilarity matrix. The SMACOF algorithm [24] was used to find the optimal coordination of all sequences in the 3D map to minimise the sum of squared errors:

$$\sum_{s_1,s_2 \in HA1} (d(s_1,s_2) - d_M(s_1,s_2))^2,$$

where s_1 and s_2 are sequences of the HA1 domain, $d(s_1,s_2)$ is the sequence dissimilarity, $d_M(s_1,s_2)$ is the Euclidean distance in the 3D map.

Amino Acid Substitution on the Trunk

A parsimony tree of HA was constructed from a total of 2,640 nucleotide sequences of the HA1 domain. The dnapars program in the PHYLIP package[24] was used to construct the parsimony tree. The main trunk of the tree was defined as the longest path from the HA of the pandemic strain in 1968 to the HA of a strain circulating in 2009. The hypothetical nucleotide sequence on each trunk was translated into an amino acid sequence. For each residue position of the HA1 domain, amino acid substitutions found on the trunk were counted, and then the mean and variance of the substitution frequency were calculated and compared with a gamma distribution having the same mean and variance.

Substitution Model

When assuming the variation of substitution rates follows a gamma distribution, the expected sequence distance between two sequences can be calculated by the formula $d = L(1-(a/(a+\bar{r}t))^a)$, where t is the difference in their isolation years and $L = 328$ is the length of the HA1 domain. To obtain non-hypothetical amino acid sequences located near the trunk, a neighbour-joining tree was constructed from their nucleotide sequences. The tree construction was done using the dnadist and neighbor programs in the PHYLIP package [40] with the Jukes-Cantor distance option. For each trunk node of the neighbour

joining tree, the amino acid sequence having shortest path to the trunk node was selected. Out of 2,640 HA sequences, 91 sequences were selected as trunk sequences (Table S3). For every pair of these selected sequences, their sequence dissimilarity (d) and the difference in isolation years (t) was recorded. By fitting the formula $d = L(1-(a/(a+\bar{r}t))^a)$to the observed relationship between $d$and t, the gamma parameter (a) and mean substitution rate ($\bar{r}$) for our substitution model were estimated. In order to estimate the effect of the selection of trunk sequences on the Figure 3B, we performed a bootstrap resampling analysis. We made 100 datasets each containing 91 trunk sequences obtained by random resampling of the original 91 trunk sequences. Then, errors on the mean number of different amino acids were estimated using the 100 bootstrap datasets. As shown in the Figure S3, the errors of the means were estimated to be around one amino acid, suggesting that the result shown in Figure 3 has moderate robustness to the selection of trunk sequences.

Dominant sequence

To define a representative sequence for each year, we adopted a strategy using the majority vote rule. For each year and each residue position, the dominant amino acid was determined as the amino acid that constituted the majority at the position in the year. The dominant sequence of a year was defined as the concatenation of the dominant amino acids of every position in the year.

Prediction and retrospective tests

For each year, a Leading Bud, which is an amino acid sequence that will be located near the trunk in the next year, was predicted as follows. Let y be a year. For a future HA sequence $s$that would appear in the year $y+1$, the expected number of different amino acids from each past trunk sequence $u$in year y' $(y-y' \geq 0)$can be denoted by the formula $d_E(s,u) = L(1-(a/(a+\bar{r}(y-y'+1)))^a)$. Thus, among sequences isolated in the year y, the sequence that is most likely to become a Leading Bud in the next year is formulated as the sequence s', such that s' has the least sum of squared errors between sequence distance and expected distance from each trunk. The sum of squared errors is calculated by the formula:

$$\sum_{u \in T_{\leq y}} \frac{(d(s',u) - d_E(s',u))^2}{d_E(s',u)},$$

where $T_{\leq y}$ is a set of trunk sequences whose isolation years are earlier than or equal to$y$. The amino acid sequence of the predicted Leading Bud was compared with the dominant sequence for the year, and a set of amino acid substitutions from the dominant sequence to the sequence of the Leading Bud was presented as predicted substitutions. After the prediction was made, the predicted substitutions were compared by the actual amino acid substitutions that occurred in the next year. Recall was calculated as the number of correctly predicted substitutions divided by the total number of actual substitutions. Precision was calculated as the number of correctly predicted substitutions divided by the total number of predicted amino acid substitutions.

Supporting Information

Figure S1 Scatter diagram of numbers of different amino acids vs. corresponding distances in the 3D map. For every pair of two sequences, the actual numbers of different amino acids (X-axis) were plotted against corresponding distances in the 3D map (Y-axis). Horizontal bars show the mean values, and vertical lines indicate ±1SD of distances in the 3D map.
(EPS)

Figure S2 Three-dimensional map of the human H1N1 influenza A viruses. A total of 1228 amino acid sequences of the HA1 domain of human H1N1 influenza A viruses isolated during 1918 to 2010 are visualized in the 3D space. Each point represents an HA sequence, colour-coded by the isolation year of the virus. The whole coordination is determined by MDS analysis. All three axes represent sequence dissimilarity (spacing between grid lines represents 10 different amino acids), and the configuration can be freely rotated and translated. Shadows represent projections of points on a coordinate plane. Bold arrows on the left, middle, and right indicate a seasonal H1N1 virus isolated in 2009, the pandemic H1N1 virus in 1918, and the pandemic H1N1 virus in 2009, respectively.
(EPS)

Figure S3 Boot strap resampling analysis of the mean substitution frequency on the trunk. A total of 100 bootstrap datasets obtained were generated by random resampling of the original 91 trunk sequences. Errors on the mean number of different amino acids were estimated to be around one amino acid. A curve using the formula $d = 328 \times (1-(a/(a+\bar{r}t))^a)$ is superimposed ($a = 0.129$, $\bar{r} = 0.0118$).
(EPS)

Table S1 The amino acid positions that were substituted one or more time. The positions on HA where amino amino acid substitution were occurred on the trunk are shown with their frequency. Each alphabet represents the antigenic domain to which the position belongs.
(DOC)

Table S2 Selected Leading Buds, predicted substitutions, and actual substitutions in the retrospective tests. Correctly predicted substitutions are shown in bold-face. The predicted substitutions that did not occur in the next years but occurred 2 or 3 years later are underlined. Different amino acids on HA between a new WHO vaccine strain and preceding vaccine strain are shown in the rightmost column.
(DOC)

Table S3 The HA sequences located near the main trunk.
(DOC)

Movie S1 Movie of 3D map of HA sequences of H3N2 human influenza A viruses.
(MP4)

Movie S2 Movie of 3D map of HA sequences of H1N1 human influenza A viruses.
(MP4)

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Author Contributions

Conceived and designed the experiments: AT HK KI. Analyzed the data: KI MI YM. Contributed reagents/materials/analysis tools: SI TM. Wrote the paper: KI AT.

References

1. Laver WG, Air GM, Webster RG (1981) Mechanism of antigenic drift in influenza virus : Amino acid sequence changes in an antigenically active region of Hong-Kong (H3N2) influenza virus hemagglutinin. J Mol Biol 145: 339–361.
2. Both GW, Sleigh MJ, Cox NJ, Kendal AP (1983) Antigenic drift in influenza virus H3 hemagglutinin from 1968 to 1980: Multiple evolutionary pathways and sequential amino acid changes at key antigenic sites. J Virol 48: 52–60.
3. Wilson IA, Cox NJ (1990) Structural basis of immune recognition of influenza virus hemagglutinin. Annu Rev Immunol 8: 737–771.
4. Cox NJ, Bender CA (1995) The molecular epidemiology of influenza viruses. Semin Virol 6: 359–370.
5. Smith DJ, Lapedes AS, de Jong JC, Bestebroer TM, Rimmelzwaan GF, et al. (2004) Mapping the antigenic and genetic evolution of influenza virus. Science 305: 371–376.
6. Wright PF, Neumann G, Kawaoka Y (2007) Orthomyxoviruses. In: Knipe DM, Howley PM, eds. Fields Virology, 5 ed Philadelphia: Lippincott Williams & Wilkins. pp 1691–1740.
7. de Jong JC, Beyer WEP, Palache AM, Rimmelzwaan GF, Osterhaus ADME (2000) Mismatch between the 1997/1998 influenza vaccine and the major epidemic A(H3N2) virus strain as the cause of an inadequate vaccine-induced antibody response to this strain in the elderly. J Med Virol 61: 94–99.
8. Treanor J (2004) Weathering the influenza vaccine crisis. N Engl J Med 351: 2037–2040.
9. Webster RG, Laver WG (1980) Determination of the number of nonoverlapping antigenic areas on Hong Kong (H3N2) influenza virus hemagglutinin with monoclonal antibodies and the selection of variants with potential epidemiological significance. Virology 104: 139–148.
10. Bush RM, Bender CA, Subbarao K, Cox NJ, Fitch WM (1999) Predicting the evolution of human influenza A. Science 286: 1921–1925.
11. Ferguson NM, Anderson RM (2002) Predicting evolutionary change in the influenza A virus. Nat Med 8: 562–563.
12. Plotkin JB, Dushoff J, Levin SA (2002) Hemagglutinin sequence clusters and the antigenic evolution of influenza A virus. Proc Natl Acad Sci U S A 99: 6263–6268.
13. Nelson MI, Simonsen L, Viboud C, Miller MA, Taylor J, et al. (2006) Stochastic processes are key determinants of short-term evolution in influenza A virus. PLoS Pathog 2: 1144–1151.
14. Wolf YI, Viboud C, Holmes EC, Koonin EV, Lipman DJ (2006) Long intervals of stasis punctuated by bursts of positive selection in the seasonal evolution of influenza A virus. Biol Direct 1: 34.
15. He J, Deem MW (2010) Low-dimensional clustering detects incipient dominant influenza strain clusters. Protein Eng Des Sel 23: 935–946.
16. Fitch WM, Leiter JME, Li XQ, Palese P (1991) Positive Darwinian evolution in human influenza A viruses. Proc Natl Acad Sci U S A 88: 4270–4274.
17. Fitch WM, Bush RM, Bender CA, Cox NJ (1997) Long term trends in the evolution of H(3) HA1 human influenza type A. Proc Natl Acad Sci U S A 94: 7712–7718.
18. Nelson MI, Holmes EC (2007) The evolution of epidemic influenza. Nat Rev Genet 8: 196–205.
19. Holmes EC, Ghedin E, Miller N, Taylor J, Bao YM, et al. (2005) Whole-genome analysis of human influenza A virus reveals multiple persistent lineages and reassortment among recent H3N2 viruses. PLoS Biol 3: 1579–1589.
20. Lavenu A, Leruez-Ville M, Chaix ML, Boelle PY, Rogez S, et al. (2006) Detailed analysis of the genetic evolution of influenza virus during the course of an epidemic. Epidemiol Infect 134: 514–520.
21. Ferguson NM, Galvani AP, Bush RM (2003) Ecological and immunological determinants of influenza evolution. Nature 422: 428–433.
22. Koelle K, Cobey S, Grenfell B, Pascual M (2006) Epochal evolution shapes the phylodynamics of interpandemic influenza A (H3N2) in humans. Science 314: 1898–1903.
23. Pybus OG, Rambaut A (2009) Evolutionary analysis of the dynamics of viral infectious disease. Nat Rev Genet 10: 540–550.
24. Borg I, Groenen PJF (2005) Modern multidimensional scaling: Theory and applications. New York: Springer.
25. Palese P, Shaw ML (2007) Orthomyxoviridae: the viruses and their replication. In: Knipe DM, Howley PM, eds. Fields Virology, 5 ed Philadelphia: Lippincott Williams & Wilkins. pp 1647–1689.
26. Neumann G, Noda T, Kawaoka Y (2009) Emergence and pandemic potential of swine-origin H1N1 influenza virus. Nature 459: 931–939.
27. Shinde V, Bridges CB, Uyeki TM, Shu B, Balish A, et al. (2009) Triple-reassortant swine influenza A (H1) in humans in the United States, 2005-2009. New Eng J Med 360: 2616–2625.
28. Bush RM, Fitch WM, Bender CA, Cox NJ (1999) Positive selection on the H3 hemagglutinin gene of human influenza virus A. Mol Biol Evol 16: 1457–1465.
29. Nei M, Chakraborty R, Fuerst PA (1976) Infinite allele model with varying mutation rate. Proc Natl Acad Sci U S A 73: 4164–4168.
30. Ota T, Nei M (1994) Estimation of the number of amino acid substitutions per site when the substitution rate varies among sites. J Mol Evol 38: 642–643.
31. Suzuki Y (2004) Three-dimensional window analysis for detecting positive selection at structural regions of proteins. Mol Biol Evol 21: 2352–2359.
32. Kryazhimskiy S, Bazykin GA, Plotkin J, Dushoff J (2008) Directionality in the evolution of influenza A haemagglutinin. Proc R Soc Lond B 275: 2455–2464.
33. Blackburne BP, Hay AJ, Goldstein RA (2008) Changing selective pressure during antigenic changes in human influenza H3. PLoS Pathog 4: e1000058.
34. Russell CA, Jones TC, Barr IG, Cox NJ, Garten RJ, et al. (2008) The global circulation of seasonal influenza A (H3N2) viruses. Science 320: 340–346.
35. Gupta V, Earl DJ, Deem MW (2006) Quantifying influenza vaccine efficacy and antigenic distance. Vaccine 24: 3881–3888.
36. Liao YC, Lee MS, Ko CY, Hsiung CA (2008) Bioinformatics models for predicting antigenic variants of influenza A/H3N2 virus. Bioinformatics 24: 505–512.
37. Nakajima K, Nobusawa E, Tonegawa K, Nakajima S (2003) Restriction of amino acid change in influenza A virus H3HA: Comparison of amino acid changes observed in nature and in vitro. J Virol 77: 10088–10098.
38. Tokuriki N, Tawfik DS (2009) Chaperonin overexpression promotes genetic variation and enzyme evolution. Nature 459: 668-U671.
39. Bao Y, Bolotov P, Dernovoy D, Kiryutin B, Zaslavsky L, et al. (2008) The influenza virus resource at the National Center for Biotechnology Information. J Virol 82: 596–601.
40. Felsenstein J (1989) PHYLIP-phylogeny inference package (version 3.2). Cladistics 5: 164–166.

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Current Opinion in Virology

Innate and adaptive immune responses to viral infection and vaccination

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Recent accumulating evidence suggests that the human immune system possesses a variety of innate receptors that recognize, distinguish, and respond to viral infections and to vaccination. These include Toll-like receptors, C-type lectin receptors, RIG-I-like receptors, Nod-like receptors and possibly AIM2-like receptors. However, the precise mechanisms by which these receptors exert their critical roles in the induction of virus-specific adaptive immune responses have not been fully elucidated. In this review, we discuss recent advances in our understanding of the innate immune recognition of viruses and the differential connection to the adaptive immune responses induced by infection or vaccination, with a particular focus on the influenza virus.

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Introduction

Several families of innate immune receptors, including Toll-like receptors (TLRs) [1], C-type lectin receptors [2], RIG-I-like receptors (RLRs) [3], Nod-like receptors (NLRs) [4], and AIM2-like receptors (ALRs) [5] have been identified over the last decade. Generally, these germ-line-encoded receptors recognize 'non-self' molecules derived from a variety of microbes. Some of these receptors also recognize danger signals sent out by damaged cells/tissues [6]. These innate immune receptors are critical for the initiation and regulation of host immune responses against infection and autoimmunity [7]. Furthermore, it is evident that innate immune responses are extremely important for establishing effective adaptive immune responses to infection and vaccination [8•,9,10]; although it is still not clear whether all innate responses contribute equally to the induction of adaptive responses [8•,11•,12•]. In the following sections, we briefly review the current knowledge about virus recognition by innate immune receptors, and discuss the connections between the innate and adaptive immune responses, using influenza virus as an example.

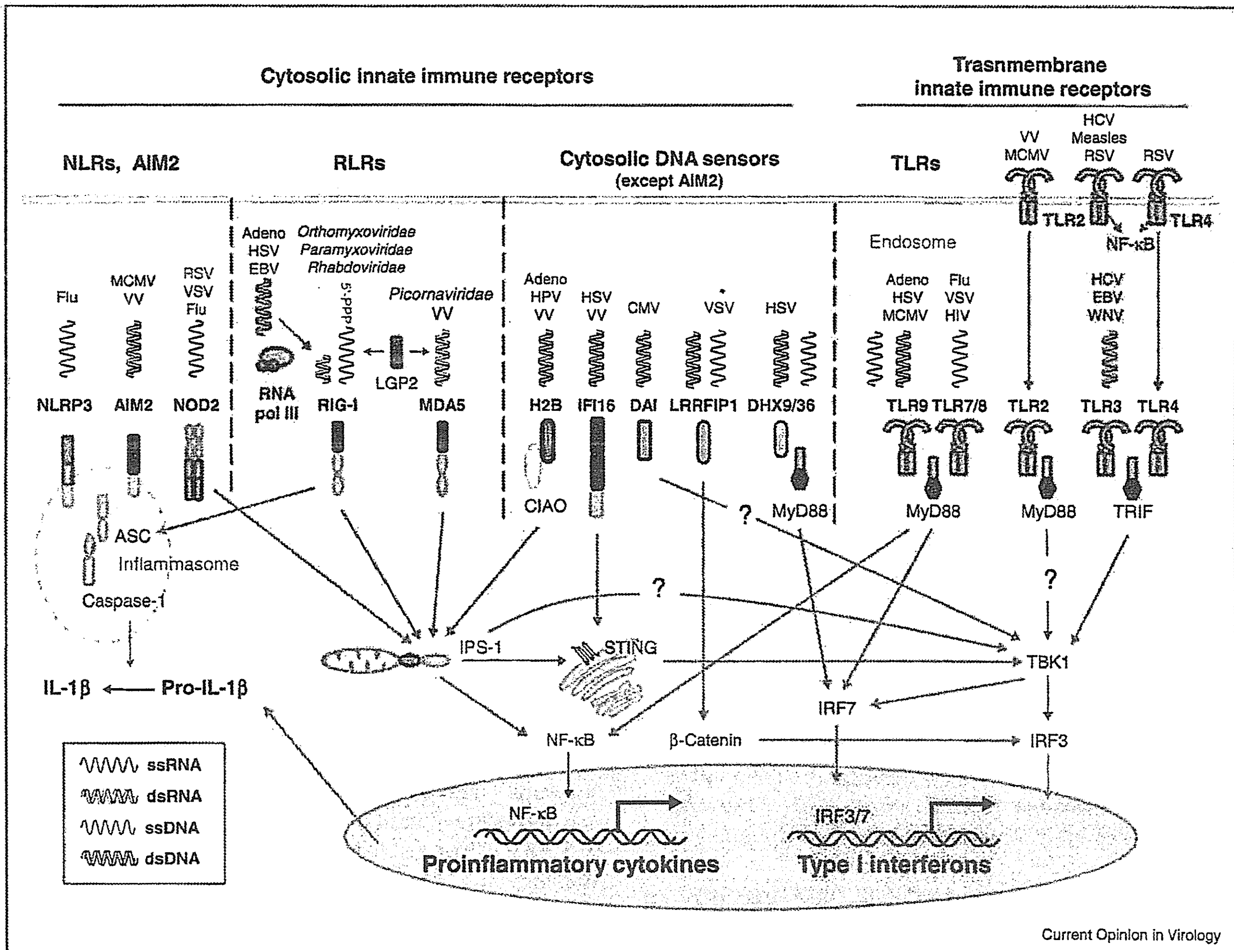
The innate immune system may distinguish between the presence of a virus and viral infection

In addition to bacteria and parasites, viruses are a major cause of infectious diseases. Because of their diverse organ/tissue tropisms, genomic structure (positive or negative stranded, single or double stranded, RNA or DNA) and pathogenic lifecycles, host cells can recognize viruses through a variety of innate immune receptors. Extracellular viruses are detected by transmembrane receptors such as TLRs, and cytosolic viral infections are detected by cytosolic receptors such as RLRs, NLRs, and ALRs (Figure 1). This diverse set of innate receptors may also allow the host immune system to determine viral status — live or dead, replicating or not replicating, pathogenic or non-pathogenic — in a manner similar to that recently proposed for bacterial infection [13]. These innate immune receptors trigger signaling cascades that are generally integrated with innate responses, such as nuclear factor kappa B (NF-κB)-dependent cytokine responses, interferon regulatory factor (IRF)-dependent IFN-α/β responses, and inflammasome/caspase-1-dependent IL-1β responses. IFN-α/β are the major cytokines that limit viral replication, while other cytokines, including IL-6, TNF-α and IL-1β, recruit immune cells to the site of infection and elicit inflammation. NF-κB-dependent and IRF-dependent cytokines are transcriptionally regulated, whereas inflammasome-dependent IL-1β secretion is regulated both transcriptionally and post-transcriptionally (Figure 1). Importantly, many viruses can suppress these innate responses at the 'sensing' and/or transcriptional level upon replication within infected cells [14].

Immune recognition of viruses by transmembrane innate receptors

Transmembrane innate receptors, such as TLRs, recognize extracellular viruses, and their activation does not

Figure 1



Innate immune receptors involved in virus recognition. **NLR and AIM2 pathways:** NLRP3 is activated by a wide variety of stimuli, including RNA viruses. Foreign cytoplasmic dsDNA is also detected by AIM2 via the HIN200 domain. Their activation induces the recruitment of the adaptor protein, ASC, via the pyrin domain. Procaspase-1 is also recruited to ASC via the CARD domain (inflammasome formation). This interaction leads to the auto-cleavage of caspase-1 and results in the activation of caspase-1, which cleaves pro-IL-1β. NOD2 is involved in the recognition of ssRNA viruses. NOD2 activates IPS-1, a mitochondrial membrane-anchored protein, through the NBD and LRR domains, which leads to IRF3 activation. **RLR pathway**: RIG-I is essential for IFN responses to several ssRNA viruses such as *Orthomyxoviridae* and *Paramyxoviridae*. However, MDA5 is necessary for responses to a different set of viruses, such as *Picornaviridae*. LGP2 can act as a positive regulator, making viral RNP complexes more accessible to RIG-I and MDA5. Some viral DNAs are transcribed into 5′ tri-phosphate RNA (the RIG-I ligand) by cytosolic RNA polymerase III (pol III). RIG-I and MDA5 signal via the adaptor protein, IPS-1, which leads to type I IFN production through the TBK1-IRF3-dependent pathway, and proinflammatory cytokine production through NF-κB translocation. RIG-I can activate the inflammasome by interacting with the CARD domains of RIG-I and ASC, and produce IL-1β. **Cytosolic DNA sensor pathways**: extra-chromosomal histone H2B binds DNA virus-like HPV through its α-helical region and interacts with IPS-1 via association with the adaptor protein CIAO. IFI16 binds DNA viruses via the HIN200 domains. They then activate the STING-TBK1-IRF3-dependent signaling pathway, resulting in the production of type I IFN. DAI detects DNA viruses and induces TBK1-IRF3-dependent type I IFN production. LRRFIP1 detects both bacterial DNA and viral RNA from VSV and induces type I IFN production via the β-catenin-IRF3 transactivator pathway. The DExD/H box helicase, DHX9/36, detects CpG-ODNs and DNA viruses such as HSV, leading to MyD88-IRF7-dependent type I IFN production. **TLR pathway**: some RNA viruses are detected by cell surface TLR2 and TLR4, which induce MyD88-dependent NF-κB activation. TLR4 is also recruited to the endosome, leading to TRIF-dependent type I IFN production. TLR3 and TLR7/8 recognize dsRNA and ssRNA, respectively, from RNA viruses. TLR3 induces TRIF-TBK1-dependent type I IFN production, whereas TLR7/8 induces NF-κB and IRF7 activation via MyD88. TLR9 detects CpG-ODNs and DNAs derived from DNA viruses, leading to NF-κB and IRF7 activation via MyD88. Some DNA viruses are also recognized by TLR2 in the endosome, which then induces IRF3/7-dependent type I IFN production.

necessarily require infection of the receptor-expressing cells. Based on cellular localization, TLRs can be grouped in two types: cell surface TLRs (TLR1,2,4,5,6) and endosomal TLRs (TLR3,7,8,9) [1]. Cell surface TLRs recognize bacterial/fungal cell wall components. However, many reports show that some viral proteins are also recognized by cell surface TLR2 and TLR4 [15,16]. A recent report by Barbalat *et al.* identified another interesting example of viral recognition by cell surface TLRs. Mouse cytomegalovirus and vaccinia virus (both dsDNA viruses) were recognized via TLR2. This led to the production of IFN-β, which was not observed upon stimulation with Pam3SK4 (a well-known bacterial TLR2 agonist) [17••]. Interestingly, this TLR2-mediated IFN-β production was restricted in Ly6C(hi) inflammatory monocytes, and was dependent on TLR2 recruitment from the cell surface to the endosome [17••]. However, the exact molecular mechanism(s) underlying virus recognition by cell surface TLRs is the subject of future research. The endosomal TLRs, TLR3, TLR7/8, and TLR9 recognize virus-derived dsRNA, ssRNA, and DNA, respectively [18]. Many viruses are recognized by these endosomal TLRs (Figure 1). TLR3 signaling is mediated by the adaptor molecule TRIF, which induces IRF3 phosphorylation leading to IFN-β production. TLR7/8/9 signaling is mediated by another adaptor molecule, MyD88 (an adaptor commonly used by other TLRs, except TLR3) leading to IRF7-mediated IFN-α production. Importantly, expression of these endosomal TLRs is restricted to certain types of dendritic cells (DCs). TLR3 is preferentially expressed by CD8α(+)DCs, and TLR7/9 is preferentially expressed by plasmacytoid DCs (pDCs). Overall, the recognition of the presence of viruses seems to be mediated by limited types of host cells that express these transmembrane innate immune receptors.

Immune recognition of viruses by cytosolic innate receptors

In contrast to transmembrane receptors, cytosolic innate receptors are expressed by all host cells. RLRs and NLRs mainly recognize viral RNAs, and the recently identified ALRs (and other cytosolic DNA sensors) detect viral DNA in the cytosol of infected cells. This cytosolic receptor-mediated virus recognition is critically important for the host innate immune responses to contain viral replication within the infected cells before the adaptive immune responses are fully developed. In contrast, the contribution of this form of cytosolic virus recognition to adaptive responses is varied and more controversial, as discussed later in this review.

RLRs comprise retinoic acid inducible gene-I (RIG-I), melanoma differentiation associated gene 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2). Both RIG-I and MDA5 recognize viral RNAs within the cytoplasm of infected cells. However, the exact molecular signatures of the RIG-I and MDA5 ligands are still not fully understood [19–22]. Owing to the lack of a caspase recruitment domain (CARD), which is important for interactions with IPS-1, LGP2 was assumed to function as a negative regulator of RIG-I and MDA5. However, a recent study suggests that LGP2 positively regulates RIG-I and MDA5 signaling, possibly by modifying the viral RNA structure before detection by these two receptors [23]. Virus recognition by RIG-I and MDA5 is mediated by a single adaptor molecule, IPS-1 (or MAVS), and leads to NF-κB and IRF3/IRF7 activation. Interestingly, a recent report demonstrated that RIG-I can directly activate ASC in an NLRP3-independent manner, leading to caspase-1-dependent IL-1β production during VSV (ssRNA virus) infection [24••].

NLRs comprise a large number of family member proteins that contain a conserved NOD motif [25], and can be classified into two groups. Activation of Nod1 and Nod2 leads to the activation of NF-κB and IRF. Although Nod2 was initially characterized as a cytosolic sensor for the bacterial cell wall component, muramyl dipeptide, which induces NF-κB activation, a recent report suggests that Nod2 also functions as a virus sensor [26••] and activates a non-classical NLR signaling pathway [27]. Sabbah *et al.* showed that Nod2 can directly sense cytosolic ssRNA from RSV and influenza virus, leading to MAVS(IPS-1)-IRF3-mediated IFN-β responses [26••]. Activation of NLRs, such as NLRP1, NLRP3, and NLRC4, leads to inflammasome formation, which results in caspase-1-mediated IL-1β and IL-18 secretion (Figure 1). The NLRP3 inflammasome is one of the best characterized inflammasomes, and is activated by bacterial toxins, LPS, and viral RNAs, as well as uric acid and alum [11•,28]. Interestingly, it appears that many RLRs and NLRs sense virus infections by detecting viral genomic, or replication-intermediate, RNA. This might indicate that the presence of viral nucleic acids provides the stronger proof of active viral infection, rather than general danger signals.

Cytosolic DNA sensors

AIM2 and IFI16 are both recently identified cytosolic DNA sensors and are involved in DNA-dependent inflammasome activation and IFN-β production, respectively [5,29–34]. Because both proteins contain a PYHIN domain [35–37], it has been proposed that they be referred to as ALRs [5]. However, several other molecules are also known to be involved in DNA sensing within the cytosol. DAI (ZBP-1) is the first reported DNA sensor molecule that triggers TBK1-IRF3-dependent IFN-β induction *in vitro* [38]; however, gene knockout mice do not show the same phenotype, suggesting the presence of redundant DNA sensor mechanisms [39]. Lrrfip1 recognizes cytosolic dsDNA (and dsRNA), subsequently interacting with β-catenin and enhancing IRF3-mediated IFN-β expression [40]. DHX36 and DHX9, present in human pDC, are cytosolic CpG-A and CpG-B binding

proteins, respectively. These proteins mediate the MyD88/IRF7-dependent production of IFN-α [41]. AT-rich DNA is also recognized indirectly by RNA polymerase III. AT-rich DNA is transcribed into 5-triphosphate dsRNA, which is then recognized by the RIG-I pathway [42,43]. The cytosolic histone, H2B, is also involved in DNA sensing. The dsDNA/H2B complex activates IPS-1 via CIAO (an adaptor molecule that links histone H2B and IPS-1) in human cell lines leading to IFN-β expression. However, this H2B-mediated dsDNA-dependent IFN-β production is not observed in mice, most likely because of the lack of the interaction between mouse CIAO and mouse IPS-1 [44].

Signaling via which innate immune receptors leads to adaptive immune responses: TLRs, RLRs, NLRs, or others?

Activation of the innate immune system is critical for establishing adaptive immune responses. This is simply demonstrated by the fact that immunization with a highly purified recombinant protein is usually unsuccessful owing to the lack of innate responses [45]. On the other hand, viral infections are usually sensed by multiple innate receptors. The live attenuated yellow fever vaccine 17D has been shown to activate multiple TLRs, resulted in CD8T and a mixed Th1/Th2 immune responses [46,47]. In the case of influenza virus, infection can be detected by three different receptors: TLR7, RIG-I, and NLRP3 [48*,49]. However, conflicting results have been reported, particularly in terms of the adaptive immune responses examined in these receptors/adaptors deficient mice (Table 1).

The involvement of TLR7/MyD88 has been examined by four independent studies [50–53]. It is very difficult to generalize the results, which range from almost nothing to identifying a prominent phenotype, except that all of the studies consistently agreed that CD8T responses were not affected by the absence of the TLR7/MyD88 pathway. However, two independent studies consistently demonstrated that, in contrast to live virus, the immunogenicity of a chemically killed (inactivated) whole virus was completely dependent on TLR7/MyD88 signaling [48*,54*].

The RIG-I/IPS-1 pathway was also examined in two independent studies [52,53]. They concluded that although RIG-I/IPS-1 signaling induces almost overlapping cytokine responses to those induced by TLR7/MyD88 (Figure 1), IPS-1-deficiency had no substantial effect upon adaptive responses to influenza virus infection [52,53]. This may reflect differential cellular expression of these receptors. RIG-I is ubiquitously expressed by most cells, whereas TLR7 is preferentially expressed by pDCs. It may also reflect the fact that RIG-I sensing requires viral replication within the cell, whereas TLR7 recognizes viruses in the endosome, which is not dependent upon virus infection (Figure 1). Differential regulation of adaptive immune responses by TLRs and RLRs has also been reported in another virus infection system. Jung *et al.* demonstrated that during LCMV infection, CD8T responses in MyD88-deficient mice were significantly reduced, whereas IPS-1-deficient mice showed comparable CD8T responses to those of wild-type mice [55].

NLRP3 can be triggered by viral RNA [56] and/or ionic perturbation caused by the influenza M2 protein [57]. NLRP3 triggers ASC-mediated NLRP3 inflammasome formation, leading to caspase-1-dependent IL-1β and IL-18 secretion. Inflammasome involvement in influenza virus infection has been studied by four independent

Table 1

Adaptive immune responses in mice deficient in innate immune receptors/adaptors against influenza virus infection and vaccination.

	Virus	TLRs(Myd88) deficiency	RLRs(IPS-1) deficiency	NLRs(NLRP3, ASC, caspase-1) deficiency
		IFN-I, TNFα/IL-6		Inflammasome (IL-1/IL-18)
Lopez *et al.* [50]	A/PR8	CD4(IFNγ)→, CD4(IL-4)↑, CD8→, Ab→	Not examined	Not examined
Heer *et al.* [51]	A/PR8	CD4→, CD8→, Ab(IgG2a)↓	Not examined	Not examined
Koyama *et al.* [52]	A/PR8, A/NC	CD4(IFNγ)↓, CD8→, Ab(IgG2a)↓	CD4(IFNγ)→, CD8→, Ab→	Not examined
Seo *et al.* [53]	A/PR8	CD4(Th1)↓, CD4(Th2)↑, CD8→, Ab→	CD4(Th1)→, CD8→, Ab→	Not examined
Ichinohe *et al.* [58]	A/PR8	Not examined	Not examined	CD4(IFNγ)↓ CD8↓, Ab(IgG, IgA)↓
Allen *et al.* [59]	A/PR8	Not examined	Not examined	Intact adaptive responses (CD8→, Ab→)
Thomas *et al.* [60]	A/PR8	Not examined	Not examined	CD8→, Ab→
Koyama *et al.* [48*]	A/NC	Not examined	Not examined	CD4(IFNγ)→, CD8→, Ab(IgG1)↓
	Inactivated WV(A/NC)	CD4→, Ab↓	CD4→, Ab→	CD4→, Ab→
Geeraedts *et al.* [54*]	Inactivated WV(H5N1)	CD4↓, Ab↓	Not examined	Not examined

groups. Ichinohe *et al.* demonstrated that NLRP3-independent, but ASC-dependent, inflammasome responses were important for both CD4T and CD8T responses, as well as IgA and IgG responses [58] (it is noteworthy that Poeck *et al.* also reported NLRP3-independent, but RIG-I and ASC-dependent, inflammasome activation by VSV [24••]). In contrast, Allen *et al.* and Thomas *et al.* showed that NLRP3 inflammasome responses were not involved in adaptive responses, but play a more important role in the innate phase of host defense and in tissue healing [59,60]. We also examined ASC-deficient mice and found that inflammasome activation had almost no impact on the adaptive response to live influenza virus infection [48•]. At present, the reason for these contradictory results is not clear [11•,61].

Viral subversion of innate immune responses may affect adaptive immune responses

These controversies may be explained by differences in the types of virus used; especially the different subversion mechanisms used by the viruses. Influenza virus (and other viruses) possesses an immune evasion protein that modulates the innate immune signaling cascades of the host [14]. Even though most studies used a mouse-adapted PR8 virus, Heynisch *et al.* reported that two variants of A/PuertoRico/8/34 show very different activation patterns for cellular signaling molecules in MDCK cells [62]. This most likely reflects the fact that these variant viruses modulate cytosolic signaling systems in different ways. Influenza NS-1 is the most well-characterized of the proteins that subvert RIG-I mediated IFN-α/β responses at multiple steps [63]. A recent report suggests that the inflammasome is also an evasion target of a herpes virus [64]. Intriguingly, no direct viral mechanism that antagonizes TLR signaling has been described for influenza A virus [63]. Taken together, these data suggest that the same PR8 virus may induce very different host immune responses. Furthermore, they may also suggest that subverting the infection-dependent cytosolic innate system may be easier than subverting the infection-independent TLR system. In line with this hypothesis, once the virus is fixed with formalin (and killed), the host immune response is consistently TLR7/MyD88-dependent [48•,54•].

Conclusions

The existence of diverse innate immune receptors may reflect a redundancy that ensures sensitive detection of viruses in a variety of tissue and cell types, and the subsequent induction of host defense mechanisms. TLRs can detect extracellular viruses (either live or dead), and do not require viral infection of receptor-expressing cells. By contrast, detection by cytosolic receptors requires viral infection and replication, which can be easier evasion targets for many viruses. The innate immune response plays two roles in host defense: (1) it limits (or at least controls) viral replication during initial infection; and (2) it induces adaptive immune responses responsible for viral clearance and maintenance (memory). However, it is still not clear to what extent each innate immune receptor contributes to the adaptive immune responses. Owing to sophisticated immune evasion mechanisms, infection by live viruses may not provide a clear answer. However, immunization with an inactivated whole virion influenza vaccine clearly demonstrates that TLR-mediated innate signaling alone is sufficient to induce adaptive immune responses. Currently, it is difficult to examine the individual contribution of each RLR and NLR to the adaptive immune response because of the lack of selective activators. Recently, Kasturi *et al.* demonstrated that synthetic nanoparticle based vaccines composed of multiple TLR ligands induced persistent antibody and CD8T responses than single TLR activating vaccine [65]. It suggests that activations of multiple innate immune receptors may be required for long lasting memory responses but not necessarily required for mounting temporal effector responses. Further studies will clarify the more detailed coordination between innate and adaptive immune responses, and provide a more rational way of vaccine design.

Conflicts of interest statement

The authors have no conflicts of interest to declare.

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References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Kawai T, Akira S: **The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors.** *Nat Immunol* 2010, **11**:373-384.

2. Geijtenbeek TBH, Gringhuis SI: **Signalling through C-type lectin receptors: shaping immune responses.** *Nat Rev Immunol* 2009, **9**:465-479.

3. Yoneyama M, Fujita T: **RNA recognition and signal transduction by RIG-I-like receptors.** *Immunol Rev* 2009, **227**:54-65.

4. Franchi L, Warner N, Viani K, Nunez G: **Function of Nod-like receptors in microbial recognition and host defense.** *Immunol Rev* 2009, **227**:106-128.

5. Unterholzner L, Keating SE, Baran M, Horan KA, Jensen SB, Sharma S, Sirois CM, Jin TC, Latz E, Xiao TS *et al.*: **IFI16 is an innate immune sensor for intracellular DNA.** *Nat Immunol* 2010, **11**:997-1004.

6. Chen GY, Nunez G: **Sterile inflammation: sensing and reacting to damage.** *Nat Rev Immunol* 2010, **10**:826-837.

7. Takeuchi O, Akira S: **Pattern recognition receptors and inflammation.** *Cell* 2010, **140**:805-820.

8. Palm NW, Medzhitov R: **Pattern recognition receptors and • control of adaptive immunity.** *Immunol Rev* 2009, **227**:221-233.
This (also Ref [11•,12•]) is an insightful review that discusses the connection between innate and adaptive immune responses.

9. Manicassamy S, Pulendran B: **Modulation of adaptive immunity with Toll-like receptors.** *Semin Immunol* 2009, **21**:185-193.

10. Koyama S, Coban C, Aoshi T, Horii T, Akira S, Ishii KJ: **Innate immune control of nucleic acid-based vaccine immunogenicity.** *Expert Rev Vaccines* 2009, **8**:1099-1107.

11. Williams A, Flavell RA, Eisenbarth SC: **The role of NOD-like • receptors in shaping adaptive immunity.** *Curr Opin Immunol* 2010, **22**:34-40.
See annotation to Ref [8•].

12. Joffre O, Nolte MA, Sporri R, Sousa CRE: **Inflammatory signals in • dendritic cell activation and the induction of adaptive immunity.** *Immunol Rev* 2009, **227**:234-247.
See annotation to Ref [8•].

13. Vance RE, Isberg RR, Portnoy DA: **Patterns of pathogenesis: discrimination of pathogenic and nonpathogenic microbes by the innate immune system.** *Cell Host Microbe* 2009, **6**:10-21.

14. Versteeg GA, Garcia-Sastre A: **Viral tricks to grid-lock the type I interferon system.** *Curr Opin Microbiol* 2010, **13**:508-516.

15. Finberg RW, Wang JP, Kurt-Jones EA: **Toll like receptors and viruses.** *Rev Med Virol* 2007, **17**:35-43.

16. Carty M, Bowie AG: **Recent insights into the role of Toll-like receptors in viral infection.** *Clin Exp Immunol* 2010, **161**:397-406.

17. Barbalat R, Lau L, Locksley RM, Barton GM: **Toll-like receptor 2 •• on inflammatory monocytes induces type I interferon in response to viral but not bacterial ligands.** *Nat Immunol* 2009, **10**:1200-1207.
This paper showed that mouse cytomegalovirus and vaccinia virus infection can induce MyD88-mediated IFN responses in Ly6C(hi)CD11b(+)CD11c(–)B220(–) inflammatory monocytes.

18. Blasius AL, Beutler B: **Intracellular Toll-like receptors.** *Immunity* 2010, **32**:305-315.

19. Schlee M, Roth A, Hornung V, Hagmann CA, Wimmenauer V, Barchet W, Coch C, Janke M, Mihailovic A, Wardle G *et al.*: **Recognition of 5′ triphosphate by RIG-I helicase requires short blunt double-stranded RNA as contained in panhandle of negative-strand virus.** *Immunity* 2009, **31**:25-34.

20. Rehwinkel J, Tan CP, Goubau D, Schulz O, Pichlmair A, Bier K, Robb N, Vreede F, Barclay W, Fodor E *et al.*: **RIG-I detects viral genomic RNA during negative-strand RNA virus infection.** *Cell* 2010, **140**:397-408.

21. Pichlmair A, Schulz O, Tan CP, Rehwinkel J, Kato H, Takeuchi O, Akira S, Way M, Schiavo G, Sousa CRE: **Activation of MDA5 requires higher-order RNA structures generated during virus infection.** *J Virol* 2009, **83**:10761-10769.

22. Schmidt A, Endres S, Rothenfusser S: **Pattern recognition of viral nucleic acids by RIG-I-like helicases.** *J Mol Med* 2011, **89**:5-12.

23. Satoh T, Kato H, Kumagai Y, Yoneyama M, Sato S, Matsushita K, Tsujimura T, Fujita T, Akira S, Takeuchi O: **LGP2 is a positive regulator of RIG-I- and MDA5-mediated antiviral responses.** *Proc Natl Acad Sci U S A* 2010, **107**:1512-1517.

24. Poeck H, Bscheider M, Gross O, Finger K, Roth S, Rebsamen M, •• Hannesschlager N, Schlee M, Rothenfusser S, Barchet W *et al.*: **Recognition of RNA virus by RIG-I results in activation of CARD9 and inflammasome signaling for interleukin 1 beta production.** *Nat Immunol* 2010, **11**:63-1824.
This paper showed that cytosolic RNA from VSV sensed by RIG-I can induce ASC/inflammasome activation in an NLRP3-independent manner.

25. Kanneganti TD: **Central roles of NLRs and inflammasomes in viral infection.** *Nat Rev Immunol* 2010, **10**:688-698.

26. Sabbah A, Chang TH, Harnack R, Frohlich V, Tominaga K, •• Dube PH, Xiang Y, Bose S: **Activation of innate immune antiviral responses by Nod2.** *Nat Immunol* 2009, **10**:1073-1080.
This paper showed that Nod2 directly recognizes ssRNA and activates the MAVS(IPS-1)/IRF3/IFN-β pathway before RIG-I mediated sensing.

27. Shaw MH, Kamada N, Warner N, Kim YG, Nunez G: **The ever-expanding function of NOD2: autophagy, viral recognition, and T cell activation.** *Trends Immunol* 2011, **32**:73-79.

28. Martinon F, Mayor A, Tschopp J: **The inflammasomes: guardians of the body.** *Ann Rev Immunol* 2009, **27**:229-265.

29. Burckstummer T, Baumann C, Bluml S, Dixit E, Durnberger G, Jahn H, Planyavsky M, Bilban M, Colinge J, Bennett KL *et al.*: **An orthogonal proteomic–genomic screen identifies AIM2 as a cytoplasmic DNA sensor for the inflammasome.** *Nat Immunol* 2009, **10**:266-272.

30. Fernandes-Alnemri T, Yu JW, Juliana C, Solorzano L, Kang S, Wu JH, Datta P, McCormick M, Huang L, McDermott E *et al.*: **The AIM2 inflammasome is critical for innate immunity to *Francisella tularensis*.** *Nat Immunol* 2010, **11**:385-394.

31. Hornung V, Ablasser A, Charrel-Dennis M, Bauernfeind F, Horvath G, Caffrey DR, Latz E, Fitzgerald KA: **AIM2 recognizes cytosolic dsDNA and forms a caspase-1-activating inflammasome with ASC.** *Nature* 2009, **458**:514-518.

32. Roberts TL, Idris A, Dunn JA, Kelly GM, Burnton CM, Hodgson S, Hardy LL, Garceau V, Sweet MJ, Ross IL *et al.*: **HIN-200 proteins regulate caspase activation in response to foreign cytoplasmic DNA.** *Science* 2009, **323**:1057-1060.

33. Jones JW, Kayagaki N, Broz P, Henry T, Newton K, O'Rourke K, Chan S, Dong J, Qu Y, Roose-Girma M *et al.*: **Absent in melanoma 2 is required for innate immune recognition of *Francisella tularensis*.** *Proc Natl Acad Sci U S A* 2010, **107**:9771-9776.

34. Rathinam VAK, Jiang ZZ, Waggoner SN, Sharma S, Cole LE, Waggoner L, Vanaja SK, Monks BG, Ganesan S, Latz E *et al.*: **The AIM2 inflammasome is essential for host defense against cytosolic bacteria and DNA viruses.** *Nat Immunol* 2010, **11**:395-403.

35. Goubau D, Rehwinkel J, Sousa CRE: **PYHIN proteins: center stage in DNA sensing.** *Nat Immunol* 2010, **11**:984-986.

36. Choubey D, Duan X, Dickerson E, Ponomareva L, Panchanathan R, Shen H, Srivastava R: **Interferon-inducible p200-family proteins as novel sensors of cytoplasmic DNA: role in inflammation and autoimmunity.** *J Interferon Cytokine Res* 2010, **30**:371-380.

37. Gariglio M, Mondini M, De Andrea M, Landolfo S: **The multifaceted interferon-inducible p200 family proteins: from cell biology to human pathology.** *J Interferon Cytokine Res* 2011, **31**:159-172.

38. Takaoka A, Wang Z, Choi MK, Yanai H, Negishi H, Ban T, Lu Y, Miyagishi M, Kodama T, Honda K *et al.*: **DAI (DLM-1/ZBP1) is a cytosolic DNA sensor and an activator of innate immune response.** *Nature* 2007, **448**:501-505.

39. Ishii KJ, Kawagoe T, Koyama S, Matsui K, Kumar H, Kawai T, Uematsu S, Takeuchi O, Takeshita F, Coban C *et al.*: **TANK-binding kinase-1 delineates innate and adaptive immune responses to DNA vaccines.** *Nature* 2008, **451**:725-729.

40. Yang PY, An HZ, Liu XG, Wen MY, Zheng YY, Rui YC, Cao XT: **The cytosolic nucleic acid sensor LRRFIP1 mediates the production of type I interferon via a beta-catenin-dependent pathway.** *Nat Immunol* 2010, **11**:487-494.

41. Kim T, Pazhoor S, Bao MS, Zhang ZQ, Hanabuchi S, Facchinetti V, Bover L, Plumas J, Chaperot L, Qin J *et al.*: **Aspartate-glutamate-alanine-histidine box motif (DEAH)/RNA helicase A helicases sense microbial DNA in human plasmacytoid dendritic cells.** *Proc Natl Acad Sci U S A* 2010, **107**:15181-15186.

42. Ablasser A, Bauernfeind F, Hartmann G, Latz E, Fitzgerald KA, Hornung V: **RIG-I-dependent sensing of poly(dA:dT) through the induction of an RNA polymerase III-transcribed RNA intermediate.** *Nat Immunol* 2009, **10**:1065-1072.

43. Chiu YH, MacMillan JB, Chen ZJJ: **RNA polymerase III detects cytosolic DNA and induces type I interferons through the RIG-I pathway.** *Cell* 2009, **138**:576-591.

44. Kobiyama K, Takeshita F, Jounai N, Sakaue-Sawano A, Miyawaki A, Ishii KJ, Kawai T, Sasaki S, Hirano H, Ishii N *et al.*:

Extrachromosomal histone H2B mediates innate antiviral immune responses induced by intracellular double-stranded DNA. *J Virol* 2010, **84**:822-832.

45. Ishii KJ, Akira S: **Toll or toll-free adjuvant path toward the optimal vaccine development.** *J Clin Immunol* 2007, **27**:363-371.

46. Querec T, Bennouna S, Alkan SK, Laouar Y, Gorden K, Flavell R, Akira S, Ahmed R, Pulendran B: **Yellow fever vaccine YF-17D activates multiple dendritic cell subsets via TLR2, 7, 8, and 9 to stimulate polyvalent immunity.** *J Exp Med* 2006, **203**:413-424.

47. Querec TD, Akondy RS, Lee EK, Cao WP, Nakaya HI, Teuwen D, Pirani A, Gernert K, Deng JS, Marzolf B *et al.*: **Systems biology approach predicts immunogenicity of the yellow fever vaccine in humans.** *Nat Immunol* 2009, **10**:116-125.

48. • Koyama S, Aoshi T, Tanimoto T, Kumagai Y, Kobiyama K, Tougan T, Sakurai K, Coban C, Horii T, Akira S *et al.*: **Plasmacytoid dendritic cells delineate immunogenicity of influenza vaccine subtypes.** *Sci Transl Med* 2010, **2**:24-25.

This paper (also Ref [54•]) showed that adaptive immune responses induced by an inactivated whole influenza virus vaccine were dependent on TLR7 signaling.

49. Ichinohe T: **Respective roles of TLR, RIG-I and NLRP3 in influenza virus infection and immunity: impact on vaccine design.** *Expert Rev Vaccines* 2010, **9**:1315-1324.

50. Lopez CB, Moltedo B, Alexopoulou L, Bonifaz L, Flavell RA, Moran TM: **TLR-independent induction of dendritic cell maturation and adaptive immunity by negative-strand RNA viruses.** *J Immunol* 2004, **173**:6882-6889.

51. Heer AK, Shamshiev A, Donda A, Uematsu S, Akira S, Kopf M, Marsland BJ: **TLR signaling fine-tunes anti-influenza B cell responses without regulating effector T cell responses.** *J Immunol* 2007, **178**:2182-2191.

52. Koyama S, Ishii KJ, Kumar H, Tanimoto T, Coban C, Uematsu S, Kawai T, Akira S: **Differential role of TLR- and RLR-signaling in the immune responses to influenza a virus infection and vaccination.** *J Immunol* 2007, **179**:4711-4720.

53. Seo SU, Kwon HJ, Song JH, Byun YH, Seong BL, Kawai T, Akira S, Kweon MN: **MyD88 Signaling Is Indispensable for Primary Influenza A Virus Infection but Dispensable for Secondary Infection.** *Journal of Virology* 2010, **84**:12713-12722.

54. • Geeraedts F, Goutagny N, Hornung V, Severa M, de Haan A, Pool J, Wilschut J, Fitzgerald KA, Huckriede A: **Superior immunogenicity of inactivated whole virus H5N1 influenza vaccine is primarily controlled by Toll-like receptor signalling.** *PLoS Pathogens* 2008, **4**:e1000138.

See annotation to Ref [48•].

55. Jung A, Kato H, Kumagai Y, Kumar H, Kawai T, Takeuchi O, Akira S: **Lymphocytoid choriomeningitis virus activates plasmacytoid dendritic cells and induces a cytotoxic T-cell response via MyD88.** *J Virol* 2008, **82**:196-206.

56. Kanneganti TD, Body-Malapel M, Amer A, Park JH, Whitfield J, Franchi L, Taraporewala ZF, Miller D, Patton JT, Inohara N *et al.*: **Critical role for Cryopyrin/Nalp3 in activation of caspase-1 in response to viral infection and double-stranded RNA.** *J Biol Chem* 2006, **281**:36560-36568.

57. Ichinohe T, Pang IK, Iwasaki A: **Influenza virus activates inflammasomes via its intracellular M2 ion channel.** *Nat Immunol* 2010, **11**:404-410.

58. Ichinohe T, Lee HK, Ogura Y, Flavell R, Iwasaki A: **Inflammasome recognition of influenza virus is essential for adaptive immune responses.** *J Exp Med* 2009, **206**:79-87.

59. Allen IC, Scull MA, Moore CB, Holl EK, McElvania-TeKippe E, Taxman DJ, Guthrie EH, Pickles RJ, Ting JPY: **The NLRP3 inflammasome mediates in vivo innate immunity to influenza A virus through recognition of viral RNA.** *Immunity* 2009, **30**:556-565.

60. Thomas PG, Dash P, Aldridge JR, Ellebedy AH, Reynolds C, Funk AJ, Martin WJ, Lamkanfi M, Webby RJ, Boyd KL *et al.*: **The intracellular sensor NLRP3 mediates key innate and healing responses to influenza A virus via the regulation of caspase-1.** *Immunity* 2009, **30**:566-575.

61. Pang IK, Iwasaki A: **Inflammasomes as mediators of immunity against influenza virus.** *Trends Immunol* 2011, **32**:34-41.

62. Heynisch B, Frensing T, Heinze K, Seitz C, Genzel Y, Reichl U: **Differential activation of host cell signalling pathways through infection with two variants of influenza A/Puerto Rico/8/34 (H1N1) in MDCK cells.** *Vaccine* 2010, **28**:8210-8218.

63. Schmolke M, Garcia-Sastre A: **Evasion of innate and adaptive immune responses by influenza A virus.** *Cell Microbiol* 2010, **12**:873-880.

64. Gregory SM, Davis BK, West JA, Taxman DJ, Matsuzawa S, Reed JC, Ting JPY, Damania B: **Discovery of a viral NLR homolog that inhibits the inflammasome.** *Science* 2011, **331**:330-334.

65. Kasturi SP, Skountzou I, Albrecht RA, Koutsonanos D, Hua T, Nakaya HI, Ravindran R, Stewart S, Alam M, Kwissa M *et al.*: **Programming the magnitude and persistence of antibody responses with innate immunity.** *Nature* 2011, **470**:543-547.

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Novel Strategies to Improve DNA Vaccine Immunogenicity

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Abstract: DNA vaccines can induce both humoral and cellular immune responses in animals. Some DNA vaccines are already licensed for infectious diseases such as West Nile virus encephalitis in horses. When used in humans, however, DNA vaccines suffer from lower immunogenicity profiles. Although the reasons for this are poorly understood, various hypotheses have been proposed. This review aims to provide better understanding of the molecular and immunological mechanisms by which DNA vaccines work and how such knowledge can be used to bring about improvements in their efficacy. Recent studies have provided evidence that the 'adjuvant effect' of plasmid DNA is mediated by its double-stranded structure. This structure activates stimulator of interferon genes/TANK-binding kinase 1 (STING/TBK1)-dependent innate immune signaling pathways in the absence of Toll-like receptors. Indeed, type-I interferons (IFNs), induced *in vivo* via the STING/TBK1 pathway, were found to be crucial for both direct- and indirect-antigen presentation via distinct cell types (i.e. dendritic cells (DC) and muscle cells, respectively). Importantly, incorporation of TBK1 into a DNA vaccine was found to enhance the antigen-specific humoral immune responses targeting the *Plasmodium falciparum* serine repeat antigen (SERA), a candidate vaccine antigen expressed in the blood-stages of human malaria parasites. Thus, the results of these studies may offer new ways to develop DNA vaccines, as well as delivering novel vaccine adjuvants against infectious diseases.

Keywords: Adjuvant, CpG motifs, DNA vaccine, innate immunity, STING, TBK1, TLR9, type I interferon.

INTRODUCTION

DNA vaccines represent a major advance in the fight against infectious diseases. Their mechanism of action enables the antigen of interest to be delivered to the host immune system in a manner that is similar to natural exposure to the pathogen. DNA vaccines comprise plasmid DNA encoding the antigen of interest whose expression is controlled by a mammalian promoter; following administration of the vaccine, the host immune system is exposed to the expressed antigen. Such *in vivo* introduction of genetic material usually elicits strong humoral and cellular (Th1 type CD4+ T cells and CD8+ cytotoxic T cells) responses, making DNA vaccines distinct from conventional protein or peptide vaccines. Indeed, DNA vaccines have shown remarkable success in most animal studies and clinical trials in humans [1-2]. For example, DNA vaccines for horses, salmonid fish and dogs have been licensed since 2005 against West Nile virus, Infectious hematopoietic necrosis virus and melanoma, respectively [3-5]. DNA vaccine clinical trials against HIV and malaria, at least showed good safety and tolerability profile in humans [6-7].

However, low immunogenicity has proved a significant obstacle to efficacy for DNA vaccines, especially in higher primates and humans. To date, various approaches have been taken to improve the immunogenicity of such vaccines. For example, modifying the microenvironment of the vaccination site by co-administration of various genes (i.e. genes encoding the co-stimulatory molecules, cytokines and chemokines [8-9], and/or genes that induce apoptosis [10-12]), proteins or other immunologically active molecules is not unusual. Some other approaches that have been tried to improve DNA vaccine immunogenicity include: 'Prime-Boost' immunization with DNA followed by a viral vector encoding the antigen (or the protein antigen) [13]; various immunization techniques and DNA delivery systems such as electroporation [14-16]; and microparticles and tattoo-immunization [17-18]. However, we are a long way from a complete understanding of how DNA vaccines work.

Recent studies are starting to shed light on this subject, however. Crucial to this is the recent discovery of many innate immune system receptors such as Toll-like receptors (TLRs), Nucleotide binding oligomerization domain (NOD)-like receptors, and Retinoic acid inducible gene (RIG)-like receptors, which have revealed that the main components of successful vaccines (adjuvants) are the ligands for many innate immune system receptors. Use of such molecules has great potential to improve vaccine design and development

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[19-21]. Indeed, many of the innate immune system receptor ligands are protein, nucleic acid, lipid, and carbohydrate in nature (i.e. lipid-A and poly-IC and CpG oligodeoxynucleotides (CpG ODNs) and have been used as adjuvants in vaccine trials for many years. Similarly, recent intensive research efforts searching for a cytosolic DNA sensor has improved our understanding [22-23]. Hence, this review will discuss some of the recent developments in this field and consider some of the strategies available for improving the immunogenicity of DNA vaccines. Attention will be directed towards the use of malarial antigens as anti-malaria vaccines.

1.1. Strategies to Improve DNA Vaccine Immunogenicity; Manipulation of Intrinsic Adjuvant Properties

It was shown that bacterial DNA contains immunostimulatory CpG motifs, which comprise an unmethylated C followed by G and specific flanking sequences that are recognized by TLR9 [24-26]. These findings suggest that DNA vaccine immunogenicity might be attributed to CpG motifs present in the plasmid backbone, which could act as an intrinsic "built in adjuvant" for DNA vaccines [27-28]. Therefore, incorporation of TLR9 and related TLR pathways has been a promising approach that could improve DNA vaccine immunogenicity.

Indeed, addition of several CpG motifs into a plasmid backbone has been shown to improve the immunogenicity of DNA vaccines [29-31]. In one study, DNA plasmid backbones were modified by the addition of two distinct types of human-specific CpG motifs (D and K types ODNs), and the effects of such modifications on various types of human immune cells (and in mice) were investigated for use in humans against malaria [31]. It was found that the modified plasmid DNA could induce maturation of human monocytes into DCs via activation of plasmocytoid DC (pDC); both modified plasmids (containing as few as three to five human CpG motifs) resulted in differential DC maturation in comparison with an unmodified plasmid. In addition, although expression of the encoded antigen (*P. falciparum* surface protein 25 (Pfs25), a *P. falciparum* transmission-blocking vaccine candidate) was not affected by introduction of additional immunostimulatory CpG motifs into the plasmid backbone, at least one of the plasmids (D type ODN incorporated Pfs25) induced higher levels of Pfs25-specific IgG with subtle differences in antibody isotypes. However, later studies found that TLR9-deficient mice had comparable levels of antigen-specific IgG, IgG1 and IgG2a antibody responses (including IFNγ and cytotoxic T lymphocyte (CTL) responses) as their wild-type counterparts [32-33], although others suggest that TLR9 signaling is critical for inducing CD8+ T cell responses after DNA vaccination at least after priming [34-35].

Other approaches to improve DNA vaccine immunogenicity include targeting TLR pathway signaling molecules, which can mimic a microbial infection in the absence of any microbial components. One such study investigated an over-expression strategy using adapter molecules such as the Myeloid Differentiation Primary Response Gene (MyD88) or Toll/IL-1 receptor (TIR)-domain-containing adaptor inducing interferon-β (TRIF) and found that greater enhancement of humoral responses was achieved when the DNA vaccine incorporated the MyD88 genetic adjuvant [36]. In contrast, incorporation of the TRIF genetic adjuvant greatly enhanced cellular immune responses resulting in superior protection against influenza virus or tumor progression. Similarly, incorporation of the interferon regulatory factor (IRF) 1, 3 and 7 into DNA vaccines improved both humoral and cellular immune responses against viral infection [37-38].

Overall, these studies suggest that although the immunogenicity of DNA vaccines can be enhanced by additional CpG motifs, or over expression of TLR-mediated signaling molecules in the plasmid backbone, the 'basal' adjuvant effects of DNA vaccines are independent of TLR-mediated recognition. The results of another study using MyD88/TRIF-double deficient mice are consistent with this [39].

1.2. Double-Stranded Plasmid DNA is Recognized Via the DNA Recognition Machinery, STING and TBK1

If plasmid DNA is not recognized by TLRs (TLR9) and its adapter molecules, what is it recognized by? Recent work has shown that bacterial or synthetic CpG-DNAs are not the only molecules that can activate the innate immune system. In fact, double-stranded (ds) DNA derived from host cells in the B form (a right-handed helical structure) can also do this [40]. When transfected into the cytosol, dsDNA activated fibroblasts, macrophages and dendritic cells to produce robust amounts of type-I interferons (IFNs) independent of TLRs; but in this case, signaling is mediated through TANK-binding kinase-1 (TBK1) [40]. TBK1 is crucial for type-I IFN induction via TLR-dependent and TLR-independent pathways [21,41]. However, within the last few years, several studies have identified various cytosolic DNA-recognition molecules (recently reviewed by [23]). Briefly, STING (stimulator of interferon genes, alternatively designated MITA, MPYS and ERIS), which is localized in the endoplasmic reticulum (ER), was found to activate NF-kB and IRF3 to stimulate type I IFN production after intracellular dsDNA stimulation [42]. Upon dsDNA stimulation, STING relocalizes with TBK1 from the endoplasmic reticulum to perinuclear vesicles containing the subunit of the exocyst complex 5 (Sec5) (also called EXOC2) [43]. Quite recently, a new molecule designated the interferon-inducible tripartite-motif (TRIM) 56 was identified as an interferon-inducible E3 ubiquitin ligase that modulates STING dimerization upstream of TBK1 to confer double-stranded DNA-mediated innate immune responses [44]. At the final stage, TBK1 comprises inducible IκB kinase (IKK-i) and these two kinases directly phosphorylate interferon regulatory factor 3 (IRF3) and IRF7 [45-46], resulting the production of type I interferons (IFNs).

These components of the DNA recognition machinery (STING and TBK1) have collectively indicated that a double-stranded structure could be essential for DNA vaccine-induced immunogenicity. Accordingly, use of TBK1-deficient mice revealed that DNA vaccine immunogenicity was completely dependent on TBK1 [39]. Moreover, such immunogenicity occurs through the activation of type-I IFN-mediated innate immunity resulting in the adjuvant effect for the encoded antigen [39,41] Fig. (1). Furthermore, type I IFNs were found to be essential for optimal DNA vaccine immunogenicity (antigen-specific T and B cell induction) by

Table 1. Summary of DNA Vaccine Studies Using Mice Deficient for Innate Immune System Molecules

Knockout Mice	DNA Vaccine-Induced Antigen Specific-			Reference
	Ab Responses	CD4+ T Cells	CD8+ T Cells	
TLR9	→	→	→*	[33,35]
MyD88/TRIF	→	→	→	[39]
IPS	→	→	→	[39]
IFNAR	↓	↓	↓	[39]
STING	↓	↓	↓	[47]
TBK1	↓	↓	↓	[39]
IRF3	→	↓	↓	[48]

*Some groups suggest that TLR9 signaling is critical for inducing CD8+ T cell responses after priming [30].

using interferon (alpha and beta) receptor 2 (IFNAR2)-deficient mice, but not attributable to TLR signaling (i.e. because MyD88/TRIF-double deficient mice normally respond to DNA vaccines [39]). Bone-marrow transfer experiments revealed that TBK1-mediated signaling in dendritic cells mainly involved the induction of both antigen-specific B cells and CD4+ T cells. Moreover, DNA-transfected non-immune cells, such as muscle cells, were found to cross-prime CD8+ T cells, which following DNA vaccination promoted adaptive immune responses Fig. (**1**).

Similar studies were performed with STING-deficient mice [47], which revealed the importance of STING for plasmid DNA immunogenicity Fig. (**1**). One possible drawback of this pathway is that RNA could be generated during DNA vaccination, which might act as an adjuvant by activating TBK1-dependent signaling [41]. Because ss- and dsRNA is recognized via TLR3/7/8, RIG-I and Melanoma differentiation-associated gene 5 (MDA5) and utilizes the adapter molecules TRIF, MyD88 and interferon-beta promoter stimulator 1 (IPS-1), respectively [21], this possibility was excluded using TRIF-, MyD88- and IPS-1-deficient mice; the results showed that DNA vaccine-induced immune responses against all of the adaptors were comparable with their WT counterparts [41].

These DNA vaccination studies in mice collectively revealed that DNA vaccination induces both antigen-specific B cells as well as CD4+ and CD8+ T cell responses entirely dependent on TBK1. However, interestingly, IRF3-deficient mice elicited strong antigen-specific humoral responses after DNA vaccinations, while CD4 and CD8 T cell responses (including the production of Th1, Th2 and Th17 cytokines) were severely impaired [48], suggesting other down-stream molecules of TBK1 leading production of antibodies, requires further investigation (see Table **1** for a summary of DNA vaccine studies as of current day by using mice deficient for immune system receptors).

1.3. Does the Inflammasome Play a Role in DNA Vaccine Immunogenicity?

In addition to TLR-independent and STING/TBK1-dependent cytosolic DNA recognition, another innate immune signaling mechanism has been suggested for cytosolic DNA recognition. That is, cytosolic DNA mediates the Apoptotic speck protein containing a caspase recruitment domain (ASC)/caspase-1-mediated secretion of IL-1β by AIM2 (absent in melanoma 2), a human IFN-inducible gene (HIN)-200 family member [49-52]. However, it should be noted that the AIM2 inflammasome is essential for caspase-1 activation, but not for type I IFN production in response to cytosolic dsDNA Fig. (**1**).

Although several studies have shown that DNA vaccine immunogenicity could be improved by incorporation of IL-1 and caspases [12,53], we do not know whether the inflammasome pathways, AIM2 and ASC, are required for DNA vaccine immunogenicity. However, recent evidence suggests that the AIM2/ASC inflammasome presumably has little impact on the immunogenicity/adjuvanticity of a DNA vaccine. We have recently shown that alum adjuvant (the only adjuvant licensed for human use) induces cell death and release of DNA; this in turn induces IL-1β release, which could be responsible for alum's adjuvanticity, independent of ASC or Caspase-1 ([1]Marichal T *et al,* in press). This hypothesis could be tested using AIM2 and/or ASC-deficient mice for DNA vaccination studies.

1.4. Can we Improve DNA Vaccine Immunogenicity by Incorporating TBK1 as a Novel adjuvant?

Having shown that plasmid DNA-induced immunogenicity was due to its adjuvant properties (mediated via the TBK1 kinase), we evaluated whether such immunogenicity could be improved by incorporating overexpressed TBK1 as an adjuvant. First, we confirmed that TBK1 protein expression was successful using *in vitro* transient transfection (*T. Aoshi, unpublished observations*). However, after many attempts to improve immunogenicity (i.e. mixing the TBK1 encoding plasmid with the antigen encoding plasmid, or using a plasmid co-expressing model of antigen plus TBK1 in a single backbone) we finally concluded that co-expressing

[1] Marichal T, Ohata K, Bedoret D, Mesnil C, Sabatel C, Kobiyama K, Lekeux P, Coban C, Akira S, Ishii KJ, Bureau F, Desmet CJ. DNA released from dying host cells mediates aluminum adjuvant activity. Nat Med In press 2011.

TBK1 in the same backbone do not enhance immunogenicity for both humoral and cellular immunity (*T. Aoshi, unpublished observations*). It is possible that the immunization route used (intramuscular electroporation) may not have been ideal (i.e. electroporation itself may release DNA) for evaluating the effect of the TBK1 molecule. Therefore, we optimized the plasmid doses and immunization routes using plasmids encoding one of the leading vaccine candidate antigens from the blood stages of *P. falciparum*, the serine repeat antigen 36 (PfSERA36) [54]. Targeting *Plasmodium* blood-stage antigens might have several benefits for humans living in malaria endemic regions [55-56]. Younger children would primarily benefit from such vaccines because they are disproportionately affected by the severity of the disease, which can result in death.

We tested intramuscular (i.m.) injection route which is milder way of introducing DNA vaccine. Equal amounts of the plasmids (50 µg) were introduced intramuscularly (i.m.)

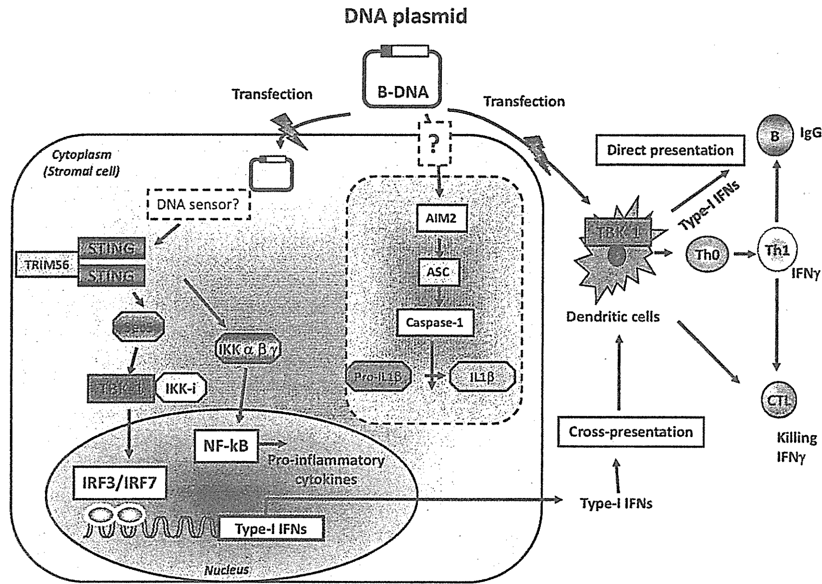

Fig. (1). Double-stranded plasmid DNA utilizes STING and TBK1 for production of type I IFN. The induction of both antigen-specific B cells and CD4+ T cells in hematopoietic cells (i.e. dendritic cells) and CD8+ T cells in non-hematopoietic cells (i.e. muscle cells) is mediated via type I IFNs controlled by STING/TBK1. Therefore, both direct priming and cross priming of the adaptive immunity occur after DNA vaccination. (Abbreviations: B-DNA; B form right-handed helical structure deoxyribonucleic acid, STING; stimulator of interferon genes, Sec5; subunit of the exocyst complex 5, TRIM56; interferon-inducible tripartite-motif 56, TBK1; TANK-binding kinase 1, IKK; IkB kinase, IKK-i; inducible IKK, IRF; interferon regulatory factor, Nf-κB; nuclear factor κB, AIM-2; absent in melanoma 2, ASC; Apoptotic speck protein containing a caspase recruitment domain).

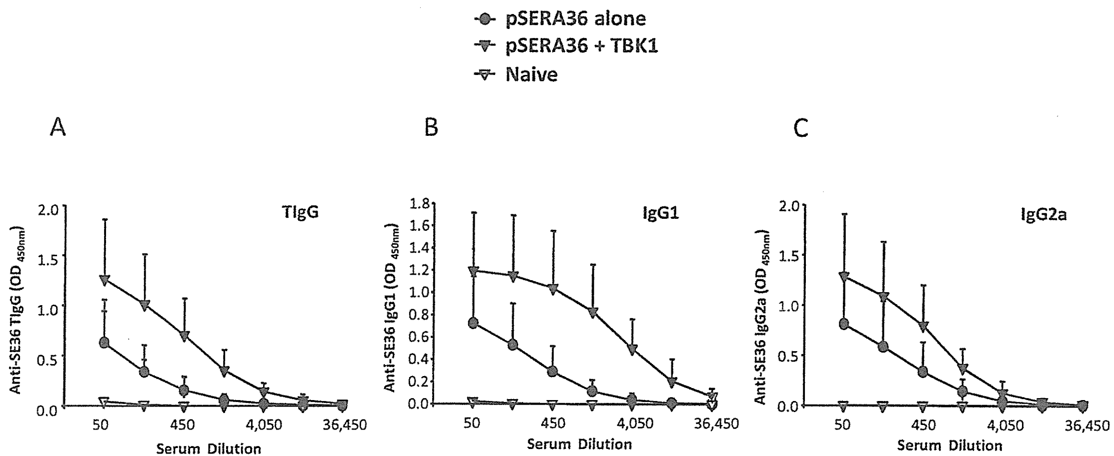

Fig. (2). Co-immunization with a TBK1-encoding plasmid improves DNA vaccine immunogenicity. Mice were immunized with 50 µg of each plasmid (encoding either the *P. falciparum* SERA36 or TBK1) via intramuscular (i.m.) immunization and boosted 4 weeks later with PfSERA36 antigen (1 µg) formulated in alum intradermally (i.d.). Total IgG (A), IgG1 (B), and IgG2a (C) responses for the *P. falciparum* SERA36 antigen were analyzed by ELISA 2 weeks after the booster immunizations.

into mice and boosted 4 weeks later with a PfSERA36 antigen intradermally (i.d.). The TBK1-encoding plasmids, while successfully improving antigen-specific antibody responses, both IgG1 and IgG2a isotypes, Fig. (**2 A-C**) in the plasmid cocktails of SERA36, failed to improve cellular immune responses (*K. Kobiyama, unpublished observations*). Our results suggested that simple injection of TBK1 expressing plasmid in DNA vaccine plasmid cocktail may improve, at least anti-malarial humoral immunogenicity.

CONCLUSION

Recent attempts to identify the double-stranded DNA sensor have provided great insight into the molecular and cellular mechanisms contributing to DNA vaccine immunogenicity (Table **1**). Our current understanding highlights the importance of type-I IFN mediated innate immune activation via the STING/TBK1 intracellular detection machinery. Such activation confers the adjuvant effect to the encoded antigen. Evidence from our studies suggests that the TBK1-encoded DNA vaccine plasmids used for targeting intracellular signaling pathways might have enormous potential to modulate the innate immune system and increase the immunogenicity of DNA vaccines. A major goal now is to translate the findings of these studies into medical applications (i.e. develop a blood-stage malaria vaccine against *P. falciparum*). It is our hope that studies of this type may deliver new ways to develop safe and effective vaccine adjuvants.

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REFERENCES

[1] Liu MA. DNA vaccines: an historical perspective and view to the future. Immunol Rev 2011; 239: 62-84.

[2] Liu MA, Ulmer JB. Human clinical trials of plasmid DNA vaccines. Adv Genet 2005; 55: 25-40.

[3] Davis BS, Chang GJ, Cropp B, *et al.* West Nile virus recombinant DNA vaccine protects mouse and horse from virus challenge and expresses *in vitro* a noninfectious recombinant antigen that can be used in enzyme-linked immunosorbent assays. J Virol 2001; 75: 4040-7.

[4] Corbeil S, Lapatra SE, Anderson ED, *et al.* Evaluation of the protective immunogenicity of the N, P, M, NV and G proteins of infectious hematopoietic necrosis virus in rainbow trout oncorhynchus mykiss using DNA vaccines. Dis Aquat Organ 1999; 39: 29-36.

[5] Bergman PJ, McKnight J, Novosad A, *et al.* Long-term survival of dogs with advanced malignant melanoma after DNA vaccination with xenogeneic human tyrosinase: a phase I trial. Clin Cancer Res 2003; 9: 1284-90.

[6] Kibuuka H, Kimutai R, Maboko L, *et al.* A phase 1/2 study of a multiclade HIV-1 DNA plasmid prime and recombinant adenovirus serotype 5 boost vaccine in HIV-Uninfected East Africans (RV 172). J Infect Dis 2010; 201: 600-7.

[7] Moorthy VS, Imoukhuede EB, Milligan P, *et al.* A randomised, double-blind, controlled vaccine efficacy trial of DNA/MVA ME-TRAP against malaria infection in Gambian adults. PLoS Med 2004; 1: e33.

[8] Ishii KJ, Weiss WR, Ichino M, Verthelyi D, Klinman DM. Activity and safety of DNA plasmids encoding IL-4 and IFN gamma. Gene Ther 1999; 6: 237-44.

[9] Applequist SE, Rollman E, Wareing MD, *et al.* Activation of innate immunity, inflammation, and potentiation of DNA vaccination through mammalian expression of the TLR5 agonist flagellin. J Immunol 2005; 175: 3882-91.

[10] Stevenson FK. DNA vaccines and adjuvants. Immunol Rev 2004; 199: 5-8.

[11] Sasaki S, Xin KQ, Okudela K, Okuda K, Ishii N. Immunomodulation by apoptosis-inducing caspases for an influenza DNA vaccine delivered by gene gun. Gene Ther 2002; 9: 828-31.

[12] Sasaki S, Amara RR, Oran AE, Smith JM, Robinson HL. Apoptosis-mediated enhancement of DNA-raised immune responses by mutant caspases. Nat Biotechnol 2001; 19: 543-7.

[13] Moore AC, Hill AV. Progress in DNA-based heterologous prime-boost immunization strategies for malaria. Immunol Rev 2004; 199: 126-43.

[14] Trollet C, Bloquel C, Scherman D, Bigey P. Electrotransfer into skeletal muscle for protein expression. Curr Gene Ther 2006; 6: 561-78.

[15] Sardesai NY, Weiner DB. Electroporation delivery of DNA vaccines: prospects for success. Curr Opin Immunol 2011.

[16] Broderick KE, Shen X, Soderholm J, *et al.* Prototype development and preclinical immunogenicity analysis of a novel minimally invasive electroporation device. Gene Ther 2011; 18: 258-65.

[17] Singh M, Chakrapani A, O'Hagan D. Nanoparticles and microparticles as vaccine-delivery systems. Expert Rev Vaccines 2007; 6: 797-808.

[18] Pokorna D, Rubio I, Muller M. DNA-vaccination via tattooing induces stronger humoral and cellular immune responses than intramuscular delivery supported by molecular adjuvants. Genet Vaccines Ther 2008; 6: 4.

[19] Kaisho T, Akira S. Toll-like receptors as adjuvant receptors. Biochim Biophys Acta 2002; 1589: 1-13.

[20] Ishii KJ, Akira S. Toll or toll-free adjuvant path toward the optimal vaccine development. J Clin Immunol 2007; 27: 363-71.

[21] Ishii KJ, Koyama S, Nakagawa A, Coban C, Akira S. Host innate immune receptors and beyond: making sense of microbial infections. Cell Host Microbe 2008; 3: 352-63.

[22] Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat Immunol 2010; 11: 373-84.

[23] Barber GN. Innate immune DNA sensing pathways: STING, AIMII and the regulation of interferon production and inflammatory responses. Curr Opin Immunol 2011; 23: 10-20.

[24] Klinman DM. Immunotherapeutic uses of CpG oligodeoxynucleotides. Nat Rev Immunol 2004; 4: 249-58.

[25] Krieg AM. Therapeutic potential of Toll-like receptor 9 activation. Nat Rev Drug Discov 2006; 5: 471-84.

[26] Hemmi H, Takeuchi O, Kawai T, *et al.* A Toll-like receptor recognizes bacterial DNA. Nature 2000; 408: 740-5.

[27] Sato Y, Roman M, Tighe H, *et al.* Immunostimulatory DNA sequences necessary for effective intradermal gene immunization. Science 1996; 273: 352-4.

[28] Klinman DM, Yamshchikov G, Ishigatsubo Y. Contribution of CpG motifs to the immunogenicity of DNA vaccines. J Immunol 1997; 158: 3635-9.

[29] Kojima Y, Xin KQ, Ooki T, *et al.* Adjuvant effect of multi-CpG motifs on an HIV-1 DNA vaccine. Vaccine 2002; 20: 2857-65.

[30] Pontarollo RA, Babiuk LA, Hecker R, Van Drunen Littel-Van Den Hurk S. Augmentation of cellular immune responses to bovine herpesvirus-1 glycoprotein D by vaccination with CpG-enhanced plasmid vectors. J Gen Virol 2002; 83: 2973-81.

[31] Coban C, Ishii KJ, Gursel M, Klinman DM, Kumar N. Effect of plasmid backbone modification by different human CpG motifs on the immunogenicity of DNA vaccine vectors. J Leukoc Biol 2005; 78: 647-55.

[32] Spies B, Hochrein H, Vabulas M, *et al.* Vaccination with plasmid DNA activates dendritic cells via Toll-like receptor 9 (TLR9) but functions in TLR9-deficient mice. J Immunol 2003; 171: 5908-12.

[33] Babiuk S, Mookherjee N, Pontarollo R, *et al.* TLR9-/- and TLR9+/+ mice display similar immune responses to a DNA vaccine. Immunology 2004; 113: 114-20.

[34] Pavlenko M, Leder C, Moreno S, Levitsky V, Pisa P. Priming of CD8+ T-cell responses after DNA immunization is impaired in TLR9- and MyD88-deficient mice. Vaccine 2007; 25: 6341-7.

[35] Rottembourg D, Filippi CM, Bresson D, *et al.* Essential role for TLR9 in prime but not prime-boost plasmid DNA vaccination to activate dendritic cells and protect from lethal viral infection. J Immunol 2010; 184: 7100-7.

[36] Takeshita F, Tanaka T, Matsuda T, *et al.* Toll-like receptor adaptor molecules enhance DNA-raised adaptive immune responses against influenza and tumors through activation of innate immunity. J Virol 2006; 80: 6218-24.

[37] Sasaki S, Amara RR, Yeow WS, Pitha PM, Robinson HL. Regulation of DNA-raised immune responses by cotransfected interferon regulatory factors. J Virol 2002; 76: 6652-9.

[38] Castaldello A, Sgarbanti M, Marsili G, *et al.* Interferon regulatory factor-1 acts as a powerful adjuvant in tat DNA based vaccination. J Cell Physiol 2010; 224: 702-9.

[39] Ishii KJ, Kawagoe T, Koyama S, *et al.* TANK-binding kinase-1 delineates innate and adaptive immune responses to DNA vaccines. Nature 2008; 451: 725-9.

[40] Ishii KJ, Coban C, Kato H, *et al.* A Toll-like receptor-independent antiviral response induced by double-stranded B-form DNA. Nat Immunol 2006; 7: 40-8.

[41] Coban C, Koyama S, Takeshita F, Akira S, Ishii KJ. Molecular and cellular mechanisms of DNA vaccines. Hum Vaccin 2008; 4: 453-6.

[42] Ishikawa H, Barber GN. STING is an endoplasmic reticulum adaptor that facilitates innate immune signalling. Nature 2008; 455: 674-8.

[43] Chien Y, Kim S, Bumeister R, *et al.* RalB GTPase-mediated activation of the IkappaB family kinase TBK1 couples innate immune signaling to tumor cell survival. Cell 2006; 127: 157-70.

[44] Tsuchida T, Zou J, Saitoh T, *et al.* The ubiquitin ligase TRIM56 regulates innate immune responses to intracellular double-stranded DNA. Immunity 2010; 33: 765-76.

[45] Fitzgerald KA, McWhirter SM, Faia KL, *et al.* IKKepsilon and TBK1 are essential components of the IRF3 signaling pathway. Nat Immunol 2003; 4: 491-6.

[46] Sharma S, tenOever BR, Grandvaux N, *et al.* Triggering the interferon antiviral response through an IKK-related pathway. Science 2003; 300: 1148-51.

[47] Ishikawa H, Ma Z, Barber GN. STING regulates intracellular DNA-mediated, type I interferon-dependent innate immunity. Nature 2009; 461: 788-92.

[48] Shirota H, Petrenko L, Hattori T, Klinman DM. Contribution of IRF-3 mediated IFNbeta production to DNA vaccine dependent cellular immune responses. Vaccine 2009; 27: 2144-9.

[49] Roberts TL, Idris A, Dunn JA, *et al.* HIN-200 proteins regulate caspase activation in response to foreign cytoplasmic DNA. Science 2009; 323: 1057-60.

[50] Fernandes-Alnemri T, Yu JW, Datta P, Wu J, Alnemri ES. AIM2 activates the inflammasome and cell death in response to cytoplasmic DNA. Nature 2009; 458: 509-13.

[51] Burckstummer T, Baumann C, Bluml S, *et al.* An orthogonal proteomic-genomic screen identifies AIM2 as a cytoplasmic DNA sensor for the inflammasome. Nat Immunol 2009; 10: 266-72.

[52] Hornung V, Ablasser A, Charrel-Dennis M, *et al.* AIM2 recognizes cytosolic dsDNA and forms a caspase-1-activating inflammasome with ASC. Nature 2009; 458: 514-8.

[53] Okuda K, Kawamoto S, Fukushima J. Cytokine and costimulatory factor-encoding plasmids as adjuvants for DNA vaccination. Methods Mol Med 2000; 29: 197-204.

[54] Horii T, Shirai H, Jie L, *et al.* Evidences of protection against blood-stage infection of Plasmodium falciparum by the novel protein vaccine SE36. Parasitol Int 2010; 59: 380-6.

[55] Coban C, Igari Y, Yagi M, *et al.* Immunogenicity of whole-parasite vaccines against Plasmodium falciparum involves malarial hemozoin and host TLR9. Cell Host Microbe 2010; 7: 50-61.

[56] Coban C, Horii T, Akira S, Ishii KJ. TLR9 and endogenous adjuvants of the whole blood-stage malaria vaccine. Expert Rev Vaccines 2010; 9: 775-84.

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