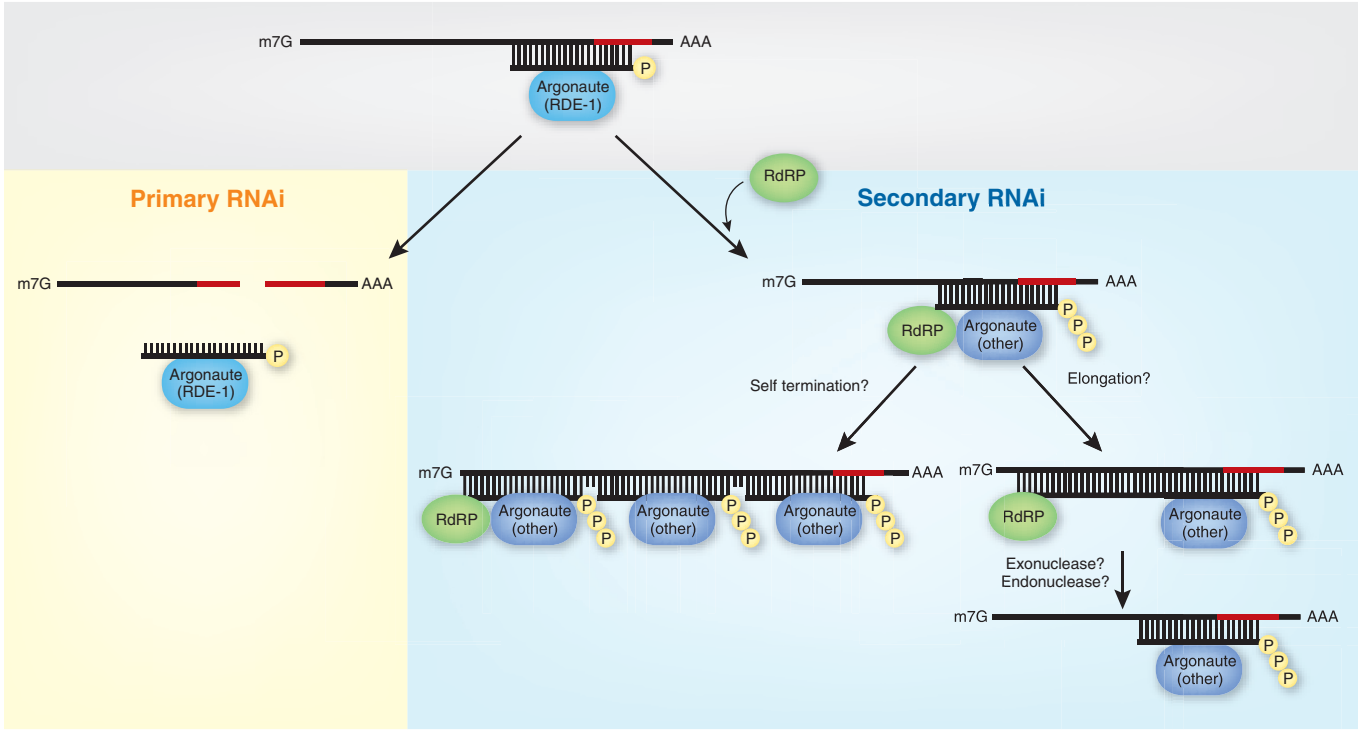

Figure 1 In *C. elegans*, RNAi has two pathways: primary RNAi and secondary RNAi. Primary siRNAs, which are 21–23 nucleotides long and are 5′-monophosphorylated, bind in complex with the Argonaute protein RDE-1 to a target mRNA, resulting in cleavage of the target mRNA cleavage. Secondary siRNAs are synthesized by an RNA-dependent RNA polymerase (RdRP) and are 22 or 23 nucleotides in length. Their length may be determined by self-termination of the RdRP, endonuclease cleavage or exonuclease cleavage. Secondary siRNAs are 5′-triphosphorylated and associate with non-RDE-1 Argonautes. It is unclear whether secondary siRNAs lead to direct target cleavage, indirect destabilization of the target or transcriptional gene silencing.

RNAi amplification. This might be beneficial, as extensive spreading would be more likely to lead to production of secondary siRNAs that could target messages related in sequence. This may be particularly pertinent for *C. elegans*, where exogenous dsRNA induces systemic RNAi[8]. Does the absence of RdRPs in *D. melanogaster* and mammals suggest that these species do not have a secondary RNAi pathway or an alternative one? Perhaps the interferon response in mammals is an alternative mechanism for preventing an excessive RNAi response[9].

Pak & Fire and Sijen *et al.* have introduced some order into the emerging siRNA zoo. Of key importance now is to find out how secondary siRNAs contribute to exogenous RNAi-induced silencing and their roles in endogenous gene regulation. Although it is too early to assess whether secondary RNAi pathways could be exploited as new tools for manipulating gene expression for biotechnology, the elucidation of these pathways should offer insight into this question.

COMPETING INTERESTS STATEMENT
The authors declare that they have no competing financial interests.

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Toll-free vaccines?

Arthur M Krieg

Strong immune responses can be activated in the absence of a major immune pathway.

In a recent report in *Nature*, Nemazee and colleagues[1] put forward a proposal that verges on heresy to some immunologists involved in vaccine development. Working with mice, they show that it is possible to stimulate strong antibody responses using an experimental antigen and common vaccine adjuvants without any contribution from Toll-like receptor (TLR) pathways. Although TLR ligands have been an important focus of recent vaccine research, the authors propose that their exclusion from vaccines may avoid unwanted side effects.

Vaccination has been the single greatest success of biomedical science, enabling eradication or control of smallpox, polio and many other diseases. Nevertheless, there is still much room for further development as we have no effective vaccines against malaria, AIDS and many other infectious diseases. Recent research on vaccine development has focused on improving adjuvants (from the Latin *adjuvare,* to

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help), immune boosters that trigger an effective response against the vaccine antigen.

Vaccine efficacy is generally linked to B cell–dependent production of protective antibodies that block pathogen infection. B cells normally require help from antigen-specific T cells to differentiate into antibody-secreting cells (**Fig. 1**). For some types of vaccines under development, such as therapeutic HIV or cancer vaccines, different antigen-specific T cells, killer T cells, are desired for their ability to directly and specifically kill infected or tumor cells. A natural infection normally triggers a protective immune response—a person can get infected with the measles virus only once, because the immune system develops 'memory' against the virus (antibodies against measles antigens) that prevents reinfection with the same virus. In addition, memory is specific—a person immune to measles could still be infected by a different virus, such as chickenpox. Immune memory and specificity characterize 'adaptive' immune responses, in contrast to 'innate' immune responses, which act to control most infections until adaptive immunity is generated.

Early vaccines were attenuated, or weakened, strains of the pathogen that stimulate a protective response without causing sickness. However, because attenuated vaccines can be lethal for people with weakened immune systems, the trend in vaccination has been to move to the use of 'subunit vaccines', which are composed of highly purified antigens that can be targeted specifically by the immune system. The role of the adjuvant is to stimulate the immune system to trigger an effective response to the purified subunit, which by itself is ignored by the immune system. The mechanisms of action of adjuvants remain unclear.

For many years, the gold-standard adjuvant was an emulsion of killed mycobacteria in oil, known as complete Freund's adjuvant (CFA). Although effective, CFA is too toxic for human use, especially if injected repeatedly, and even the oil component alone (incomplete Freund's adjuvant, IFA) causes injection-site reactions severe enough to preclude its use except in cancer vaccines. Adjuvant discovery was a matter of trial and error for many years, which may explain why alum, an aluminum salt developed in the early 1920s, is still the most widely used adjuvant. An especially promising class of adjuvants in development today is TLR ligands, which are thought to work by binding to TLRs, a family of ten immune proteins in humans.

A pivotal advance in understanding how vaccines work was the realization that the optimal development of an adaptive immune response requires that the vaccine activate a concomitant innate immune response. One of the most effective vaccines known, the live attenuated yellow fever vaccine 17D, stimulates multiple TLRs, the activation of which is essential for the generation of the adaptive immune response[2]. Another study even suggested that the production of antibody responses requires TLR activation in B cells[3].

It has become widely accepted that innate immunity can easily be triggered by stimulating TLRs and will lead to strong adaptive immunity. Indeed, synthetic ligands for almost all of the TLRs have been reported to be effective vaccine adjuvants in animal studies, and ligands for TLR4 and TLR9 have enhanced vaccine responses in human clinical trials.

A TLR4 agonist, monophosphoryl lipid A (MPL; often called 'Ribi adjuvant'when mixed in trehalose dicorynomycolate, a formulation that provides additional adjuvant activity), boosts the percentage of people who develop protective antibody levels to a hepatitis B vaccine within three months of initial vaccination from ~60% of subjects receiving the TLR-free commercial vaccine to 100% of the subjects receiving the MPL-containing vaccine[4]. The hepatitis B vaccine containing MPL was recently licensed in Europe (Fendrix).

Two TLR9-agonist adjuvants, oligodeoxynucleotides containing unmethylated CpG motifs (CpG-ODN), have been tested in clinical trials as adjuvants to the hepatitis B vaccine[5,6] and induce protective antibodies in most normal subjects within just two weeks of the first vaccine dose, compared with none in the subjects receiving a conventional vaccine. For one of the CpG-ODN, CPG 7909, 100% of the vaccinated subjects achieved protective antibody levels within just six weeks[5]. Remarkably, hepatitis B vaccination with CPG 7909 rapidly induced long-lasting (>three years) protective antibody titers even in HIV-infected subjects who had previously failed to respond[7]. Co-administration of CPG 7909 with a one-tenth normal dose of an influenza vaccine in normal volunteers restored the full level of antigen-specific IFN-γ secretion[8]. In a tumor vaccine, CPG 7909 with

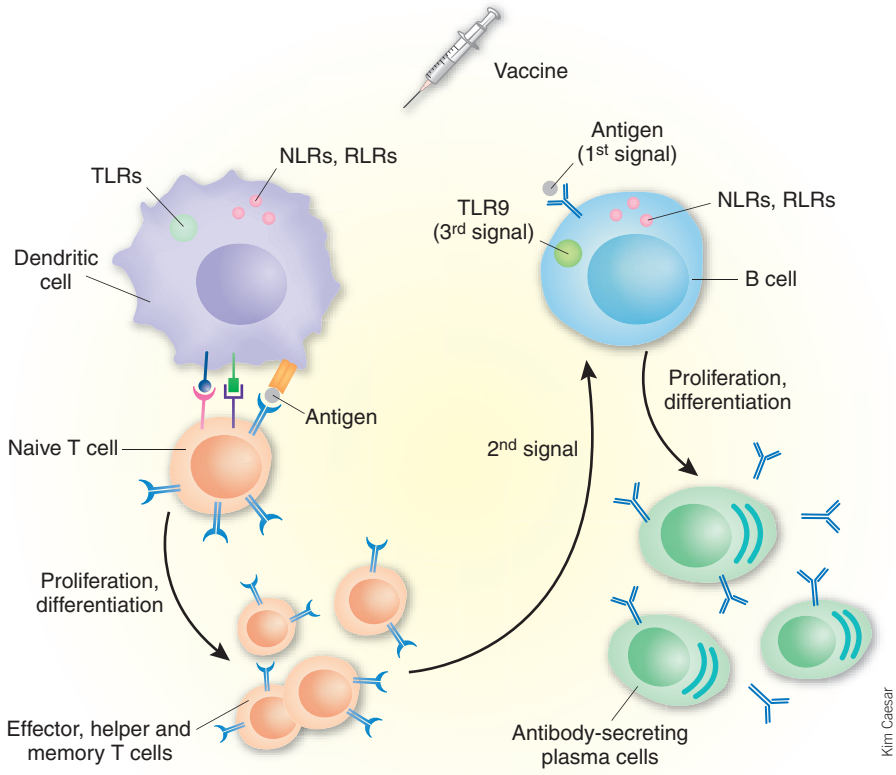

Figure 1 Development of an effective vaccine. At a minimum, a vaccine must have an antigen and an adjuvant (the adjuvant is inherent in the case of live, attenuated vaccines[2]). To produce antibodies to the vaccine, B cells are thought to require two signals: (i) binding to the antigen through the surface receptor and (ii) a co-stimulatory signal provided by T cells activated against the same antigen by dendritic cells. Adjuvants are essential for the provision of the second signal, but some adjuvants, such as TLR9 agonists, also can provide a third stimulatory signal through TLR9 expressed in the B cell. Some NOD-like receptors (NLRs) and RIG-I-like receptors (RLRs) are thought to be expressed within all immune cell types and can trigger cell activation[11], making them interesting candidate targets for the development of new vaccine adjuvants.

IFA increased by ~tenfold the frequencies of antigen-specific $CD8^+$ T cells in melanoma patients, making this the strongest adjuvant yet reported for inducing this type of immune response[9].

However, the use of MPL or CpG ODN vaccine adjuvants can increase the frequency of the usual vaccine-associated toxicities: injection-site reactions and transient flu-like symptoms, such as muscle and joint aches, fatigue, nausea, malaise and headache, which are usually graded as minimal to mild[4–9].

Nemazee and colleagues used mice that were genetically deficient in all the known TLR pathways to determine whether these pathways were needed for the mice to make an antibody response against a chemically modified antigen (not an actual vaccine) in combination with any of four of the standard adjuvants mentioned above: alum, CFA, IFA and the Ribi adjuvant. Both CFA and Ribi adjuvant contain TLR ligands (along with other components that could have TLR-independent adjuvant activity), and therefore might have been expected to be inactive in TLR-deficient mice. Surprisingly, all these adjuvants drove strong antigen-specific antibody responses in the absence of any functional TLR pathways.

However, no assays were performed to examine the T-cell response to the antigen, and the duration of the immunity was not tested. In addition, there was no challenge experiment to determine whether the antibodies would be equally effective against a pathogen—previous studies in monkeys vaccinated with the FDA-approved anthrax vaccine, AVA, with or without CPG 7909 have shown that the TLR9-induced antibodies provide improved protection against lethal anthrax challenge upon passive transfer of the immune serum into mice[10].

What do these results mean for investigators developing new vaccines? The authors did not address the question of the safety and efficacy of TLR agonists as vaccine adjuvants. In fact, based on the limited human clinical trials performed to date, it appears likely that adding a specific TLR4 or TLR9 agonist can dramatically enhance the efficacy of a vaccine without adding major toxicity. However, TLRs are no longer the only game in town—the new findings point to the existence of other pathways for the activation of specific antibody production that might also be harnessed for the development of new generations of adjuvants and vaccines (**Fig. 1**). At present, it is unclear whether these TLR-independent pathways are independent of the B cell, whether they are mediated by soluble factors produced by some other cell type or whether they involve cooperative interactions of several immune cells. Candidates for these other pathways are the recently described families of NOD-like receptors (NLRs) and RIG-I-like receptors (RLRs)[11], but further studies are now needed to clarify the possible contributions of these and other pathways in vaccination.

COMPETING INTERESTS STATEMENT
The author declares competing financial interests (see the *Nature Biotechnology* website for details).

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Erratum: Billion dollar babies—biotech drugs as blockbusters

Stacy Lawrence
Nature Biotechnology **25, 380–382 (2007); published online 03 April 2007; corrected after print 1 June 2007.**

In the version of this article initially published, the subheadings for Table 1, columns three and four should have said ($ billions) rather than ($ millions).

Corrigendum: Toll-free vaccines?

Arthur M Krieg
Nature Biotechnology **25, 303–305 (2007); published online 7 March 2007; corrected after print 1 June 2007.**

In the version of this article initially published, reference 1 is incorrect. The correct reference is Gavin, A.L. *et al. Science* 314, 1936–1938 (2006). The incorrect reference has been replaced in the HTML and PDF versions of the article.

Addendum: Response to "Blame factory farming, not organic food"

***Nature Biotechnology* responds**
Nature Biotechnology **25, 165–166 (2007); published online 7 February 2007; corrected after print 1 June 2007.**

The following information was not available when this correspondence response was initially published. At a California Senate hearing held on Tuesday, February 28, 2007, California Department of Health Services officials, under direct questioning by Senator Maldonado, indicated that the outbreak of *E. coli* O157:H7 in spinach originated from an organic farm, contrary to the statements made by Craig Holdrege. The entire hearing can be found at http://www.calchannel.com/archive.php by looking under February 2007, 022807, "Food-Borne Illness"; the comments relating to the organic source of the outbreak occur ~10 minutes into the hearing (after formalities). This addendum has been added to the HTML and PDF versions of the article.