Activation and programming of adaptive immune responses by vaccine adjuvants

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Introduction

The principle of vaccination has been known for over 2000 years. The ancient Greeks were aware that individuals who recovered from the plague had immunity, or diminished susceptibility, when exposed for a second time to the disease. However, it was not until the end of the 18th century that Edward Jenner provided the first scientific evidence of the vaccination principle [10]. Jenner was a country doctor who inoculated an 8 year-old boy with pustule material from cowpox and showed that this protected him against smallpox. Benjamin Jesty, an English cattle breeder, had observed this phenomenon before, but had not investigated it.

The term vaccination came from the cowpox virus, *vaccinia*, which in turn derived its name from the Latin *vacca*, meaning cow. It was only after Louis Pasteur's successful immunisation experiments, in 1885, that the tremendous potential of prophylactic immunisation was fully realized by the public and scientific community. The inoculates he used were accidentally weakened forms of chicken cholera and intentionally attenuated rabies virus, but the mechanisms responsible for immunity were not understood at that time, and thus vaccination attempts were based on trial and error. The worldwide application of vaccines in the last century has accomplished an almost complete control of many life threatening infectious diseases affecting man, e.g. poliomyelitis, diphtheria, measles, mumps, rubella and pertussis. Similarly, routine vaccinations used in veterinary practice have had a highly beneficial impact on the health and welfare of livestock and companion animals. Prophylactic immunisation has a long history of success and there can be no doubt that it represents the most effective approach to immune modulation. However, despite the tremendous increase in knowledge about immunological pathways, much still remains to be clarified before the outcome of immune interventions can be predicted.

The causal relationship between disease and infection has led microbiologists to focus their efforts on understanding the replication strategies and pathogenesis of microbes. They hoped to identify key pathological events, and to isolate microbial components suitable for incorporation into vaccines. However, injection of inactivated pathogens or isolated split products usually provides minimal or poor protection. The discovery of so-called helper substances, known as adjuvants (Latin *adjuvare* = to help) has dramatically changed this situation, and in many cases they significantly increased the levels of protection afforded by a vaccine. In 1925, Ramon [13] was first to recognize that a variety of substances could increase antigen-specific antibody production when added to diphtheria and tetanus toxoids prior to vaccination. Despite the recognition of many different types of adjuvant, however, there is still little is known about their mode of action. Janeway [7] called adjuvants 'the immunologists' dirty little secret'; 'dirty' because adjuvants contaminated the purified vaccine antigen recognized by T and B-lymphocytes, and 'secret' because their mode of action remains a mystery.

The primary objective developing a vaccine is to generate a high antibody titre in the vaccine – the only measurable immune parameter currently available. Historically, the process of vaccine development started with the search for an effective antigen, preferably an inactivated microbe, which carried protective epitopes. It was subsequently formulated into a vaccine containing an empirically selected, immunostimulatory adjuvant. However, protection against various pathogens requires different types of immune response, and hence in the past the efficacy of a chosen antigen-adjuvant combination was often unpredictable. Nowadays the physico-chemical information of a vaccine formulation is available prior to injection, and antibody production with concomitant reduction in clinical symptoms can be monitored. Nevertheless, little is known about the key immunological events that lead to

vaccination-induced immunity. For primary target species such as man and animals of veterinary importance, progress in this field has been hampered by ethical restrictions and by a paucity of research tools, respectively. Even for the mouse, the immune system of either the adjuvant components or the physico-chemical parameters of the vaccine formulation. This knowledge must be acquired, however, because it will help in the development of rational strategies for vaccine design.

This review describes established and novel immunological events to explain the mode of action used by vaccine delivery systems and adjuvants – the most powerful immunostimulatory molecules known today.

Immune effector mechanisms

The immune system has evolved to protect the host against invading pathogenic microorganisms, such as bacteria, viruses, parasites and fungi. It is now well recognized that a range of primary immune responses is required by the host for the successful elimination of these microbes, each one of which has its own unique life style (Table 1). The efficacy of vaccines is determined largely by their ability to induce anti-microbial antibodies. While elimination of extracellular pathogens requires the production of antibodies by B cells, the control of intracellular pathogens is dependent upon the destruction of infected host cells by cytolytic T cells, by Fas-mediated lysis, or by the microbicidal action of activated macrophages. Each of these immune effector mechanisms depends largely on help from antigen-specific T helper cells, which must first be activated in secondary lymphoid tissues (the local draining lymph nodes and spleen). Vaccination aims to prophylactically induce effector molecules and cells that are capable of eliminating a pathogen as quickly as possible. The most efficient protection against a variety of bacterial, viral and parasitic infections is provided by long-lived, antigen-specific, neutralizing antibodies that have sufficient affinity [1]. Cellular immunity, on the other hand, is critical for the control of certain intracellular pathogens, including HIV, malaria, tuberculosis, etc. Compared to naive lymphocytes, immunisation-induced memory B and T cells have lower reactivation thresholds and are less dependent on the activation of dendritic cells (DCs) and co-stimulatory molecules.

Immunity	Effector response	Effector cells/molecules	Action	T helper (TH) activity	Cytokines
Cell-mediated	DTH (delayed type hyper-sensitivity)	CD4+	macrophage activation	TH1 associated	IFN-γ
	CTL (cytolytic T cell activity)	CD4+	release granzyme perforin cytokines		
		CD8+			
Humoral	lysis	IgG2a	opsonizing (ADCC)		
			complement binding		
	neutralizing	IgG1	neutralizing (receptor blockage)	TH2 associated	IL-4
		IgE	allergy helminths		
		IgA	mucosal	TH3 associated	TGF-β

Table 1. Classification of adaptive effector immune responses.

Development of the immune response

Activation of the immune system requires appropriate presentation and recognition of an antigen. In mammals, immune activation is initiated in the lymph nodes that drain the non-lymphoid tissue in which antigen has been either newly expressed or introduced via the afferent lymph vessels. Naive lymphocytes capable of recognizing the antigen circulate continuously between these lymph nodes and the spleen. Activation of these cells, which carry the cognate receptor for the antigen, requires delivery of the antigen into the lymph node. Most often it is antigen-loaded DCs that serve as transport vehicles. Immature DCs act as sentinels in peripheral tissues where they capture incoming antigen, self-antigens in cell debris, and vaccine components by fluid-phase pinocytosis or receptor-mediated uptake. They are called 'professional' antigen presenting cells (APCs) 'nature's adjuvant' [15], because of their unique ability to activate the scarce number of naive T helper cells present in the lymph node. Naive T cells express the T cell receptor (TCR) that recognizes antigen presented on the surface of the DCs, which at the same time lose their ability to capture antigen as they mature during their voyage through the afferent lymphatic vessels. The DCs process the captured antigen and present antigen-peptide fragments in association with empty or newly formed major histocompatibility complex (MHC) class II molecules, or in association with newly synthesized MHC class I molecules. These MHC peptide complexes accumulate on the surface of the APC and are recognized by the TCRs of peptide-specific, naive T lymphocytes.

Contact between APCs and T cells occurs where and when a transient central cluster of TCRs is surrounded by a ring of integrin family adhesion molecules. The complex of receptors plus adhesion molecules is termed 'immunological synapse' [5]. Activation of the TCR upon interaction with MHC-peptide complexes on APCs is defined as Signal 1. On its own, a limited amount of Signal 1 is insufficient to activate T cells; it leads to tolerance and clonal deletion via apoptosis – a process that is necessary in situations of self-antigen presentation by APCs. Naive T lymphocytes require a second stimulus – Signal 2 – to become fully activated. During maturation DCs increase their expression of membrane-bound, so-called co-stimulatory molecules, such as B7.1 and B7.2, which are recognized by naive T cells via molecules like CD28. DCs also up-regulate the synthesis and surface expression of cytokines, including interleukin(IL)-1, IL-12, tumour necrosis factor(TNF)-α and interferon(IFN)-α. These soluble mediators together with the membrane-bound B7 molecules are collectively termed Signal 2. This signal amplifies the up-regulation of CD40-ligand on the newly activated antigen-specific lymphocytes, which in turn activate the DCs via CD40 (the CD40-ligand receptor) enforcing activation of the T cell in a bi-directional fashion. This APC/T cell cross talk takes place exclusively in the T cell areas of the lymph node and results in T cell proliferation (by clonal expansion), memory cell formation (in case of re-infection) and finally T cell differentiation.

Activated T helper cells lose expression of the chemokine receptor CCR7, found commonly on naive lymphocytes and needed for the homing of the lymphocytes to lymph nodes, and instead express other chemokine receptors responsible for recruitment to peripheral sites of inflammation via chemokine gradients. Within the lymph node, the newly activated T cells can provide help for T cell-dependent antibody synthesis by B cells further down-stream the cascade. Alternatively, they can help cytolytic T cells, which recognize MHC peptide complexes of cells infected by intracellular pathogens. They may also activate macrophages to become microbicidal. The diverse functions of T helper cells are largely determined by different cytokine expression patterns, their chemokine driven migration, and the expression of membrane-bound co-stimulation molecules, for example ICOS (inducible co-stimulatory molecule) on B cells. The type of DC/APC, the duration of TCR stimulation, and the concentration, time span of local presence and type of antigen and cytokines influences the differentiation of activated T cells; most importantly for our topic, differentiation is heavily influenced by the type of adjuvant present during initial antigen uptake by the DCs.

Immunological concepts of adjuvant activity

Immunological adjuvants form essential components in the design of safe and non-replicating vaccines and are responsible for activating and programming the immune response to co-administered antigens. However, the events triggered by these highly successful immunomodulators are poorly understood, and it is still unknown what exactly is essential in an

adjuvant formulation to trigger APC or accessory innate immune cell activation. A unique structural motif has yet to be identified in any of the known adjuvants.

If antigen delivery is the key, what is the key to delivery?

Zinkernagel and his co-workers [16] have argued that the immune response is regulated by two variables: antigen localisation and concentration. Mice that lack secondary lymph nodes, as a result of genetic mutation or surgical ablation, are severely immunocompromised [11]. Occlusion of afferent lymphatic vessels before antigen has reached the lymph nodes also precludes an immune reaction. Adjuvant-induced enhancement of an immune response may therefore be ascribed to improved delivery of vaccine antigen into the draining lymph nodes. This may be achieved by facilitating antigen uptake by APCs, or by increased influx of APCs into the injection site or lymph node. Whichever is the case, the result is the same: an increase in the provision of antigen-loaded APCs for cognate naive T cells.

Another theory related to the concept of antigen transport and presentation in the lymph nodes (the geographical concept) is the so-called depot theory [4]. Antigen dissolved in saline is either quickly taken up by professional APCs or removed by neutrophils and macrophages, and they are subsequently unable to prime naive T cells (Figure 1). The amount of antigen presented and the number of available antigen-specific T and B cells at the moment of immunisation are both dependent on the extent and duration of T and B cell activation. However, after initial immune activation, the dwindling antigen concentration becomes the limiting factor in sustaining T cell stimulation and proliferation. Thus, following the antigen's disappearance the immune response declines, and after a while is barely detectable. Repository adjuvants, such as oil-emulsions, antigen-absorbing aluminium salts, and polymer or lipid particles, may retain antigen at the injection site, from where it is released in minute quantities over a prolonged period of time. This process sustains T cell activation and extends an otherwise short-lived response.

Both the geographical concept and depot theory of antigen delivery reveal the importance of antigen localisation and controlled release of antigen (Signal 1), without the requirement for up-regulation of co-stimulatory (Signal 2) molecules. Adjuvants chosen for their ability to regulate antigen delivery might also include liposomes, cochleates, and nano- or microparticles, consisting of lipid vesicles, polymers, etc (Figure 2).

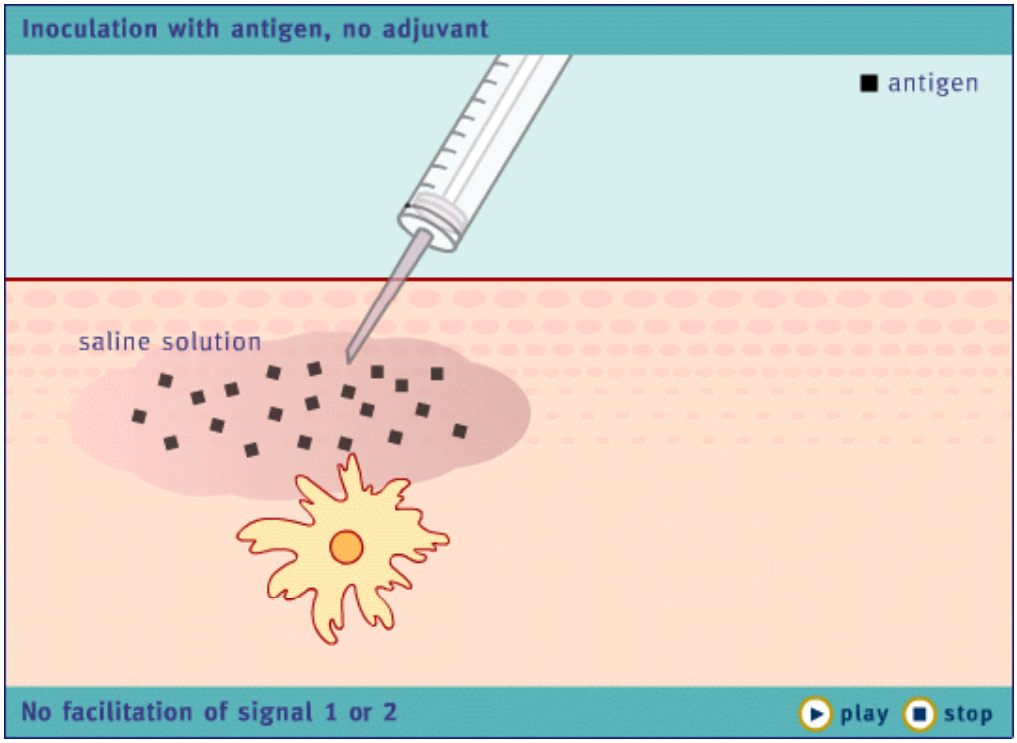

Figure 1. Animation demonstrating the inoculation of antigen in the absence of adjuvant. Dendritic cells (DCs) in the vicinity of the injection site bind to antigen and transport it to the local draining lymph nodes, via the afferent lymphatic vessels, where they present processed antigen to naive T cells. Only limited amounts of antigen are removed from the injection site, if at all, and only for a limited time span. This process is insufficient to facilitate Signal 1 and Signal 2 and hence leads to T cell tolerance not activation.

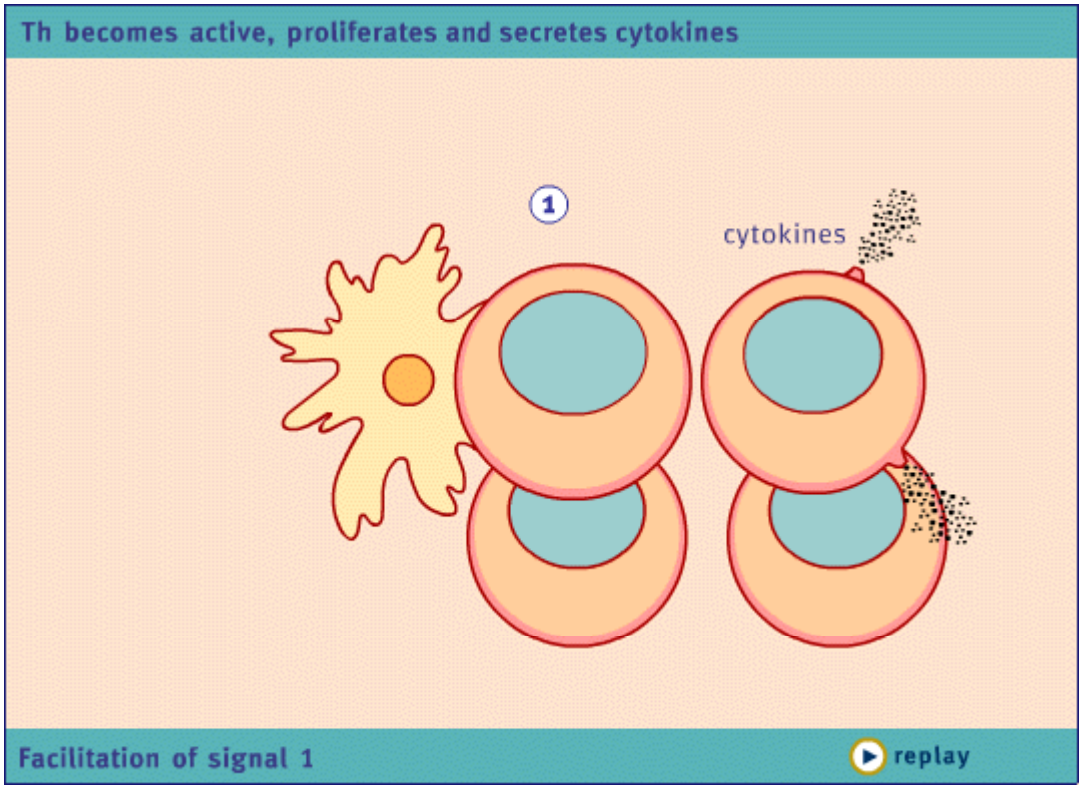

Figure 2. Animation demonstrating inoculation of antigen in the presence of adjuvant, which enhances the influx of Dendritic cells to the injection site and facilitates antigen presentation to naive T cells (Signal 1) in the draining lymph nodes. This process is sufficient to prime reactive T cells and lead to their clonal expansion, and to memory and effector cell formation.

Adjuvants stimulate innate immune cells

The immune system evolved to respond only when necessary, i.e. in situations of stress. According to Janeway [7] this stress stimulus may represent the signature of a pathogenic organism. Matzinger, on the other hand, thought it might be the result of signals from damaged cells [12]. Both authors state that T and B cell activation requires co-stimulatory Signal 2 molecules, and thus it is important to understand exactly how the expression of these molecules is regulated. Janeway reported that potentially noxious substances could be seen only by receptors on innate immune cells [7], which he called pathogen recognition receptors (PRRs). These receptors would recognize pathogen-associated microbial patterns (PAMPS) present on microorganisms (non-self) but not on host (self) cells. He called this recognition event 'Signal 0' because it precedes the induction of the co-stimulatory Signal 2 and Signal 1 molecules. Signal 2 is essential for the activation of naive T helper cells, the master coordinators of subsequent T cell-dependent immune responses. In this way, then, the non-antigen-specific, innate immune system determines the regulation of Signal 2 and thereby dictates when, where, to what, and how the adaptive immune system responds. Microbial components like lipopolysaccharide (LPS) and CpG motifs in bacterial DNA are recognized by Toll-like receptor (TLR)4 and TLR9, respectively, on innate immune cells [6]. Similarly, pertussis toxoid and Mycobacteria are well-known adjuvants. One wonders whether non-microbial adjuvants like oil emulsions, aluminium-based adjuvants or saponins, are recognized by PRRs or other receptors on innate immune cells (Figure 3A).

According to the 'danger model' [3,12] immune activation occurs upon recognition of host-derived danger signals from stressed cells in damaged tissues – not from an invading pathogen. These signals have yet to be defined molecularly, they may include cell debris from necrotic (not apoptotic) cells, heat shock proteins (HSPs), nucleotides, reactive oxygen intermediates, cytokines (e.g. interferon), etc. They activate DCs to up regulate Signal 2 expression and may be described as endogenous adjuvants [3]. Indeed, transient tissue damage at the injection site has been observed for many different adjuvants; it is recognized as an inevitable side effect of vaccination, and some scientists even believe its extent proportional to the ensuing immune response. Ideally, an adjuvant should be a precision drug that activates the desired immune reaction, with minimal effect on the health of the injected tissue (Figure 3B).

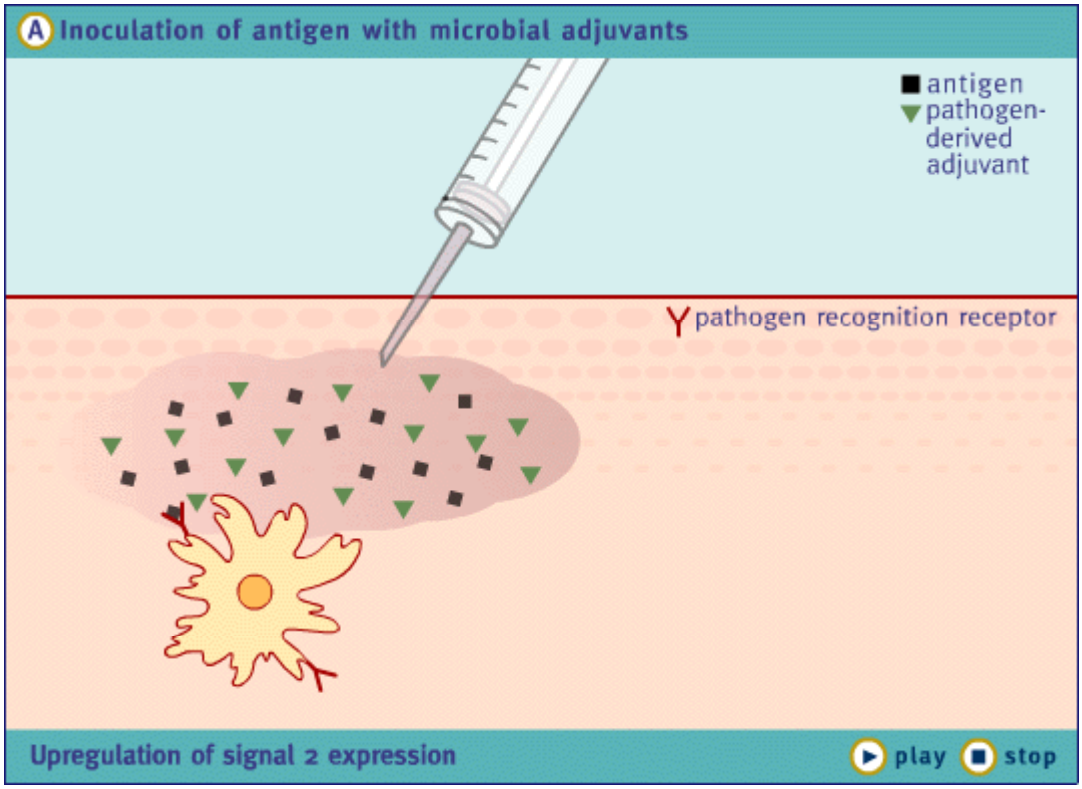

Figure 3. Animation showing inoculation of antigen in the context of stress, provided either by recognition of a microbial structure, Signal 0 (Part A), or by damaged cells (Part B). The process leads to up regulation of Signal 2 molecules, which are considered essential for T cell activation.

Host-derived biological immune stimulants

As outlined above, many adjuvants may act by inducing extracellular communication signals and intracellular signalling cascades, which lead ultimately to Signal 2 molecule expression in the form of critical go/no-go signals. This may explain why artificial stimulation or exogenous provision of cytokines and membrane-bound B7 molecules of the CD40-ligand family enable improved immune responses during immunisation [2]. Because there is competition among receptors for endogenous ligands, the additional supply of exogenous ligands, in the form of recombinant molecules, is likely to prime, enable or polarize immune reactions, similar to or better than traditional adjuvants. When administered at the correct dose and targeted at the relevant site of action, cytokines, chemokines and co-stimulatory molecules may act as precision instruments with minimal side effects.

The future

Adjuvant activity may be categorised according to one of the mechanisms described above [14], although more than one mechanism is likely to be in operation for they are not mutually exclusive. A normal immune response occurs in a step-wise fashion, which means that its progress is limited by many factors, such as the amount of antigen, the duration of the stimulus, the number of APCs present, the presence or not of certain cytokines, etc. An adjuvant may augment immune responses by influencing any stage of this complex cascade of events and this explains why unifying concept is lacking.

Elimination or neutralization of a pathogen require different types of immune reaction, and it is therefore important to know the immunological correlates of protection and the requirements for generating such a response prophylactically. To design a vaccine the essential formulation parameters must be investigated with respect to the desired immune reactions. Research necessary for the development of vaccines is performed either in mice - the most thoroughly studied *in vivo* immune system - or in relevant veterinary species [9]. Veterinary vaccinology has the advantage that a vaccine's efficacy may be tested in the target species. There are many examples of adjuvants that had shown good efficacy in the mouse model but proved disappointing when tested in an animal species or in man. Data from species of veterinary importance, however, may provide information for the design of human vaccines, which the mouse model could not.

Despite the lack of tools to monitor immune responses in most species of farm and companion animals, recent advances in molecular biology have enabled identification and isolation of genes that encode some important immunoregulatory molecules. Thus, microarray screening to identify immune response-related gene expression profiles (see article by Douglas Call in this issue) may result in molecular movies showing the cellular interactions of adjuvant-induced immune responses (W. Degen, personal communication). Apart from their fundamental importance, these studies will have far reaching implications for the hitherto undervalued fields of adjuvant and vaccine design.

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