Emerging concepts in the science of vaccine adjuvants

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Abstract | Adjuvants are vaccine components that enhance the magnitude, breadth and durability of the immune response. Following its introduction in the 1920s, alum remained the only adjuvant licensed for human use for the next 70 years. Since the 1990s, a further five adjuvants have been included in licensed vaccines, but the molecular mechanisms by which these adjuvants work remain only partially understood. However, a revolution in our understanding of the activation of the innate immune system through pattern recognition receptors (PRRs) is improving the mechanistic understanding of adjuvants, and recent conceptual advances highlight the notion that tissue damage, different forms of cell death, and metabolic and nutrient sensors can all modulate the innate immune system to activate adaptive immunity. Furthermore, recent advances in the use of systems biology to probe the molecular networks driving immune response to vaccines ('systems vaccinology') are revealing mechanistic insights and providing a new paradigm for the vaccine discovery and development process. Here, we review the 'known knowns' and 'known unknowns' of adjuvants, discuss these emerging concepts and highlight how our expanding knowledge about innate immunity and systems vaccinology are revitalizing the science and development of novel adjuvants for use in vaccines against COVID-19 and future pandemics.

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An adjuvant is a substance that is added to a vaccine to stimulate and enhance the magnitude and durability of the immune response (BOX 1). The traditional development of new vaccine adjuvants has been described as one of the slowest processes in the history of medicine[1]. For more than seven decades since initial licensure in the 1920s, insoluble aluminium salts (alum) remained the only adjuvant included in licensed products, such as vaccines against hepatitis B, diphtheria, tetanus and pertussis or human papilloma virus. However, in the late 1990s, the oil-in-water emulsion adjuvant MF59 was first included in a licensed product in Europe known as Fluad, which is a trivalent inactivated vaccine against seasonal influenza licensed for adults older than 65 years of age. In the 20 years since, four other adjuvants have been included in additional products: AS01 (for the shingles vaccine Shingrix and the malaria vaccine Mosquirix), AS04 (for the hepatitis B vaccine Fendrix and the human papilloma vaccine Cervarix), AS03 (for the pandemic influenza vaccines Pandemrix and Arepanrix) and cytosine phosphoguanosine (CpG) 1018 (for the hepatitis B vaccine Heplisav-B). Although many other adjuvants have demonstrated high potency in preclinical models during this period, most have not yet achieved licensure in humans, often owing to safety or tolerability concerns. Furthermore, despite their widespread use, the molecular mechanisms by which the available adjuvants — including alum, MF59 and the Adjuvant Systems AS0 adjuvants — actually work in humans is not well understood.

Nevertheless, the past two decades have witnessed a revolution in our understanding of how the innate immune system senses microbes, which offers a huge opportunity for additional insights into adjuvant design and development. In the late 1990s, it was discovered that activation of receptors such as Toll-like receptors (TLRs) — which are typically expressed on dendritic cells (DCs) and sense highly conserved pathogen-associated molecular patterns (PAMPs)[2–4] in microbes — results in activation of DCs, which stimulates antigen-specific T and B cell responses[5–8]. These discoveries provided strong experimental evidence for the major conceptual paradigm proposed by Charlie Janeway in 1989: that the innate immune system senses microbes through pattern recognition receptors (PRRs; such as TLRs) that recognize PAMPs and lead to activation of innate immune cells and the ensuing adaptive immune response[9]. In the decade following the discovery of TLRs, other innate PRRs (such as retinoic acid-inducible gene I (RIG-I) and other RNA sensors)[10], DNA sensors (such as stimulator of interferon genes (STING) protein)[11], C-type lectins[12], nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) and cytosolic receptors — such as NLRP3, which activate the inflammasome[13,14] — were also discovered and shown to impact adaptive

Box 1 | **The elements of a vaccine**

A vaccine comprises two components: antigens and adjuvants. Antigens typically consist of proteins or carbohydrates derived from the pathogen, against which an adaptive immune response is desired. An adjuvant is a substance that is added to a vaccine to stimulate and enhance the magnitude and durability of the immune response. The word adjuvant (derived from the Latin word 'adjuvare', meaning 'to help') was coined by the French veterinarian Gaston Ramon, who while working at the Pasteur Institute in 1920 discovered that horses vaccinated against diphtheria had stronger antibody titres if they developed inflammatory abscesses at the site of injection[234]. Ramon was able to show that adding various substances, such as breadcrumbs or starch, to the inactivated diphtheria toxin caused inflammation at the injection site and also boosted antibody production in response to the vaccine[235]. At around the same time, the British immunologist Alexander Thomas Glenny, at the Wellcome Physiological Research Laboratory in London, serendipitously discovered the adjuvant properties of alum and, in 1925–1926, developed the alum-precipitated diphtheria toxoid[236]. Glenny et al. were trying to purify diphtheria toxoid (inactive toxin) using potassium aluminium sulfate to develop a vaccine. Surprisingly, vaccines developed using aluminium salt precipitation led to better antibody responses than soluble toxoids. Since then, aluminium-containing adjuvants have been incorporated into billions of doses of vaccines and administered to millions of people every year.

immunity. Molecules that target these receptors are also being pursued as potential vaccine adjuvants[15].

Despite these major recent advances, our mechanistic understanding of how the currently available adjuvants included in licensed vaccines actually function remains underdeveloped. However, two major advances that have occurred during the past decade are beginning to yield deeper insights into the mechanism of action of adjuvants and are revitalizing the process of adjuvant discovery and development, which has been the status quo for decades. First, immunologists' view of how the immune system can be stimulated has evolved beyond the paradigm of pattern recognition by PAMPs originally proposed by Janeway. Thus, evidence has emerged that DCs and other cells of the innate immune system can become activated not only by pathogen sense via PRRs but also by tissue damage that results in the release of damage-associated molecular patterns (DAMPs)[16,17] and cell death via necroptosis or pyroptosis[18,19], as well as other stress signals such as amino acid starvation (via ancient stress and nutrient sensing pathways)[6,20]. Many of these stimuli have been shown to induce adaptive immunity, thus providing support for the concept of the 'Danger Model' proposed by Polly Matzinger in 1994 (REF.[21]). Second, the use of systems biology approaches to probe the innate and adaptive immune responses to vaccines and define the molecular networks that drive innate and adaptive immune response to vaccination in humans is beginning to yield fundamental new mechanistic insights about how immune responses are initiated and controlled[22]. This 'systems vaccinology' approach is now being increasingly used to assess immune responses to vaccination in humans, to identify molecular signatures that can predict vaccine efficacy and to obtain mechanistic insights. In this Review, we discuss how these advances are revitalizing the science of adjuvants. First, we discuss the known facts ('known knowns') and the known gaps ('known unknowns') of the adjuvants in licensed products. Second, we discuss several emerging concepts about the immunological mechanisms of action of adjuvants. In the final section, we provide a conceptual framework in which we highlight how systems-based approaches are beginning to revitalize adjuvant design and development.

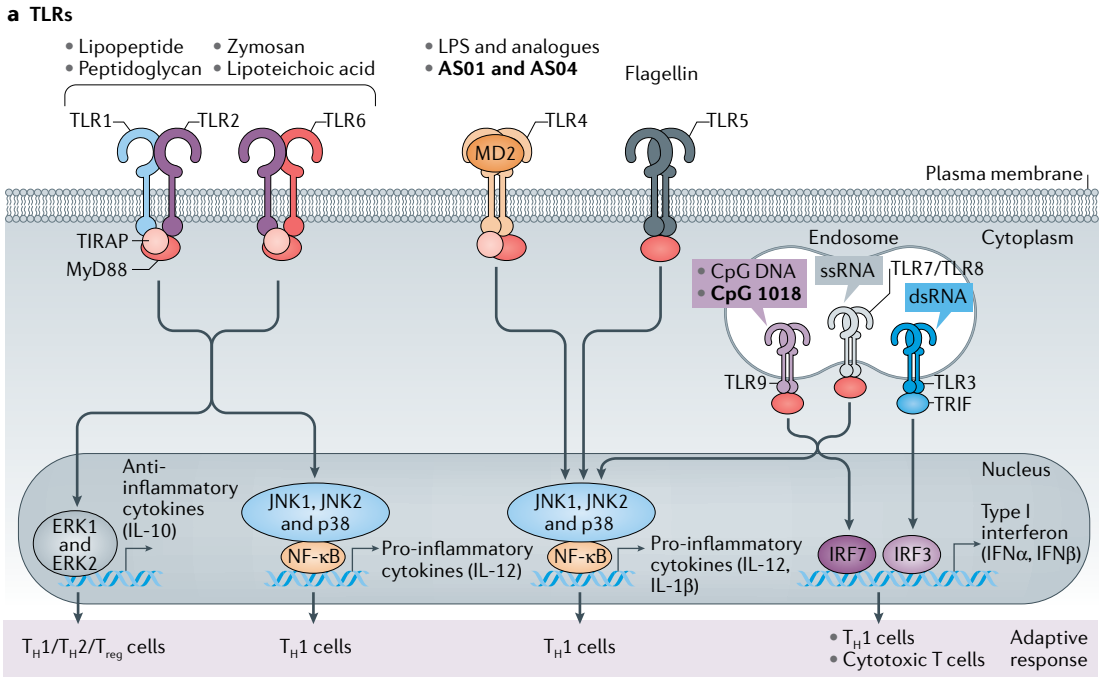
Germinal centre
(GC). A dynamic microenvironment in lymphoid organs where maturation of B cell responses occurs.

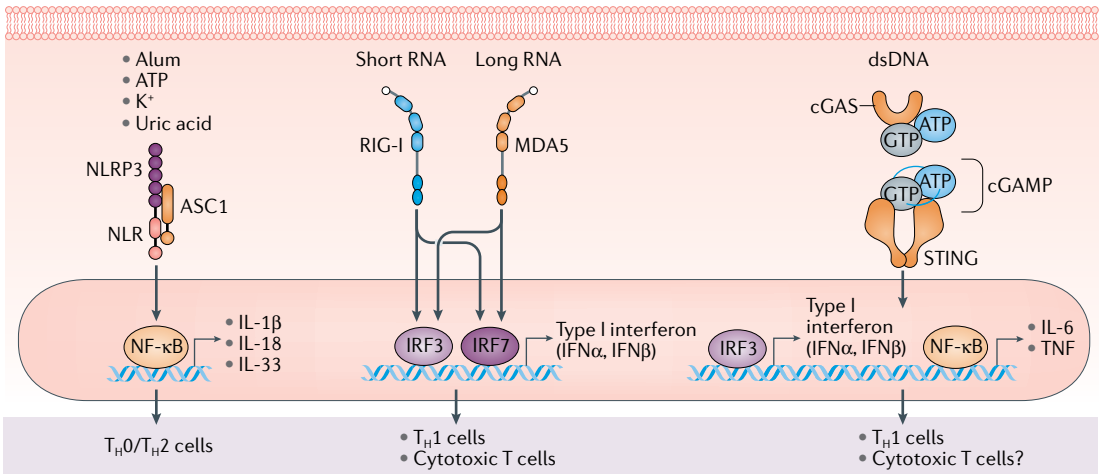
Adjuvants in licensed vaccines

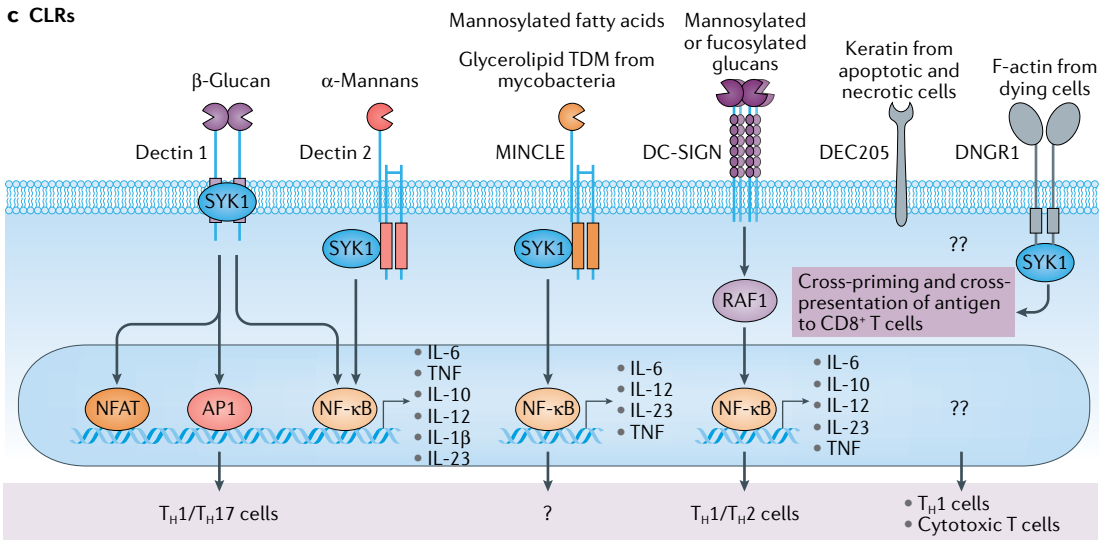
Endogenous adjuvants in live vaccines

Although only a handful of adjuvants are available for clinical use, it is now clear that many vaccines that have been used safely in billions of humans contain endogenous adjuvants. Significant conceptual advances in innate immunity and delineation of the critical innate receptors, the PRRs, have led to the realization that many live vaccines that are widely used induce immune responses partly by activating specific PRRs[23]. These vaccines consist of live attenuated pathogens that activate innate immunity through the expression of various PAMPs. For example, the live attenuated yellow fever vaccine (YF-17D) — one of the most potent vaccines ever developed and administered to 600 million people globally — was shown to activate the innate immune system by signalling through multiple TLRs (TLR2, TLR3 and TLR7–TLR9) as well as through RIG-I and melanoma differentiation-associated protein 5 (MDA5)[24] (FIG. 1). Importantly, TLR signalling is also essential for the immunogenicity of YF-17D (REFS[24,25]), although the additional impact of sustained antigen expression through replication of the attenuated virus should not be underappreciated. Similarly, the Bacillus Calmette–Guérin (BCG) vaccine against tuberculosis, of which more than 4 billion doses have been administered, activates TLR2, TLR4, TLR9 and CD209 antigen (also known as DC-SIGN)[26,27]. In addition, certain inactivated vaccines also trigger TLRs, such as the seasonal influenza vaccines that activate the innate immune system via TLR7 and myeloid differentiation primary response 88 (MyD88) signalling pathways[28]. These findings have further pointed to the continued rational development of TLR agonists as critical targets in adjuvant design, especially in the context of subunit vaccines, which do not contain endogenous adjuvants. Numerous studies have demonstrated the efficacy of synthetic TLR ligands as vaccine adjuvants in mice and in non-human primates (NHPs) in promoting enhanced and more durable antibody responses to vaccination with recombinant protein antigens, virus-like particles or DNA-encoded antigens[29,30].

The fact that live viral vaccines typically induce robust and persistent antibody responses in humans[31,32] raised the possibility that adjuvants given with inactivated vaccines could induce such robust and persistent antibody responses too. On the basis of the discovery that the yellow fever vaccine engages multiple DC subsets via multiple TLR ligands[24], we designed synthetic nanoparticles that contain TLR4 and TLR7/TLR8 ligands as adjuvants, which were used in combination with soluble protein antigens, such as chicken ovalbumin or influenza haemagglutinin[33]. Experiments in mice showed that these nanoparticle vaccines induced robust and durable antigen-specific antibody responses, robust and persistent antigen-specific germinal centre (GC) reactions, T follicular helper (T_{FH}) cell responses and long-lived plasma cells (LLPCs)[33]. Of note, these robust responses induced by the combination of TLR ligands lasted a lifetime in mice and required activation


a TLRs
• Lipopeptide
• Peptidoglycan
• Zymosan
• Lipoteichoic acid
• LPS and analogues
• AS01 and AS04
Flagellin
TLR1
TLR2
TLR6
MD2
TLR4
TLR5
Plasma membrane
TIRAP
MyD88
Endosome
Cytoplasm
• CpG DNA
• CpG 1018
ssRNA
TLR7/TLR8
dsRNA
TLR9
TLR3
TRIF
Nucleus
Anti-inflammatory cytokines (IL-10)
ERK1 and ERK2
JNK1, JNK2 and p38
NF-κB
Pro-inflammatory cytokines (IL-12)
JNK1, JNK2 and p38
NF-κB
Pro-inflammatory cytokines (IL-12, IL-1β)
IRF7
IRF3
Type I interferon (IFNα, IFNβ)
TH1/TH2/Treg cells
TH1 cells
TH1 cells
• TH1 cells
• Cytotoxic T cells
Adaptive response


b Cytosolic PRRs
• Alum
• ATP
• K+
• Uric acid
Short RNA
Long RNA
dsDNA
NLRP3
ASC1
NLR
RIG-I
MDA5
cGAS
ATP
GTP
cGAMP
STING
NF-κB
• IL-1β
• IL-18
• IL-33
IRF3
IRF7
Type I interferon (IFNα, IFNβ)
IRF3
Type I interferon (IFNα, IFNβ)
NF-κB
• IL-6
• TNF
TH0/TH2 cells
• TH1 cells
• Cytotoxic T cells
• TH1 cells
• Cytotoxic T cells?


c CLRs
Mannosylated fatty acids
Glycerolipid TDM from mycobacteria
Mannosylated or fucosylated glucans
Keratin from apoptotic and necrotic cells
F-actin from dying cells
β-Glucan
α-Mannans
Dectin 1
Dectin 2
MINCLE
DC-SIGN
DEC205
DNGR1
SYK1
SYK1
SYK1
??
SYK1
RAF1
Cross-priming and cross-presentation of antigen to CD8+ T cells
NFAT
AP1
NF-κB
• IL-6
• TNF
• IL-10
• IL-12
• IL-1β
• IL-23
NF-κB
• IL-6
• IL-12
• IL-23
• TNF
NF-κB
• IL-6
• IL-10
• IL-12
• IL-23
• TNF
??
TH1/TH17 cells
?
TH1/TH2 cells
• TH1 cells
• Cytotoxic T cells

◀ Fig. 1 | **Molecular targets of adjuvants. a** | Toll-like receptors (TLRs) TLR1, TLR2, TLR4, TLR5 and TLR6 are expressed on the cell surface, whereas TLR3, TLR7, TLR8 and TLR9 are expressed in endosomes. TLR1 and TLR6 heterodimerize with TLR2 and signal through the myeloid differentiation primary response 88 (MyD88) pathway to activate NF-κB and MAP kinases, leading to secretion of pro-inflammatory and anti-inflammatory cytokines. TLR4 and TLR5 function as homodimers and signal through the MyD88 pathway. TLR7 and TLR9 also use the MyD88 pathway, but rapidly activate IRF7 to induce type I interferons. TLR3 uses TIR domain-containing adapter-inducing IFNβ (TRIF) signalling to induce type I interferons through IRF3. **b** | Cytosolic pattern recognition receptors (PRRs) are sensors of pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) present inside the cytoplasm of the cell. Nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) are cytosolic sensors of bacterial PAMPs but also recognize multiple cellular products including ATP, uric acid and K^+ to activate the NF-κB pathway and induce cytokines driving T helper 2 (T_H2) cell differentiation. Retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated protein 5 (MDA5) are intracellular viral sensors that drive type I interferon response through IRF3 and IRF7. The cGAS–stimulator of interferon genes (STING) pathway recognized double-stranded DNA (dsDNA) to induce the NF-κB pathway. **c** | C-type lectin receptors (CLRs) are cell surface molecules expressed on multiple myeloid cell subsets. Dectins 1 and 2 and MINCLE recruit SYK1 and activate NF-κB through the CARD9–BCL-10–MALT1 complex. Furthermore, dectin 1 has been shown to induce the NFAT and AP1 pathways in macrophages and dendritic cells (DCs) and in in vitro experimental models, respectively. Dectins are also specialized in inducing antifungal immunity. Dendritic cell-specific ICAM3-grabbing non-integrin 1 (DC-SIGN) activates NF-κB via acetylation of p65; however, the resulting gene expression is poorly understood although IL-10 expression has been shown to be induced. DEC205 and DNGR1 are known to induce cross-presentation but the signalling pathways are unknown. ASC, apoptosis-associated speck-like protein containing a CARD; CpG, cytosine phosphoguanosine; dsRNA, double-stranded RNA; LPS, lipopolysaccharide; ssRNA, single-stranded RNA; TDM, trehalose-6,6-dimycolate; T_{reg} cell, regulatory T cell.

of multiple DC subsets and direct activation of B cells[33]. Subsequent studies in NHPs showed that such nanoparticle-encapsulated TLR ligands were potent adjuvants in stimulating robust and persistent antibody responses, GC and T_{FH} cell responses and a high frequency of LLPCs that persisted until the termination of the study at 70 weeks[30,34,35]. It seems that generating LLPCs may be the key to generating durability of antibody responses, and adjuvants driving differentiation of LLPCs will be of particular interest in vaccinology[36,37]. Thus, developing adjuvants that promote LLPCs represents a major challenge in vaccinology.

Recent work suggests that slow-release delivery systems (such as osmotic pumps) that release the antigen with delayed kinetics can enhance the magnitude, quality and persistence of antibody responses[38,39]. Although this has been attributed to enhanced GC reactions owing to longer antigen retention in lymph nodes, the effect of sustained activation of innate cells by the adjuvants has not been thoroughly evaluated. In this context, we and others have found that 3M-052, a novel TLR7/TLR8 agonist that is released slowly from the site of administration[40], results in activation of monocytes and DCs that lasts for 3–4 weeks[30], and induces persistent antibody responses and LLPCs of a much higher magnitude (up to 100-fold greater) than those observed with alum[30,35] that lasted until the termination of the study at 70 weeks post immunization.

Alum

Alum is the most commonly used adjuvant and induces antibody responses and $CD4^+$ T helper cell responses in humans[41–43] (TABLE 1). These T helper responses are T helper 2 (T_H2) cell-biased in mice[44–47], but this bias is less clear in humans[48]. It was long thought that alum mediated its adjuvant effects predominantly through a 'depot effect' mechanism that involved the slow release of antigens from the site of immunization[49]. Consistent with this, chemical modification of antigens with short peptides composed of repeating phosphoserine (pSer) residues enhances binding to alum — which results in prolonged immunogen bioavailability — and greatly enhances GC and antibody responses, compared with conventional alum-adsorbed antigens[50]. In addition, alum actually exerts several effects on the immune system[51]. As discussed above, although TLRs seemed to be essential for the immunogenicity of live viral vaccine such as the yellow fever vaccine YF-17D[24,25], the immune responses stimulated by immunization with alum plus antigen were unaffected in mice lacking the critical adaptor proteins involved in TLR signalling: MyD88 or TIR domain-containing adapter-inducing IFNβ (TRIF)[52]. This suggested that the adjuvant effects of alum occurred through a mechanism independent of TLR signalling. Later studies showed that alum activated the NLRP3 inflammasome[53–56], although the data are contradictory regarding the relative importance of this in mediating alum's adjuvant effect[57,58]. Experiments assessing alum-enhanced immune responses in mice deficient in NLRP3 signalling seem to give conflicting results on whether NLRP3 inflammasome activation is necessary for adaptive immune responses (TABLE 1).

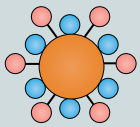
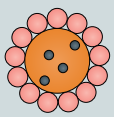
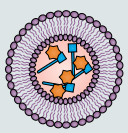
Alum can also enhance adaptive immunity by causing tissue damage that induces uric acid-mediated activation of inflammatory DCs[59]. Injection of alum rapidly recruited various cells, including neutrophils, which released neutrophil extracellular traps (NETs) composed of chromatin[60,61]. The DNA released in NETs partially mediated the adjuvant activity of alum[62,63]. For example, in mice, immunization with antigen plus alum induced cell death and the subsequent release of host cell DNA, which stimulated an antigen-specific IgE response and T_H2 cell responses. T_H2 cell responses were associated with IgE isotype switching via mechanisms dependent on the TANK-binding kinase 1 (TBK1) and the transcription factor IRF3, which are critical components of the signalling pathway mediated by the cytosolic DNA sensor STING[62] (TABLE 1). In some of those studies, co-injection of DNase preparations with alum and antigen reduced the immune response to vaccination. However, a subsequent study reported that commercial DNase preparations are contaminated with proteases, which were responsible for some of the inhibitory effects of DNase preparations on the adjuvant activity of alum[64]. Nevertheless, DNase lowers responses induced by alum to some extent, but the effect of DNase seemed to be independent of its ability to cleave DNA[64]. Thus, future work should be aimed at clarifying the relative importance of DNA sensing in mediating the induction of antibody and $CD4^+$ T helper cell responses induced by immunization with alum plus antigen. Alum is also used in inactivated virus COVID-19 vaccines that have been approved for limited or emergency use in certain countries[65,66] (TABLE 2). Hence, the mechanism of action of alum is complex, with likely several factors contributing simultaneously in small animals, and the contribution of the formulation cannot be ignored[67].

Isotype switching
A process in which antigen-experienced B cells switch from making IgM to other isotypes.

MF59

MF59 is an oil-in-water emulsion adjuvant that has been included in a licensed influenza vaccine in Europe since 1997 and has now been administered to more than 100 million people in more than 30 countries. This adjuvant comprises droplets of squalene oil, a biodegradable, biocompatible oil that is a normal component of the human body, stabilized in an aqueous buffer by the surfactants Tween 80 and Span 85, which are commonly used non-ionic surfactants[68]. Its mechanism of action has been explored and is reviewed elsewhere[69]. To summarize briefly, administration of

Table 1 | **The 'known knowns' and 'known unknowns' of adjuvants used in licensed vaccines**

Adjuvant	Known knowns	Known unknowns
Alum Aluminium hydroxide Aluminium phosphate 	Antibody response independent of TLR signalling[52] Activation of NLRP3 inflammasome in macrophages and DCs[53,54] Activation of DCs is mediated by uric acid[59] Rapid recruitment of neutrophils and formation of NETs[62] Induces cell death that releases DNA, which triggers STING–IRF3 activation, necessary for IgE antibody and T_H2 cell responsed[62]	Innate receptors and signalling that result in antibody and T helper cell responses are poorly understood The relevance of stress response signals, tissue damage, and metabolic and nutrient sensing pathways is poorly understood There is conflicting evidence for a role of NLRP3 inflammasome in mediating adjuvant activity[53,54,57]
MF59 Squalene Tween (polysorbate) 80 Span 85 	Activates macrophages and DCs at injection site[68] Induces chemokine secretion[71] Antibody and $CD4^+$ T cell responses depend on transient release of ATP by muscle cells[73] TLR-independent MyD88 activation and NLRP3-independent ASC activation[74,75] Stimulation of antigen-specific $CD8^+$ T cells in tissues is via RIPK3-dependent pathway[74]	Innate receptors and signalling that result in antibody and T helper cell responses are poorly understood The relevance of stress response signals, tissue damage, and metabolic and nutrient sensing pathways is poorly understood
AS04 Alum MPL 	Enhanced antigen presentation by AS04-activated DCs in comparison with alum[78] TLR4 activation by MPL is critical and alum prolongs TLR4-induced responses[78]	Innate receptors and signalling that sense alum and result in antibody and T helper cell responses poorly understood
AS03 Squalence and α-tocopherol Tween (polysorbate) 80 	Induction of NF-κB activity and chemokine response locally and in draining lymph nodes in mouse between 6 and 48 h (REF.[88]) α-Tocopherol activates human monocytes and macrophages[87]	Innate receptors and signalling that result in antibody and T helper cell responses are poorly understood The relevance of stress response signals tissue damage, and metabolic and nutrient sensing pathways is poorly understood
AS01 MPL QS-21 Liposomes 	Local secretion of chemokines, and IFNγ by NK cells and $CD8^+$ T cells in draining LNs within hours[101] QS-21 activates caspase 1 in SSMs[98] Induces differentiation of monocytes to DCs[213] Heterogeneous DC populations responsible for T cell activation in draining LNs[213]	Innate receptors and signalling that sense QS-21 and result in antibody and T helper cell responses are poorly understood The relevance of stress response signals, tissue damage, and metabolic and nutrient sensing pathways is poorly understood
CpG 1018 22-mer single-stranded DNA 	Activates TLR9, which results in MyD88 pathway and type I interferon response	None

ASC, apoptosis-associated speck-like protein containing a CARD; DC, dendritic cell; IFNγ, interferon-γ; LN, lymph node; MPL, 3-*O*-desacyl-4′-monophosphoryl lipid A; MyD88, myeloid differentiation primary response 88; NET, neutrophil extracellular trap; NK cell, natural killer cell; STING, stimulator of interferon genes; SSM, subcapsular sinus macrophage; T_H2 cell, T helper 2 cell; TLR, Toll-like receptor.

Table 2 | **Vaccine adjuvants in COVID-19 vaccines in limited use or in advanced clinical trials**

Adjuvant	Vaccine	Manufacturers	Status	Refs
Alum	Inactivated SARS-CoV-2 virus vaccines	Sinopharm Sinovac	Approved for limited or emergency use in certain countries	65,66
Matrix-M	Recombinant SARS-CoV-2 spike (S) protein	Novavax	Phase III	230
AS03	Recombinant SARS-CoV-2 spike (S) protein as a soluble protein or on virus-like particles	GSK (AS03) Sanofi (antigen) Medicago (antigen)	Phase I/II Phase III	85,86
CpG 1018	Recombinant SARS-CoV-2 spike (S) protein on virus-like particles	Dynavax (CpG 1018) Medicago (antigen)	Phase I/II	86
TLR7/TLR8 ligand adsorbed in alum	Inactivated SARS-CoV-2 vaccines	Bharath Biotech	Phase III/emergency use in India	233

COVID-19, coronavirus disease 2019; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; TLR, Toll-like receptor.

MF59 in muscle activates myeloid cells — such as macrophages and DCs — which respond by producing chemokines such as CC-chemokine ligand 2 (CCL2), CCL4, CCL5 and CXC-chemokine ligand 8 (CXCL8; also known as IL-8), which in turn recruit neutrophils, eosinophils and more monocytes and DCs to the injection site (TABLE 1). These cells amplify the response further and migrate to draining lymph nodes to activate B and T cells[69,70]. Studies in mice demonstrate that MF59 induces a broader range of cytokines and chemokines than alum, and rapidly recruits $CD11b^+$ inflammatory cells to the site of injection[71]. These observations based on small animal studies have been largely substantiated by similar studies carried out in NHPs[72]. Intramuscular injection of an MF59-adjuvanted influenza vaccine into mice results in the transient extracellular release of ATP, and local injections of apyrase, an ATP-hydrolysing enzyme, inhibited cell recruitment and antigen-specific $CD4^+$ T cell responses and antibody responses induced by MF59, but not by alum or incomplete Freund's adjuvant[73]. These data demonstrate that transient release of ATP induced by MF59-adjuvanted vaccines mediates the adaptive immune response.

With regards to the innate immune receptors involved in sensing MF59, Seubert et al. demonstrated that MF59-adjuvanted antibody responses were dependent on MyD88, but independent of NLRP3 inflammasome activation[74]. Immunization of *Myd88*-knockout mice with an MF59-adjuvanted vaccine against *Neisseria meningitidis* (rMenB) resulted in lower antibody titres compared with those obtained in control mice[74]. Surprisingly, however, MF59 did not activate TLR signalling in HEK293 cell lines expressing TLRs, which led the authors to conclude that MF59 induces antibody responses via an MyD88-dependent mechanism that is independent of TLRs[74]. In an independent study, immunization of an MF59-adjuvanted H5N1 subunit vaccine into mice genetically deficient in apoptosis-associated speck-like protein containing a CARD (ASC), an adaptor protein within the NLRP3 inflammasome, resulted in reduced H5-specific IgG antibody titres relative to that observed in wild-type mice[75]. Interestingly, and consistent with the study by Seubert et al., the response was intact in *Nlrp3*-knockout and *Casp1*-knockout mice[75], which led the authors to conclude that MF59-adjuvanted vaccines induce antibody responses via an ASC-dependent, but inflammasome-independent, pathway. Finally, our recent work demonstrates that subcutaneous immunization of mice with MF59 plus antigen stimulates antigen-specific $CD8^+$ T cell responses (which are stimulated at very low magnitude in the draining lymph nodes, but can be detected at much higher frequencies in tissues such as the lung and liver), via a mechanism dependent on receptor-interacting serine/threonine protein kinase 3 (RIPK3), which is a key mediator of necroptosis[76]. Thus, immunization of mice with antigen mixed with MF59 or its mimetic Addavax, but not with alum, induced rapid RIPK3-dependent necroptosis of lymph node macrophages. RIPK3-deficient mice were impaired in their capacity to mount antigen-specific $CD8^+$ T cell responses in the lung and liver, in response to subcutaneous immunization with antigen plus Addavax. However, surprisingly, such responses were normal in mice deficient in mixed-lineage kinase domain-like protein (MLKL), a downstream mediator of necroptosis, suggesting that the impaired $CD8^+$ T cell responses observed in RIPK3-deficient mice occurred through a mechanism independent of necroptosis[76]. In contrast to the effects observed on $CD8^+$ T cells, antibody responses were not affected in RIPK3-deficient mice or in caspase 1-deficient mice. However, they were impaired by administration of the pan-caspase inhibitor Z-VAD-FMK, suggesting a contribution of apoptotic caspases to the induction of antibody responses. These observations in mice provide mechanistic insights into the adjuvant effects of squalene-based adjuvants such as MF59, but the extent to which such mechanisms operate in humans remains to be determined.

The AS0 Adjuvant Systems

The Adjuvant Systems AS0 have been developed by GlaxoSmithKline over the past three decades and are based on a rational combination of classical adjuvant molecules — including alum, emulsions and liposomes — to obtain maximal adjuvant effect with acceptable tolerability, in combination with immunostimulatory molecules, such as TLR ligands and others.

AS04. AS04 consists of 3-*O*-desacyl-4′-monophosphoryl lipid A (MPL), a detoxified form of lipopolysaccharide (LPS) extracted from *Salmonella minnesota*, which is adsorbed on aluminium salts. Importantly, studies in mice have revealed that MPL retains its complete immunostimulatory activity through TLR4 activation, when adsorbed on alum. The adjuvant effect of AS04 is mediated by signalling through TLR4 on innate cells, in combination with the inherent immunomodulatory properties of alum[77] (TABLE 1). Addition of AS04 to the hepatitis B virus (HBV) and the human papillomavirus (HPV) vaccines based on recombinant antigens induced higher levels of antibodies in comparison with the same antigens adjuvanted with just alum, demonstrating the added value of the TLR4 agonist MPL in humans[78–81]. The higher immunogenicity also translated into a high and long-lasting efficacy of the HPV-16/18 vaccine. Data available now show that the efficacy of the vaccine approaches can reach 100%, more than 9 years after primary immunization[80,82]. Further studies are required to better understand the precise mechanisms behind this beneficial effect of the AS04-adjuvanted HPV vaccine. For example, the inclusion of AS04 results in a greater breadth of immune response against diverse HPV strains in humans, through mechanisms that are not yet clearly defined[83].

AS03. AS03 is a squalene oil-in-water emulsion adjuvant that is similar to MF59, but also contains α-tocopherol (vitamin E) as an additional immune-enhancing component. AS03 is licensed in combination with pandemic influenza vaccines[84] (TABLE 1). AS03 is being evaluated in clinical trials as a vaccine adjuvant for numerous different recombinant spike protein COVID-19 vaccines[85,86] (TABLE 2). AS03 enhances the magnitude and breadth of antibody responses and $CD4^+$ T cell responses, leading to enhanced protection against flu compared with non-adjuvanted vaccines[87]. Mouse[88,89] and human[90,91] studies have demonstrated that AS03 triggers transient innate immune responses, although additional mechanisms of action are likely also involved. In mice, AS03 stimulated a transient production of cytokines at the site of injection and in the draining lymph nodes[88]. α-Tocopherol modulated the expression of certain chemokines and cytokines such as CCL2, CCL3, IL-6, granulocyte colony-stimulating factor (G-CSF; encoded by *CSF3*) and CXCL1, enhanced antigen uptake in monocytes and increased the recruitment of granulocytes to the draining lymph nodes[88]. A subsequent study in mice demonstrated that intramuscular injection of AS03 elicited a rapid and transient downregulation of genes involved in lipid metabolism in the draining lymph nodes. In vitro, in myeloid cell lines, these changes were associated with changes in lipid composition and altered endoplasmic reticulum (ER) morphology, and with activation of the unfolded protein response (UPR) pathway. In vivo, treatment with a chemical inhibitor of the ER stress response or genetic deletion in myeloid cells of the ER stress sensor kinase IRE1α reduced IL-6 production[92]. In humans, within 24 h of vaccination with AS03-adjuvanted H5N1 avian influenza vaccine, there were increased serum levels of IL-6 and IP10 (also known as CXCL10) as well as transcriptional signatures of interferon signalling and antigen processing and presentation in DCs, monocytes and neutrophils[90]. Interestingly, this early upregulation of interferon signalling was found to predict antibody titres at 56 days[90]. Given the similarity of AS03 and MF59 both being squalene-based adjuvants, it is likely that they engage common pathways of innate immune activation. As described, both adjuvants stimulate innate cytokines and chemokines, but the extent to which they both depend on the IRE1α and RIPK3-dependent pathways[76] remains to be determined.

AS01. AS01 is included in a licensed vaccine against varicella zoster (Shingrix), approved for use in older adults (50 years and older), with high efficacy (97.2%)[93]. The adjuvant AS01, which is a unique combination of two different immunostimulatory components — the TLR4 ligand used in AS04 (MPL) and an isolated and purified saponin fraction (QS-21) — is also used in a licensed malaria vaccine, which is currently being implemented in Africa in a limited campaign[94]. QS-21 is a triterpene glycoside purified from the bark extracts of the tree *Quillaja saponaria* Molina. Preclinical studies using the QS-21 adjuvant showed enhanced antibody as well as cell-mediated immune responses[95–97]. However, although QS-21 alone was potent, there were significant concerns about its tolerability profile when used as a single-component adjuvant in human vaccines. In AS01, MPL and QS-21 are formulated together in liposomes in the presence of cholesterol, which is used to bind QS-21 into the liposome and to quench its reactogenicity. The MPL activates the innate immune system through TLR4, largely through TRIF-dependent signalling. In addition, studies in mice show that QS-21 activates caspase 1 in subcapsular sinus macrophages (SSMs) in the draining lymph node[98] (TABLE 1). Although caspase 1 activation by QS-21 is NLRP3-dependent in vitro, NLRP3 does not seem to have a role in adjuvanticity in vivo[98,99]. When formulated in liposomes, QS-21 enters the cells through cholesterol-dependent endocytosis and induces lysosomal destabilization, followed by tyrosine-protein kinase SYK activation[100]. Collectively, the combination of the specific activation of different innate pathways by the two individual molecules is critical for the full adjuvant effect of AS01, as depletion of TLR4, caspase 1 or SSMs individually impairs the adjuvant effect in mouse models. Hence, a striking feature of AS01 is the synergy that occurs to induce novel pathways that are not triggered by either component alone[98,101]. The key emergent pathways seem to be IFNγ-related, as blocking IFNγ in vivo abrogates the synergistic effect of MPL and QS-21. The typical immune response to AS01 adjuvant is characterized by an increase in polyfunctional $CD4^+$ T cells (such as T cells that express IL-2, IFNγ and TNF) specific to the co-administered antigen, along with enhanced functional antibodies[101]. In summary, in AS01, two well-established adjuvant molecules were combined in a novel delivery system (liposome) that resulted in a synergistic engagement of innate immunity, such that the adaptive immune response induced was greater than the individual sum of the independent components.

Immunosenescence
Ageing of the immune system.

CpG-B class oligonucleotide
An oligonucleotide containing CpG dinucleotides that directly induce B cell proliferation.

Nevertheless, although much has been learned in preclinical models, there is still much to be learned about how AS01 functions in humans, particularly in older subjects, in which it seems to have a remarkable ability, amongst licensed adjuvants, to overcome 'immunosenescence'.

Taken together, the Adjuvant Systems AS0 exert their effects by multiple mechanisms, depending on which components were used in the formulation. Using a combination of adjuvants that were already in various phases of preclinical or clinical testing, rational combinations were created to maximize potency, while ensuring that an acceptable tolerability and safety profile were also in place to enable successful product development. Nevertheless, there was a long, arduous and challenging path to licensure, which we hope to abbreviate in the future, on the basis of the lessons learned. We believe that key lessons will continue to emerge from human studies using systems biology approaches, particularly those that are focused on challenging the assumptions on mechanisms of action, which have emerged from small animal studies. We also believe that key observations will come from mechanism-based studies in large animal models, which allow more comprehensive analysis, but observations from selective small and large animal studies will still need to be substantiated in humans[102].

Cytosine phosphoguanosine 1018

There are three classes of CpG oligonucleotide ligands for TLR9, which can be distinguished by different nucleotide sequence motifs and their capacity to stimulate IFNα in plasmacytoid DCs[103] (TABLE 1). The TLR9 agonist CpG 1018, a 22-mer unmethylated CpG-B class oligonucleotide, is a potent T_H1 cell adjuvant and stimulates strong B cell and NK cell activation. CpG 1018 is currently being evaluated in clinical trials as a potential vaccine adjuvant for COVID-19 vaccines[86] (TABLE 2). Furthermore, this molecule is a component of Heplisav-B, an improved HBV vaccine licensed for use in adults (age >18 years). Although there are other HBV vaccines that were already licensed and used widely, they are typically administered in a three-dose regimen, whereas the key advantage of Heplisav-B is that it offers a simplified two-dose regimen[104]. In preclinical studies in mice, CpG nucleotides induced significantly higher antibody responses to various antigens, including the surface antigen of HBV, HBsAg, compared with unadjuvanted or alum-adjuvanted formulations[105]. However, the expression pattern of TLR9 in mice is different from that in humans and macaques. In mice, many cell types, including cells of monocyte/macrophage lineage, express TLR9, whereas in humans and macaques TLR9 expression is strictly limited to plasmacytoid DCs and B cells, which highlights the importance of understanding the molecular mechanisms of action of novel adjuvants in humans[106]. Finally, the different immunostimulatory capacities of the distinct classes of CpG molecules mentioned above seem to depend on whether they occur in monomeric or multimeric forms. Thus, monomeric CpG-B oligonucleotides localize to lysosome-associated membrane protein 1 (LAMP1)-positive endosomes and promote plasmacytoid DC maturation but little or no IFNα production; however, when complexed into microparticles, CpG-B localizes to transferrin receptor 1 (TFR1)-positive endosomes and leads to IFNα production, via a TLR9-dependent mechanism[103]. Furthermore, monomeric CpG-B can be readily taken up by B cells and stimulate their activation[107]. By contrast, CpG-A, which spontaneously forms nanoparticle-like complexes because of its palindromic structure, also localizes to $TFR1^+$ endosomes in plasmacytoid DCs and stimulates IFNα production but cannot be taken up by B cells, which do not internalize larger DNA complexes such as CpG-A[103,107]. The innate mechanisms and cell types that stimulate adaptive immunity in response to immunization with CpG plus antigen also seem to depend on the form of CpG. Thus, immunization of mice with soluble CpG-B plus antigen induces T_H1 $CD4^+$ T cells and antibody responses through a mechanism dependent on TLR9-mediated MyD88 activation in DCs[108]. Conversely, immunization with an aggregated form of CpG-B plus antigen seems not to depend critically on MyD88 signalling in DCs, arguing for a role of other innate cells in sensing aggregated forms of CpG and eliciting adaptive immunity[108].

Emerging concepts in adjuvant biology

Role of adjuvant in $CD8^+$ T cell response

Although it is well established that adjuvants can enhance antibody responses to vaccination in humans, to date no adjuvant has been shown to induce the magnitude of antigen-specific $CD8^+$ T cell responses stimulated by live viral vaccines such as YF-17D (REF.[109]). It has been argued that the initial dose and persistence of antigen are the dominant determinants of $CD8^+$ T cell responses[109]. However, analysis of the immune responses induced by live viral vaccines in humans has revealed that both the antigen load and the adjuvant signals play critical parts in inducing adaptive immune responses.

In mice, several immunogens in combination with a range of adjuvants induce potent $CD8^+$ T cell responses. By contrast, in humans, only live viral vaccines such as yellow fever and smallpox induce a very high magnitude of antigen-specific effector $CD8^+$ T cell responses and memory $CD8^+$ T cell responses[25,109,110]. As these are replicating vaccines, these studies have highlighted that the initial viral load (antigen) determines the magnitude of the $CD8^+$ T cell response in humans[109]. Experiments in mice with YF-17D have shown that the activation of DCs, via multiple TLRs and the resulting MyD88 signalling, drives the induction of $CD8^+$ T cells[24]. In addition, YF-17D signals through the RNA sensors RIG-I and MDA5 (REF.[25]). Thus, an important question to address is whether there are adjuvants that can mimic the potency of live vaccines in inducing $CD8^+$ T cell responses in humans. Accumulated experience shows that subunit vaccines, even when delivered with potent adjuvants, do not induce $CD8^+$ T cell responses in humans, at least as measured in the blood. However, TLR ligands or combinations of TLR ligands delivered with antigen can induce antigen-specific $CD8^+$ T cell responses in mice[111–113]. Antigen-specific $CD8^+$ T cells can also be induced in mice by targeting C-type lectin receptors (CLRs) such as DEC205 (REFS[114,115]) to DCs, to promote cross-presentation of antigen to $CD8^+$ T cells. These

Box 2 | **Frontiers in adjuvant design and development**

Recent advances in immunology have highlighted novel cellular and molecular targets for adjuvant development.

- **Adjuvants that induce CD8$^+$ T cells and tissue resident memory T cells (T_{RM} cells):** developing novel adjuvants that induce a high magnitude of antigen-specific CD8$^+$ T cells and T_{RM} cells in humans, similar to that induced by live viral vectors such as the yellow fever vaccine[109].
- **Adjuvants that target non-Toll-like receptor (TLR) pattern recognition receptors (PRRs):** developing novel adjuvants that target PRRs other than TLRs, including nucleic acid-sensing receptors such as retinoic acid-inducible gene I (RIG-I)[137], stimulator of interferon genes (STING) protein[139], C-type lectin receptors (CLRs)[138] and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs)[237].
- **Metabolic adjuvants:** nutrient sensors such as mTOR and GCN2 have key roles in modulating dendritic cell (DC) function and adaptive immunity[6,155–160]. Small molecules that target such metabolic regulators in DCs may represent novel adjuvants.
- **Cell death adjuvants:** it is now known that uptake of dead cells by DCs can result in cross-presentation of cellular corpse-derived antigens to CD8$^+$ T cells[148]. In particular, recent work has highlighted that receptor-interacting serine/threonine protein kinase 3 (RIPK3)-mediated necroptosis can synergize with NF-κB-dependent inflammation to promote CD8$^+$ T cell responses. Furthermore, recent work shows that the adjuvant effects of MF59 in promoting CD8$^+$ T cell response are largely mediated by the key mediator of necroptosis RIPK3 but via a necroptosis-independent pathway[76].
- **Epigenetic adjuvants:** recent work suggests that innate cells including monocytes and macrophages undergo epigenetic modifications and acquire memory-like characteristics following stimulation with pathogen-associated molecular patterns (PAMPs)[161–166]. Thus, small molecules that target such cells may represent epigenetic adjuvants that stimulate heightened activation of particular facets of the innate response (such as an antiviral state, including interferon stimulated genes (ISGs)). Such an epigenetically reprogrammed antiviral state will provide resistance to a broad array of viruses, for some period of time, perhaps a few weeks or so.

strategies have been successful in mice, but it continues to be challenging to translate these outcomes to NHPs or humans[29,30,34,116–118], despite the observation that the generation of CD8$^+$ T cell responses can be enhanced by the physical linking of TLR ligands to soluble antigens[117,118]. Furthermore, the currently available adjuvants induce much lower magnitude CD8$^+$ T cell responses relative to viral vaccines such as YF-17D or smallpox[109,110], although CD4$^+$ T cell responses are more readily induced by subunit vaccines[119,120]. DNA-based vaccines and recombinant viral vectors have been tested extensively in the form of prime-boost regimens[121–125], but still do not induce the magnitude of CD8$^+$ T cell responses observed with live viral vectors. Therefore, the discovery of adjuvants that induce potent cell-mediated immune responses to subunit vaccines remains a challenge for the future. In this context, it should be noted that recent results from the COVID-19 vaccine trials with mRNA vaccines have demonstrated a high magnitude of CD8$^+$ T cell responses in humans[126]. Thus, in the phase I/II trial of the mRNA vaccine from BioNTech/Pfizer, the mean number of IFNγ-producing CD4$^+$ T cells and CD8$^+$ T cells specific for the SARS-CoV-2 receptor binding domain (RBD) spike protein was approximately 700 and 400 per million peripheral blood mononuclear cells (PBMCs), with high responders having as many as 4,000 spots per million PBMCs. Consistent with this, by intracellular cytokine staining, the frequency of RBD-specific CD8$^+$ T cells was remarkably high, with several vaccinees having frequencies as high as 4% RBD-specific CD8$^+$ T cells of the total T cell pool[126]. The mechanistic basis of this high magnitude of CD8$^+$ T cell response induced by the mRNA vaccine is unclear, but could be due to activation of the innate immune system by nucleic acid sensing receptors, or other mechanisms, as well as antigen persistence for some period, perhaps a few days or a week or so.

Finally, there is now heightened interest in tissue resident memory T cells (T_{RM} cells), which are generally believed to reside permanently within tissues and provide a frontline defence against pathogens[127,128]. T_{RM} cells are positioned to rapidly respond upon reinfection at barrier sites. Upon pathogen entry to a mucosal site, T_{RM} cells that recognize the pathogen are rapidly activated and stimulate neighbouring cells in the local microenvironment to activate and recruit immune cells and establish an antiviral state. Our recent studies in NHPs suggest that vaccination-induced T_{RM} cells in mucosal tissues, upon reactivation, induce an antiviral gene expression programme in resident myeloid cells and CD4$^+$ T cells. Of note, the T_{RM} cell innate crosstalk significantly reduced the neutralizing antibody titres required for protection against mucosal simian-human immunodeficiency virus (SHIV) challenge[129]. Therefore, a major challenge for adjuvant research is developing adjuvants that can induce a high magnitude of T_{RM} cells (BOX 2).

Collectively, these observations suggest that induction of effective CD8$^+$ T cell responses in humans requires the optimal adjuvant signalling, in conjunction with the sustained presence of antigen, at least for a few days. Ideally, the kinetics of antigen presence and its distribution could be designed to be similar to that observed following administration of live viral vaccines. For example, the lessons from analysis of the immune responses to the yellow fever vaccine YF-17D suggest that activation of diverse innate immune receptors (TLRs, RIG-I, or MDA5) is necessary for induction of CD8$^+$ T cell responses. In addition, sustained antigen presence for a week or so, as might be expected to occur during an acute viral infection, could also promote enhanced CD8$^+$ T cell responses. Hence, the failure of adjuvants to induce CD8$^+$ T cell responses to inactivated vaccines in humans suggests that the antigen load, persistence and distribution needs to be better controlled. It is known that live replicating viral vaccines, such as YF-17D and smallpox, induce an acute viral infection in humans[109], which conceivably facilitates priming of CD8$^+$ T cells at multiple sites throughout the body, thereby contributing to a greater magnitude of CD8$^+$ T cell response. Therefore, future research should be aimed at better defining the parameters of antigen and adjuvant persistence and distribution and the optimization of such parameters to determine the potential for effective generation of CD8$^+$ T cell response, including T_{RM} cells, in humans.

It is not just about DCs

DCs have long been considered the primary cellular targets of vaccine adjuvants[5,6,8,23,130,131]. Indeed, there is clear evidence that DCs are essential for the adjuvant activity of TLR ligands[23]. Thus, in mice deficient in DCs, the adjuvant activity of TLR ligands is severely compromised[33,108]. Even for non-TLR-based adjuvants such as alum or MF59, conditional deletion of DCs or subsets of DCs

results in a major impairment of adaptive immune responses[59,76]. These studies have unambiguously placed DCs as one of the key cell types in sensing adjuvants and tuning adaptive immunity. However, the immune system is a complex network of interacting cell types, and although DCs play a central part in innate sensing and orchestrating immune responses, emerging evidence highlights key roles for other cell types in this process. For example, as described previously, the administration of alum and MF59 induces transcriptional changes in muscle cells[71], more so than in DCs. In addition, ATP release from muscle cells induced by MF59 is critical for its adjuvant activity[73]. Furthermore, monocytes and granulocytes are more readily detected at the site of administration in muscle in response to the chemokine signal induced by the adjuvant[70], and respond to in vitro stimulation more effectively[132]. In addition, direct stimulation of B cells seems to be an equally important factor in generating long-lasting plasma cell responses[33]. Thus, chimeric mice in which TLR signalling is selectively absent in B cells are severely impaired in their capacity to produce antibodies in response to immunization with TLR ligands plus antigen[33]. Consistent with this, B cell-intrinsic MyD88 signalling was shown to be essential to stimulate antigen-specific B cell and antibody response to a virus-like particle[133]. Lymph node resident macrophages also have central roles in the adjuvanticity of MF59 and QS-21 (REFS[98,134]). Finally, few studies have examined the effect of adjuvants on epithelial cells, even though these cells frequently represent the first contact of an external stimulus[135]. Therefore, future work should be aimed at a more comprehensive evaluation of the different cell types involved in the innate sensing of adjuvants, and the mechanisms by which they orchestrate the adaptive immune response.

It is not just about TLRs either

Although the major focus of adjuvant discovery during the past decade has been to target the TLR pathway, it is now clear that other PRRs can be targeted to achieve an adjuvant effect. The available receptors for potential exploitation by new-generation adjuvants include NLRs[136], RIG-I-like receptors (RLRs)[137], CLRs[138] and STING ligands[139]. Of note, signalling through most of the PRRs, including TLRs, induces some local tissue and cellular damage, and the DAMPs released by this seem to be a critical component of the adjuvant activity of several adjuvants. For instance, NLRs, the cytosolic sensors of bacterial PAMPs, recognize multiple cellular products, including ATP, uric acid and K^+ efflux, suggesting that this activation is potentially mediated by cellular damage[140,141] (FIG. 1).

Ligands of STING and RIG-I pathways are thought to be potential adjuvants that stimulate robust $CD8^+$ T cell responses in mice. For example, a recent study described the induction of potent $CD8^+$ T cells in mice by 2′,3′-cGAMP, but of particular interest was the significant tumour regression seen with 2′,3′-cGAMP[142]. Stimulation of RIG-I and RLRs offers another potential mechanism to be explored to possibly induce $CD8^+$ T cell responses[143,144]. Chen et al. recently showed that circular RNA activates RIG-I in vivo and can be used as an adjuvant to induce cell-mediated as well as antibody responses[145]. It will be interesting to further evaluate whether circular RNA encoding proteins could induce stronger and more potent $CD8^+$ T cell responses as the expressed antigen is intracellular for efficient presentation by major histocompatibility complex (MHC) class I.

Metabolism, cell death and epigenetics

As mentioned above, it has become increasingly clear over the past decade that the innate immune system can not only sense microbes via PRRs but is also capable of sensing various kinds of tissue damage and stress signals[6,20]. For example, tissue damage can be caused by trauma, autoimmunity or infections and lead to cell death and the extracellular release of a plethora of DAMPs, such as ATP or uric acid, or fragments of DNA or RNA or high mobility group box 1 (HMGB1), which can activate DCs to stimulate adaptive immunity[16,17,146,147]. This has raised the question of whether these pathways of innate activation can be harnessed in designing novel adjuvants. Indeed, there is emerging evidence that adjuvants that are already in use in the clinic may indeed stimulate immune responses via such pathways (FIG. 2a). For example, as discussed above, there is evidence that, in mice, alum causes cell death and subsequent release of host cell DNA that acts as a DAMP in stimulating immune responses[62]. In addition, as mentioned above, injection of MF59-adjuvanted vaccines into mice stimulates the extracellular release of ATP, whose inhibition by local injections of apyrase diminishes the immune response[73].

Cell death adjuvants. Cell death has also emerged as a key regulator of immune responses[19]. Early work by Bevan and colleagues showed that exogenous antigens, which normally would not be expected to gain access to the cytoplasm of DCs, could be translocated to the cytoplasm, where they could be processed or presented on MHC class I to stimulate $CD8^+$ T cell immunity[148]. Subsequent work by many laboratories helped establish this pathway of antigen presentation by DCs and demonstrated that DCs could also acquire antigens from apoptotic cells cross-presenting these to $CD8^+$ T cells[149]. However, research during the past decade has revealed multiple ways in which cells can die, and much effort has focused on understanding mechanisms of necrosis such as necroptosis and pyroptosis[150]. Necroptosis is mediated by RIPK1, which autophosphorylates and then recruits RIPK3, the kinase activity of which mediates necroptosis[151–153]. The coordinated action of the RIPK1-dependent cell death pathway and NF-κB-dependent inflammation synergize to promote enhanced cross-priming of $CD8^+$ T cells[154]. Interestingly, immunization of mice with MF59 plus antigen results in necroptosis of lymph node macrophages and stimulation of antigen-specific $CD8^+$ T cells (present at very low magnitude in the draining lymph nodes but detected at much higher frequencies in the lung and liver)[76]. Interestingly, the RIPK3-mediated stimulation of $CD8^+$ T cells occurred through a mechanism independent of MLKL, which suggests that RIPK3 mediates activation of $CD8^+$ T cells through a necroptosis-independent mechanism[76]

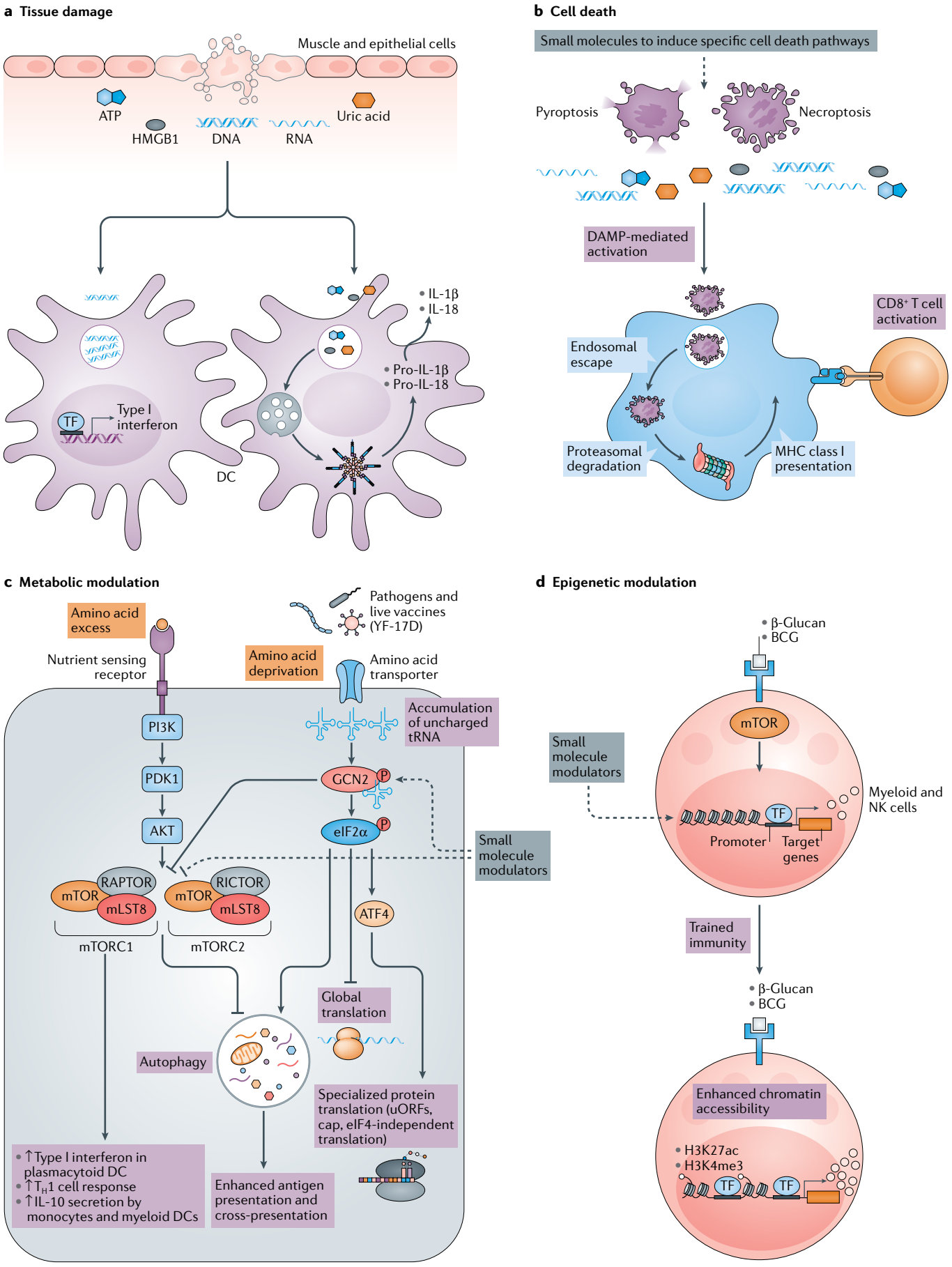

a Tissue damage
Muscle and epithelial cells
ATP
HMGB1
DNA
RNA
Uric acid
• IL-1β
• IL-18
• Pro-IL-1β
• Pro-IL-18
TF
Type I interferon
DC
b Cell death
Small molecules to induce specific cell death pathways
Pyroptosis
Necroptosis
DAMP-mediated activation
CD8+ T cell activation
Endosomal escape
Proteasomal degradation
MHC class I presentation
c Metabolic modulation
Amino acid excess
Nutrient sensing receptor
Pathogens and live vaccines (YF-17D)
Amino acid deprivation
Amino acid transporter
Accumulation of uncharged tRNA
PI3K
PDK1
AKT
GCN2
P
eIF2α
P
Small molecule modulators
RAPTOR
mTOR
mLST8
RICTOR
mTOR
mLST8
mTORC1
mTORC2
ATF4
Global translation
Autophagy
Specialized protein translation (uORFs, cap, eIF4-independent translation)
• ↑Type I interferon in plasmacytoid DC
• ↑T_H1 cell response
• ↑IL-10 secretion by monocytes and myeloid DCs
Enhanced antigen presentation and cross-presentation
d Epigenetic modulation
• β-Glucan
• BCG
mTOR
Small molecule modulators
TF
Promoter
Target genes
Myeloid and NK cells
Trained immunity
• β-Glucan
• BCG
Enhanced chromatin accessibility
• H3K27ac
• H3K4me3
TF
TF

Fig. 2 | **Beyond pattern recognition receptors.** Recent studies have yielded insights into novel pathways that could be targeted for adjuvant activity. **a** | Tissue damage caused by trauma, infection and autoimmunity results in release of a multitude of damage-associated molecular patterns (DAMPs), including nucleic acids, uric acid, ATP and proteins such as high mobility group box 1 (HMGB1), that activate the innate immune system. **b** | Cell death induced by different stimuli also releases DAMPs. Of particular interest is the mechanism of cell death induced by different stimuli. Specialized cell death pathways, such as necroptosis and pyroptosis, can activate innate immune cells. Small molecules that induce specific cell death pathways could be effective adjuvants. **c** | Cellular metabolism is the third concept that is emerging as a central regulatory network of immune responses. Immune cells, such as dendritic cells (DCs), have a distinct metabolic state in different tissues. The insights stemmed from the systems analysis of yellow fever vaccine-induced immune responses in humans, in which the amino acid sensor GCN2 emerged as an early correlate of lasting $CD8^+$ T cell responses. GCN2 activation in DCs by the yellow fever vaccine enhances antigen presentation to T cells via autophagy. Furthermore, the central metabolic regulator mTOR is shown to have various effects on innate immune responses, especially of DCs. **d** | Vaccines such as Bacillus Calmette–Guérin (BCG) and pathogen-associated molecular patterns (PAMPs) such as β-glucan induce epigenetic changes that maintain the innate immune system at an alarming state for extended periods. Small molecules targeting appropriate cell types offer attractive components of novel adjuvants. H3K4me3, histone H3 trimethylated at Lys4; H3K27ac, histone H3 acetylated at Lys27; MHC, major histocompatibility complex; NK cell, natural killer cell; P, phosphorylation; TF, transcription factor; T_H1 cell, T helper 1 cell; uORF, upstream open reading frame.

(FIG. 2b). Taken together, these findings suggest that small molecules that can be targeted to macrophages in lymph nodes to transiently induce RIPK3-mediated pathways, including cell death, might represent adjuvants that stimulate $CD8^+$ T cell responses (FIG. 2b).

Metabolic adjuvants. Another concept that has gained momentum in recent years is the notion that the metabolic state of myeloid cells, such as macrophages and DCs, can programme their innate response and capacity to stimulate T cells[6,155]. Thus, DCs at different maturation stages, or in different tissues, have distinct metabolic states[155,156]. For example, the central metabolic regulator mTOR complex has a major role in mediating TLR-induced type I interferon secretion by plasmacytoid DCs[157], affects IL-12 secretion in myeloid DCs and metabolically reprogrammes lung DCs to skew allergic inflammation from eosinophilic T_H2 to neutrophilic T_H17 cell polarity, via a mechanism that results in enhanced IL-23 and fatty acid oxidation[156].

In addition, the amino acid sensor eIF2α kinase GCN2 can modulate the innate function of myeloid cells, and their capacity to induce T cell responses. For example, YF-17D stimulates GCN2 activation in DCs, leading to enhanced autophagy and antigen presentation to $CD8^+$ and $CD4^+$ T cells[158] (FIG. 2c). By contrast, GCN2 results in suppression of inflammasome activation, via a mechanism involving the sequestration of reactive oxygen species (ROS), an activator of NLRP3, in autophagosomes[159]. Furthermore, GCN2 alters myeloid cell function and promotes immunosuppression against antitumour response through a mechanism involving increased translation of the transcription factor CREB2 (also known as ATF4), which was required for maturation and polarization of macrophages and myeloid-derived suppressor cells[160]. These results suggest that pharmacological modulation of metabolic regulators such as mTOR or GCN2 in DCs may represent adjuvant strategies to simulate immune responses.

Epigenetic adjuvants. Another concept that has emerged recently is that of innate immune memory or 'trained immunity'[161–163]. It has been proposed that myeloid cells, such as monocytes and macrophages[163] or NK cells[164], acquire memory-like characteristics following stimulation with PAMPs. This 'innate memory' depends on sustained epigenetic programming induced upon a primary stimulus that causes cells to be either hyperresponsive or hyporesponsive following a secondary stimulation[161,162]. Vaccines (such as BCG) and PAMPs (such as β-glucan) induce histone H3 trimethylated at Lys4 (H3K4me3) and H3 acetylated at Lys27 (H3K27ac) in monocytes and macrophages (FIG. 2d). These changes were maintained up to several weeks after the elimination of the stimuli, resulting in an enhanced epigenetic status. During a secondary stimulus with the same or a different PAMP, this enhanced epigenetic status induces enhanced gene expression[163,165,166]. An attractive possibility is to target small molecules to the appropriate cell types as pharmacological modulators to reprogramme the epigenetic landscape of innate immune memory[167]. In particular, one could envision small molecules that target monocytes or other myeloid cells to undergo epigenetic reprogramming that stimulates heightened activation of particular facets of the innate response, such as an antiviral state that provides heightened resistance to virus, for some period of time, perhaps a few weeks or so. Such an epigenetic adjuvant could be used to imprint an enhanced antiviral state that confers resistance against a broad array of viruses in humans. The use of such epigenetic adjuvants may be especially advantageous during a pandemic, such as the COVID-19 pandemic, in conferring enhanced antiviral resistance, for a limited period of perhaps a few weeks, in a susceptible population.

Continuing education by adjuvants

One of the dominant paradigms in immunology is that the rapidly responding innate immune system senses microbial stimuli and then programmes the ensuing adaptive immune response. However, emerging data also point to a potential continuing role for adjuvant signals in later stages of the immune response, by directly targeting cells of the adaptive immune system such as B cells. Immunization with a vaccine results in uptake of antigens by DCs, and their activation by the adjuvants contained in the vaccine, via TLRs or other PRRs[6,23] (FIG. 3). Activated DCs present antigens to naïve antigen-specific $CD4^+$ T helper cells, which are found in the T cell-rich areas of the lymphoid organs. The magnitude and type of the T helper cell response induced depends greatly on the nature of the adjuvant and the PRRs triggered. Activated T helper cells migrate to the interface between the B cell follicle and the T cell area, where they stimulate the clonal expansion of antigen-activated B cells. These activated antigen-specific B cells migrate to the medullary cords in the lymph nodes and differentiate into short-lived plasma cells[168–170]. Other activated B cells migrate into B cell follicles to form GCs. In addition, some T helper cells upregulate BCL-6, which induces expression of CXCR5 and CCR7, enabling migration to the T cell–B cell border, where they interact with antigen-specific B

cells[168,170]. These T_{FH} cells provide IL-21 and CD40L signals, which are essential for B cell proliferation and differentiation of GC cells or plasma cells. Antigen-specific B cells compete with each other for T_{FH} cell help for cytokines and co-stimulation signals at the T cell–B cell border based on the amount of peptide MHC class II antigens presented by the B cells to the T_{FH} cells. In turn, signals from B cells, including CD40L, IL-6, IL-12 and IL-23, stimulate the development of T_{FH} cells and their migration into the nascent GCs, where they play a central part in orchestrating the development of memory B cells and LLPCs[168,170]. Therefore, T_{FH} cells represent attractive targets for adjuvant design; in particular, for a deeper mechanistic understanding of the subsets of DCs and the PPRs on such subsets that need to be triggered to induce enhanced T_{FH} cell responses. In this context, our recent work in NHPs has demonstrated that the synthetic TLR7/TLR8 ligand known as 3M-052 formulated in nanoparticles or in alum induces a very high magnitude of T_{FH} cell response and GC response, as well as remarkably persistent bone marrow LLPCs that persist at very high levels for 70 weeks, which was the termination of the study[35]. On the basis of these results, 3M-052 formulated in alum is currently under evaluation as a vaccine adjuvant for HIV Env antigens in humans (ClinicalTrials.gov NCT04177355).

Although targeting particular DC subsets via specific PRRs during the early 'innate' phase of the immune response is a well-established mechanism of adjuvant action, emerging evidence suggests that adjuvants can also provide 'continuing education' to the B cell response even during the GC stage of development. Within GCs, activated antigen-specific B cells proliferate rapidly and undergo somatic hypermutation in the genes encoding their immunoglobulin receptors. As a result, new specificities of B cells are created, including those with a heightened capacity to bind the immunizing antigen[169]. GC B cells compete for antigens that are expressed as immune

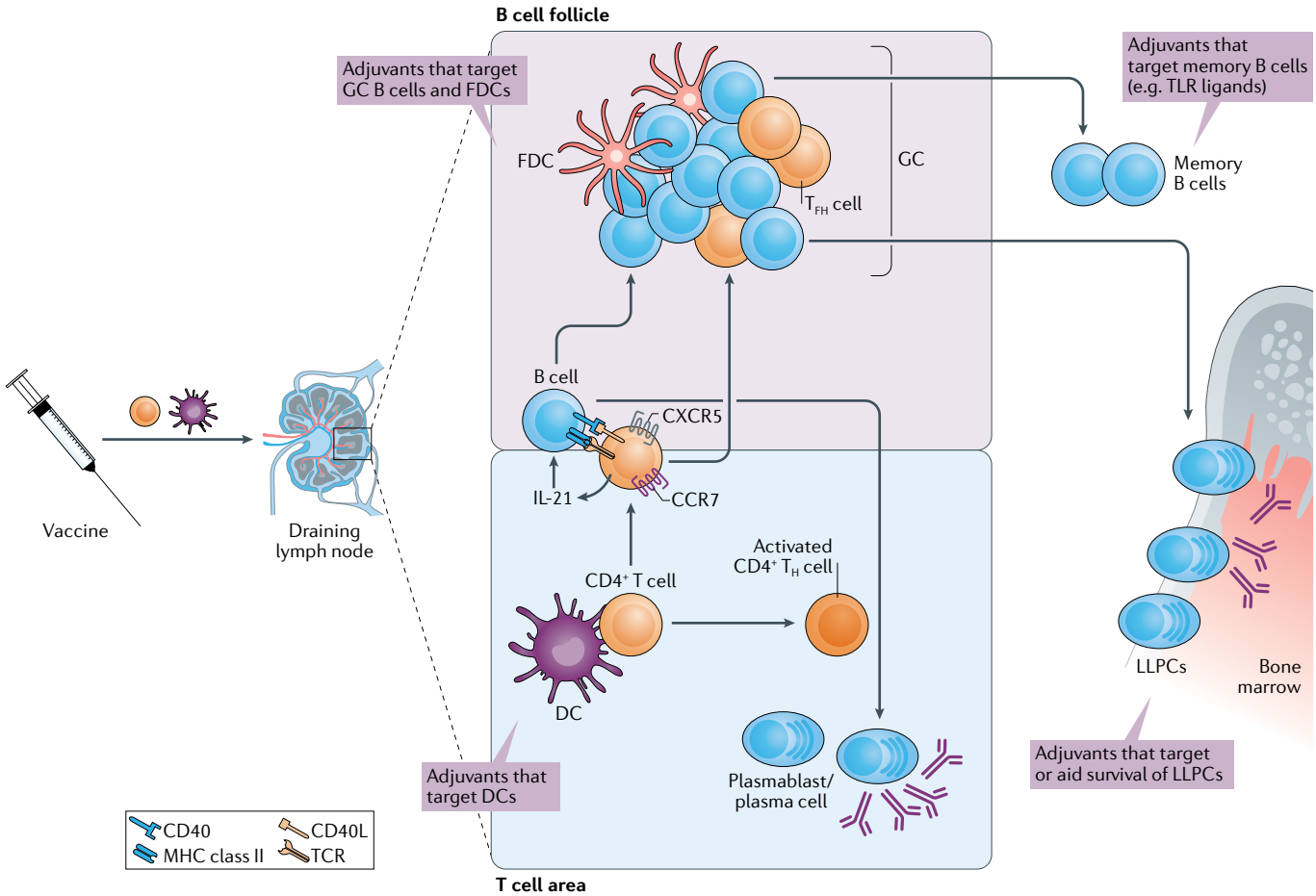


Fig. 3 | **Beyond the innate/adaptive paradigm, continuing education by adjuvants.** The innate immune system sensing adjuvants and programming the ensuing adaptive immune responses is the current model of how adjuvants function. Activated dendritic cells (DCs) present antigens to naïve antigen-specific $CD4^+$ T helper cells (T_H cells) in T cell areas. Some activated T_H cells upregulate CXCR5, which mediates their migration to the interface between the B cell follicle and the T cell area, where they express IL-21 and CD40L that stimulate the clonal expansion of antigen-activated B cells. Although some antigen-specific B cells migrate to the medullary cords and differentiate into short-lived plasma cells, other activated B cells migrate into B cell follicles to form germinal centres (GCs). B cells in GCs can subsequently differentiate into memory B cells that recirculate, or long-lived plasma cells (LLPCs) that migrate to the bone marrow. Many adjuvants are known to work primarily by targeting DCs to induce their activation and antigen presentation, but emerging studies demonstrate that adjuvants such as Toll-like receptor (TLR) ligands can also target B cells. Therefore, potentially novel adjuvant targets could include B cell subsets in GCs, bone marrow LLPCs and other cell types that aid survival of LLPCs, follicular DCs (FDCs) and T follicular helper cells (T_{FH} cells). MHC, major histocompatibility complex; TCR, T cell receptor.

complexes on follicular dendritic cells (FDCs), and those with the highest affinity for antigens are preferentially selected and differentiate into memory B cells or LLPCs that migrate to the bone marrow[169]. This process results in 'affinity maturation' of the antibody response. Activated B cells express TLRs and can respond to TLR stimulation[33,171]. Combination of specific TLRs can drive persistent GC responses[33] and, as mentioned above, B cell-intrinsic MyD88 signalling is essential to stimulate antigen-specific B cell and antibody responses[33,133]. In addition, FDCs express TLRs and TLR-mediated activation of FDCs is crucial for GC differentiation and affinity maturation[172,173]. Hence, this mechanism may be key in inducing high-affinity antibodies and the differentiation of LLPCs, which is essential to inducing sustained antibody responses, a major challenge in vaccinology. These observations suggest that adjuvants might not only target DCs during the early phase of the innate response but could also have a continuing role in impacting GC responses and memory B cell differentiation.

Immunogenicity is context-dependent

The 'rules of immunogenicity', or how the immune system responds to a given adjuvant or vaccine, depend greatly on the context. For example, the requirements for stimulating naïve T or B lymphocytes are generally more stringent than those for stimulating memory lymphocytes, and thus adjuvants may be especially needed to stimulate a naïve response[174–176]. This issue is important because vaccines against many infections such as influenza often need to stimulate recall responses, because most people have been exposed to influenza. However, sometimes a vaccine (such as a vaccine against pandemic influenza) may need to evoke responses against new antigenic epitopes, in the face of clonal competition from memory B cell clones that recognize conserved epitopes. This concept has been termed the 'original antigenic sin'[177] and proposed to be a reason for lower vaccine efficacy observed during certain seasons[178,179]. Adjuvants that selectively activate naïve B cells or APC subsets that stimulate naïve B cells may offer potential to overcome the challenges of the original antigenic sin. Adjuvants such as AS03 can overcome this; a recent study shows that vaccination of humans with the avian influenza H5N1 vaccine adjuvanted with AS03 results in enhanced stimulation of naïve B cells specific to H5N1, relative to the stimulation induced by a non-adjuvanted H5N1 vaccine[180].

Another example of context-dependency is that the immunological states of individuals within a given population, or those in geographically distinct populations, can vary because of differences in genes, the environment or the microbiome[181–183]. Within a given population, individuals at the extremes of age can have different or suboptimal immune responses to vaccination. Indeed, the efficacy of unadjuvanted influenza vaccines in older people is consistently poor[184,185], and adjuvanted vaccines increase the potency and effectiveness of influenza vaccines in this population[186,187]. Furthermore, the CpG 1018-adjuvanted hepatitis B vaccine Heplisav-B is much more effective in older adults than the alum-adjuvanted HBV vaccine, using a very similar antigen[188]. Similarly, the AS01-adjuvanted recombinant varicella zoster vaccine has a remarkably high efficacy of 97.2% in populations aged older than 50 years (REF.[93]). At the other extreme of age, the novel TLR7/TLR8 adjuvant 3M-052 overcomes hyporesponsiveness of the newborn immune system in macaques, to elicit more potent immune responses[189]. However, mechanistic insights into the innate immune system and the way it responds to different stimuli in young and older people are scarce[190,191].

There can also be differences in vaccine-induced immunity in populations living in different geographical regions of the world. For example, it is well known that vaccines against polio, rotavirus, malaria and yellow fever provide less protection for populations in Africa and Asia, relative to those in Europe or the USA[181]. The microbiome is emerging as a central player in the human immune system, and it is suggested that differences in the composition of the microbiome in distinct populations could, in part, account for these differences[181,192–194]. Indeed, a recent study suggests that ablation of the microbiota in humans with broad-spectrum antibiotics results in a marked reduction in H1N1-specific binding antibody responses and neutralization titres to seasonal influenza vaccination in humans[194]. Strikingly, this effect was only observed in subjects who had relatively low baseline titres against influenza, and who had not been vaccinated against influenza or acquired influenza in the 3 years preceding their enrolment into the study[194]. This suggests that the microbiota had provided some endogenous adjuvant signal in the context of influenza vaccination. In addition to this effect on the adaptive immune system, antibiotic administration was observed to stimulate a profound alteration in the metabolome in the serum, with a 1,000-fold reduction in secondary bile acids, which was highly correlated with enhanced inflammation, notably signatures of inflammasome activation[194]. These results reveal a key role for the microbiome in modulating vaccine responses and raise the concept of the microbiota as an endogenous vaccine adjuvant. A practical example of this is the use of probiotics in vaccination and immunotherapy[195]. However, although there are many efforts at evaluating the effect of probiotics on the immune response to vaccination, the results have been variable because of the relatively small study sizes. Therefore, future research should be aimed at devising novel adjuvants, such as small molecules, that harness the microbiome–host axis (for example, metabolites such as secondary bile acids) to regulate immunity to vaccination. Furthermore, it will be important to investigate the mechanisms of the rules of immunogenicity in different populations, such that these can be harnessed in designing adjuvants that are optimally suited to such populations.

The power of adjuvant formulation

Synthetic molecules versus natural products. Although the most successful adjuvants available right now, those included in licensed products, consist mainly of components derived from natural sources, we would not suggest that this necessarily reflects the best path to 'success' in the early twenty-first century. It must be acknowledged that the components used in the most successful adjuvants were generally available in the early 1990s and

before. Hence, they were the best materials to include in product development plans back then, as they were readily available and potent, even if their mechanism of action was often poorly understood. However, now that there is greater appreciation of the critical role of innate immunity on adjuvant action, synthetic chemistry facilitates the development of novel molecules, which can be better designed for the purpose, as pure agonists. Such an approach led to the discovery of small molecule immune potentiators (SMIPs), which are currently in various stages of preclinical and clinical evaluation as adjuvants[196–199]. Although the initial programmes focused on the discovery of TLR agonists, the search for novel agonists of other innate activation systems soon followed[200–202]. The inherent characteristics of these newly discovered adjuvants make them more suitable for product development than the original natural molecules that they will likely come to replace. Ideally, the newly discovered adjuvants are small molecules that can be designed as pure agonists, with mechanistic insights allowing modulation of the level of agonism to control activation signals.

Established formulation versus creating a novel formulation approach. Because such small molecule agonists benefit greatly from particulate presentation, chemical manipulation can render them suitable to be formulated into preferred delivery systems, with flexibility in solubility and compatibility profiles[203–205]. Recent research in animal models has highlighted the value of nanoparticle-based vaccines. Here, the physical properties of nanoparticles, such as their size and the antigen density on their surface, can influence their immunogenicity[204,205]. In addition, glycosylated antigens displayed in a multimeric form on the surface of nanoparticles can engage with innate immune defence proteins such as mannose binding lectin (MBL), which facilitates their rapid shuttling to FDCs in GCs, leading to enhanced antibody responses[206]. Experiments in mice and macaques indicate that formulations can also modulate the release kinetics of antigens, which can affect the magnitude of antibody responses[38,207].

There are several formulation approaches already established that are used for the adjuvants included in licensed products. These include insoluble aluminium salts used as an adsorbent, a low oil content that is easy to inject, oil-in-water emulsions and liposomal delivery systems. Although these systems are very different compositionally, they share some significant similarities. They are all fabricated as nanoparticles, although insoluble alum adjuvants are typically stable aggregates of nanoparticles, which aggregate into particulates of a mean size of several microns. Importantly, alum, emulsions and liposomes can also function as effective 'delivery systems' for both antigens and immune potentiators, including TLR agonists (most notably TLR4, TLR7 and TLR9). 3M-052 has also been formulated using several different approaches, including adsorption to alum and encapsulation in liposomes[208,209].

Finally, a major challenge in the development of these delivery systems is translation to humans. Controlled release of antigen through polymeric particles has always been attractive conceptually and has gained much support as there were already well-established biodegradable polymers available for use in humans. However, many challenges emerged in the early years of this approach, which were not able to be overcome despite much effort; these challenges have been summarized previously[210]. In brief, most recombinant antigens were degraded by the nanoparticle encapsulation process or were released only partially after encapsulation, and entrapment was often inefficient, resulting in high cost due to 'wasted' antigen. Although the in vivo performance in small animals was often good, there was no clear path to product development owing to several inherent liabilities. These include the fact that the particles typically required expensive aseptic manufacturing in very specialized facilities, and that the release and quality control assays were very challenging, as the entrapped antigen needed to be recovered from the particle to be assayed and quantified. Hence, a key lesson is that there are many reasons why a technology may not be viable beyond small-scale studies in research laboratories or may not be suitable for rational vaccine development.

A recent example of the success of translating a concept to humans is the ongoing clinical programme that followed on from the initial SMIP discovery[211]. After the identification of novel TLR7 agonists, although there were preclinical data showing the possibility of using multiple delivery systems, the decision taken was to clinically evaluate the molecule adsorbed to alum. The reasoning was that alum is well established, safe and effective, and is already well known to regulators. Hence, the new molecule is initially presented in a 'familiar' format, so all questions can be focused on the clinical profile of the new compound only, as it can easily be evaluated in conjunction with established safe and effective vaccines[211]. The manufacturing approach was also established for alum, and is used in many products and allows the potential of the optimal product presentation form, as liquid single vials or pre-filled syringes. Moreover, this approach had already been established as a practical way to deliver TLR agonists, owing to the success of AS04 (REF.[78]). Hence, a previously successful path to product development was followed for a vaccine incorporating a novel adjuvant. Importantly, the flexibility inherent in the SMIP approach allowed chemical modification of the molecule to ensure adsorption to alum adjuvants, allowing rapid clinical evaluation, without the need to establish a new manufacturing process. Encouragingly, so far, the TLR7 agonist adsorbed to alum looks potentially safe and well tolerated in humans[212].

Including only what is necessary. Ensuring that each component is necessary and adds clear value, while not introducing significant unjustified liabilities, enables the rational building of improved adjuvants. AS01 is currently the most successful adjuvant included in a licensed product, based on the level of efficacy achieved in the product, with efficacy >97% against varicella zoster[93]. Prior to the development of AS01, the key components, MPL and QS-21, were both already established as adjuvants and had been explored independently for quite some time. However, an insight that was key to the development of

the AS01 adjuvant was that these two molecules together resulted in a synergy of innate activation[213]. The two molecules together induced an innate activation pattern that could not be induced by either one alone, regardless of the dose level. Importantly, to enable this synergy, a liposomal delivery system was developed, which allowed the co-delivery of the two immune potentiators to the same immune cell populations. This co-delivery could not be accomplished with the established alum or emulsion approaches, and hence the development of liposomes was fully justified. Importantly, the liposomal formulation was also effective in reducing the potential of QS-21 to induce significant local and systemic reactogenicity. Hence, AS01 stands as an excellent example of how strong justifications are needed to add additional components but new approaches can be justified based on outcomes. However, as described below, novel system vaccinology approaches could be used to identify novel adjuvant pathways and formulations that could be harnessed to design the next generation of adjuvants.

A new framework

Systems vaccinology

The established approach to the development of vaccines containing novel adjuvants has been described as one of the slowest processes in medicine[1]. For decades, adjuvant development has relied on systematic testing of candidate molecules in mice, advancement of promising candidates into NHP models and eventual testing in humans (FIG. 4a). Yet, out of all the adjuvants that have shown great immunogenicity and efficacy in animal models, only a handful have proven to be safe and effective in humans. A major reason for this lack of translation from mice to humans includes their evolutionarily divergence some 62 million years ago, along with the resulting important immunological differences (such as differences in TLR7 expression in DC subsets) despite the broad similarities of their immune systems (both mice and humans have T and B lymphocytes)[183]. These main differences underscore the need to harness a human model in the testing of adjuvants. Recent advances in systems vaccinology have transformed our ability to probe the immune response to vaccination in humans, with an unprecedented degree of precision[22,25,214–216].

For decades, vaccine manufacturers have relied on a single measure, typically serum antibody titres, to assess immune responses to vaccination. A major limitation of this approach is that it does not capture the complexity of the immune response to vaccination, and therefore may fail to identify correlates and critical mechanisms of protective immunity. Systems vaccinology uses high-throughput technologies such as transcriptomics, metabolomics, high-dimensional cytometry and epigenomics to comprehensively analyse the immune responses to vaccination and to use the data generated through such analyses to delineate correlates and mechanisms of vaccine immunity[22,183]. The first studies to use systems-based approaches to study vaccine responses identified transcriptional signatures induced within 1–7 days of vaccination that correlated with the magnitude of the ensuing antigen-specific $CD8^+$ T cell response and neutralizing antibody response that was measured 60–90 days later[25]. This study revealed that the yellow fever vaccine triggered multiple TLRs, as well as RIG-I and MDA5, and identified innate signatures that predict the immunogenicity of the vaccine. Further analysis using machine learning approaches demonstrated the capacity of these signatures to predict the magnitude of the ensuing antigen-specific $CD8^+$ T cell response and neutralizing antibody response in an independent study of subjects who received the yellow fever vaccine[25]. This study provided a proof of concept that systems biological approaches could be used to predict vaccine immunity. In an independent study, Gaucher et al. identified similar signatures induced by the yellow fever vaccine that correlated with the ensuing adaptive immune response[215]. Following these studies, several groups have used this approach to investigate immune responses to vaccination against several other diseases including influenza[216–221], malaria[222–224], meningococcal and pneumococcal infections[225,226] and varicella zoster virus[227]. Importantly, systems vaccinology approaches have been used to identify common vaccine-induced signatures across multiple influenza seasons and in diverse populations (older people, people with diabetes)[217]. Such studies have identified signatures that correlate with the durability of the antibody response[217].

Systems vaccinology studies have yielded many novel mechanistic insights about vaccine response. For example, expression of *TLR5* was shown to be induced within a few days of vaccination and correlated strongly with the antibody response several weeks later[216]. Subsequent experiments with mice revealed that antibody responses to vaccination with the seasonal influenza vaccine were impaired in mice deficient in *TLR5*. This might be due to flagellin from the intestinal microbiota signalling through TLR5 and providing an adjuvant signal to enhance the antibody response. Thus, vaccination of mice treated with broad-spectrum antibiotics or germ-free mice resulted in impaired antibody responses to influenza vaccination[228]. On the basis of these studies, we performed a study in humans to assess the impact of the microbiota on immune responses to the seasonal influenza vaccine, by administering broad-spectrum antibiotics to healthy humans before and after seasonal influenza vaccination[194]. The results revealed that in subjects with low pre-existing antibody titres, microbiome loss resulted in significant impairment in the H1N1-specific neutralization and binding IgG1 and IgA antibody responses. In addition, there was an enhanced inflammatory response (including signatures of inflammasome activation) and a 1,000-fold reduction in secondary bile acids, which was highly correlated with the inflammatory signature[194]. Multi-omics integrative analysis revealed significant associations between bacterial species and metabolic phenotypes, highlighting a key role for the microbiome in human immunity. These studies reveal the power of systems vaccinology approaches: first, in identifying molecular predictors of the vaccine response in humans; then, in experimentally validating them in mouse models; and, finally, in testing these mechanistic insights in a new human study. This highlights the seamless continuum of human immunology studies and mechanistic studies in mice. Additional mechanistic insights that resulted

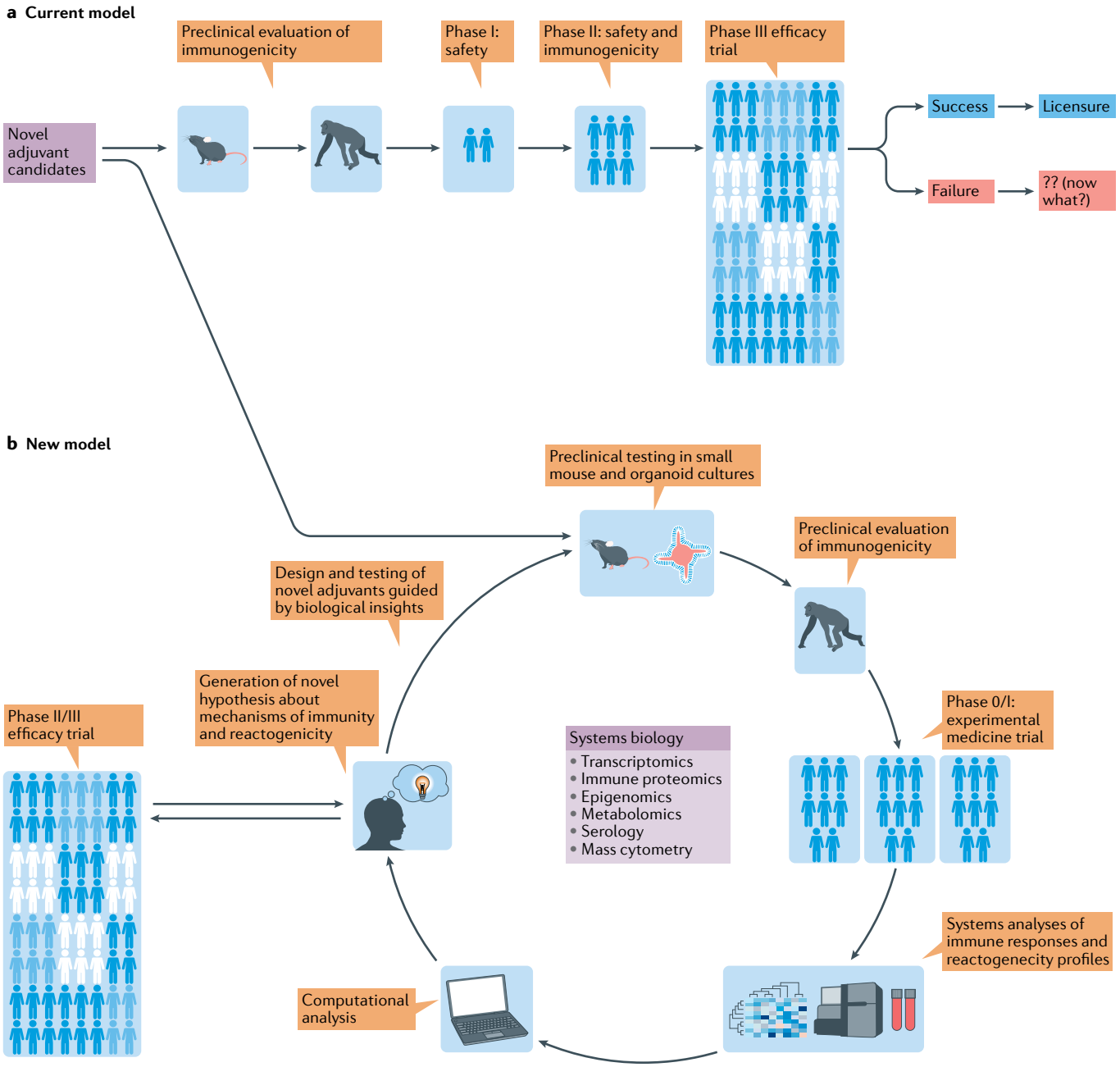


Fig. 4 | **A new framework for development of adjuvants. a** | The current model of developing vaccines containing novel adjuvants represents a linear progression from the systematic testing of novel candidates in mice, to the advancement of promising candidates to testing in non-human primates (NHPs), and the eventual testing in humans in multiple phases of clinical trials. **b** | The new model we propose relies on a process of iterative testing in mice, organoid cultures, NHPs and humans. We advise the early use of small-scale experimental human trials and the use of systems biology approaches to generate multiparametric immunological read-outs, which enable the generation of novel hypothesis and adjuvant concepts that can be retested in preclinical models.

from systems vaccinology studies include demonstration of the role of the amino acid sensing molecule GCN2 in regulating DC function to stimulate T cell responses to the yellow fever vaccine[158,159].

Advancing adjuvant development

A new framework for adjuvant development could be one that places greater emphasis on testing many potential adjuvant concepts in small clinical trials (phase 0/I), early in their developmental pipeline (BOX 3). Thus, novel adjuvants can be rapidly tested in small phase I (phase 0) human trials and systems vaccinology approaches used to obtain mechanistic insights. For example, Sékaly and colleagues studied the innate immune response in humans to synthetic double-stranded RNA (polyinosinic:polycytidylic acid (poly(I:C)) stabilized with poly-L-lysine (poly(ICLC))), an agonist for TLR3 and MDA5 (REF.[229]). Transcriptional analysis of blood samples,

Box 3 | **How systems vaccinology can advance new adjuvants**

- **Discovering the mechanisms of action of established adjuvants to discover new adjuvants.** There is a paucity of detailed understanding about the molecular and cellular mechanisms of action of adjuvants that are currently used in licensed vaccines, including squalene-based adjuvants such as MF59 and AS03 as well saponin-based adjuvants such as AS01b. Systems vaccinology approaches to probe the molecular networks induced by such adjuvants in humans can illuminate potential mechanisms of action, which can then be experimentally validated in animal models[23,182]. Such a detailed mechanistic understanding can then help design novel adjuvants.
- **Early phase 0/I testing in humans to accelerate the adjuvant development process.** For decades, adjuvant testing has progressed in a linear, unidirectional path, starting with preclinical testing in mice, followed by testing the most promising candidates in non-human primates and progressing slowly to clinical testing in humans. However, translation of promising candidate adjuvants into humans has been frustrated by differences in immunogenicity in mice and humans. Furthermore, knowledge of the failure in translation typically occurs very late in the developmental process, typically years after preclinical studies. Therefore, testing candidate adjuvants in humans earlier in the developmental process, in small phase 0/I trials, and using systems-based approaches to define signatures of immunogenicity will help accelerate adjuvant development[229,238]. This will also help benchmark adjuvant and formulation signatures with other successful vaccines[30,218,225].
- **Identifying mechanisms and correlates of reactogenicity.** A major consideration in adjuvant development is safety. Sometimes, adjuvants can induce undesirable reactogenicity, which typically occurs within a few days of vaccination, and can be local (such as erythema, swelling or pain) or systemic (such as fever, myalgia, headache and flu-like symptoms). In addition to such reactogenicity, in rare cases, adverse events such as anaphylactic reactions after administration of an adjuvanted vaccine can also occur[239]. Retrospective, nested, case–control studies can be used to analyse samples from such adverse events compared with controls to identify mechanisms underlying the host response to adverse events.

after subcutaneous administration of poly(ICLC), showed upregulation of genes involved in multiple innate immune pathways in all subjects, interferon and inflammasome signalling, similar to the signature observed with the yellow fever vaccine. Comparative transcriptional analysis showed that several innate immune pathways were similarly induced in volunteers immunized with the highly efficacious yellow fever vaccine[229]. Therefore, a chemically defined PRR agonist such as poly(ICLC) can be a reliable and authentic microbial mimic for inducing innate immune responses in humans. Similar studies can be done in humans vaccinated with novel adjuvants that can be 'benchmarked' against successful vaccines. In addition, systems vaccinology studies can provide insights into the mechanisms of action of adjuvants in humans. This is of value even for adjuvants that are currently used in licensed vaccines, including squalene-based adjuvants, such as AS03 and MF59, and saponin-based adjuvants, such as AS01b, which do not seem to activate the canonical TLR-dependent or other PRR-dependent pathways of innate activation. Regarding saponin-based adjuvants, the results from a recent clinical trial for a COVID-19 vaccine with another saponin-based adjuvant known as Matrix-M1 (BOX 2) demonstrate a high magnitude of neutralizing antibody titres[230], but there is a lack of detailed knowledge about the molecular and cellular mechanisms by which such saponin-based adjuvants mediate their effects[231]. Furthermore, systems vaccinology approaches could be used to define not only mechanisms of action of adjuvants but also the underlying mechanisms by which formulations work, the underlying mechanisms of adverse reactions that occur soon after vaccination and the rational design of optimal formulations for vaccine delivery[30]. The results obtained from such phase 0/I studies will enable the formulation of mechanistic hypothesis about adjuvants that can then be tested in animal models or in vitro human organoid cultures[232] (FIG. 4b).

Here, we propose an improved new model for rational design and iterative testing of novel adjuvants — an interdisciplinary approach based on systems vaccinology to accelerate adjuvant discovery and development in the clinic (FIG. 4). Based on our experiences in managing new adjuvants through to commercial approval in vaccine products, we would like to highlight the value of the early use of human studies. The 'omics' data generated from such studies can catalyse the formulation of novel hypotheses about the mechanisms by which the candidate adjuvants stimulate robust and durable antigen-specific T and B cell responses. Such hypotheses can then be retested in animal models, and the mechanistic insights that ensue can then be used to design novel adjuvant concepts. Such an approach that harnesses the human model combined with the field of systems vaccinology at an early stage of the adjuvant development pipeline has the transformative potential required to revitalize the science of adjuvants.

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Competing interests
D.T.O. is a paid employee of GSK. B.P. serves on the External Immunology Network of GSK and is on the scientific advisory board of Medicago.

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