Innate Immunity and Vaccine Adjuvants: From Concepts to the Development of a Unique Adjuvant System AS04 Used for the Formulation of a Human Papillomavirus (HPV) Vaccine

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Abstract: New vaccine technology has led to vaccines containing highly purified antigens with improved safety profiles, but increased antigen purity often results in weakened immunogenicity. A better understanding of innate and adaptive immunity and their interaction at the molecular level has led to the use of innovative adjuvants combined with careful antigen selection. Adjuvants can be used to amplify the immune response, and the combination of antigens with more than one adjuvant, the Adjuvant System approach, allows the development of vaccines which generate specific and effective immune responses adapted to both the pathogen and the target population. One of those Adjuvant Systems is AS04, a combination of the TLR4 agonist MPL (3-O-desacyl-4'-monophosphoryl lipid A) and aluminum salt. The added value of MPL in AS04-based formulation above Aluminium was evidenced for a prophylactic human papillomavirus (HPV)-16/18 vaccine by higher vaccine-elicited antibody responses, as well as the induction of higher levels of memory B-cells. This review focuses on the role of AS04 for development of *Cervarix*™, a vaccine for the prevention of cervical cancer.

Key Words: Adjuvant system, HPV vaccine, MPL, improved vaccines.

INTRODUCTION

Tremendous progresses have been made during the past decade in understanding the immune response mechanisms, particularly the complex interaction between innate and adaptive immunity and their close interaction at the molecular level. Antigen-presenting cells (APCs) play a crucial role in pathogen recognition and trigger the different immune effectors according to the particular microbial type. APCs are activated by innate receptors on their membrane which recognize pathogen specific molecules called pathogen-associated molecular patterns (PAMPs). These PAMPs represent microbial structures that can be expressed by both replicating or non-replicating attenuated pathogens or whole inactivated micro-organisms and provide the APCs with specific danger signals that guide the subsequent immune response [1]. The family of Toll-like receptors (TLRs) with currently 10 members identified in humans are the best understood pathogen-specific receptors to date [2,3].

Modern vaccines are increasingly based on highly purified subunit antigens and/or antigens produced by recombinant DNA technology. Although these new approaches have led to improved vaccine tolerability and safety profiles, the loss of pathogen-derived structures has considerably reduced their intrinsic immunogenicity *in vivo*. Additional substances, referred to as adjuvants, need to be included in the vaccine formulation to enhance the immune response. Adjuvants act as substitute of danger signals, but they provide this information in a safe manner. It is becoming more and more evident that an appropriate use of adjuvants is required and that their accurate selection may help to tailor better the desired immune response to vaccine antigens [4]. The use of aluminium salts as adjuvants has been known for more than 80 years [5,6], but they might be insufficient to address the new challenges of modern vaccines. In fact the classical vaccine formulation with aluminium has often been proven to be less effective, or to fail completely for vaccines targeted against challenging diseases such as malaria, HIV, or for challenging populations such as elderly and immunosuppressed individuals. A better understanding of the immune system's ability to identify and respond to PAMPs has allowed researchers to identify how novel adjuvants can be used as a vaccine component to enhance a pathogen specific immune response that is strong enough to prevent future infection. In addition, the advances in vaccine technology have led to an improved ability to select and characterize substances with adjuvant features.

Adjuvants can act as delivery systems, enhance the uptake of antigens by APCs, allow for progressive release of antigens, delayed clearance and better exposure to the immune system. They may also increase the ability of antigens to activate signalling pathways controlling the induction of innate and adaptive immunity, predominantly targeting the APCs.

New adjuvants have been developed that are able to act on APCs to activate more specifically the desired arms of the immune system. These new adjuvants can also be combined to leverage the effect of each single component.

In this review we present the challenges, the vision, and the experience of designing a vaccine against human papillomavirus containing a new adjuvant combination, the Adjuvant System AS04.

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HPV, A CHALLENGING PATHOGEN

Infection

The second most common cancer in women worldwide is cervical carcinoma with about 500,000 new cases every year worldwide [7]. Persistent HPV infection is considered to be the necessary cause of this disease [8,9]. Approximately 130 different HPV types have been identified [10] and about 40 of these infect the human genital tract [11]. Among the 15 HPV types classified as oncogenic, HPV types 16 and 18 cause approximately 70% [12,13] of all cervical cancer cases worldwide, and HPV types 45, 31, 33 an additional 10% [14]. After transmission by sexual contact, the virus remains local in the epithelial cells of the mucosa or the skin, and has just little, if any, exposure to the immune system. About 80% of all women will acquire a HPV infection during their lifetime [15]. Most adult women clear spontaneously HPV infection, but 5–10% will not clear the virus and develop persistent infection. A persistent infection with oncogenic HPV may lead to the development of low grade cervical intraepithelial lesions (CIN1), high grade cervical intraepithelial neoplasias (CIN2 and CIN3), also referred to as cancer in situ (CIS) and ultimately to invasive cervical carcinoma (CC) [16].

Natural Immune Response

The natural infectious cycle of HPV is adapted to the differentiation program of keratinocytes it infects and allows the virus to evade detection by the immune system. The time from infection to viral release is approximately 3 weeks, which coincides with the time for basal keratinocytes to undergo complete differentiation, desquamation and natural cell death. HPV by itself is not cytolytic for the infected cells and natural cell death does not present a danger signal to the immune system and is not accompanied by inflammation [11]. During maturation, late proteins L1 and L2 are expressed to form capsids and the viruses are only shed externally [17].

HPV proteins are expressed at low levels and not secreted, hence the virus is not visible to the immune system [18]. As HPV does not cause viraemia or systemic infection, HPV antigens are barely exposed to the systemic immune system and the host is unable to mount a strong antibody response. Innate and cell-mediated immune responses are the first line of defence against an HPV infection. Proinflammatory cytokines are produced locally in response to infection, while specific helper and cytotoxic T lymphocytes, directed against the early E2 and E6 HPV proteins, are often detected in infected individuals [19-21]. Low levels of neutralising antibodies to the major capsid protein L1 may also appear in the serum of infected individuals, while specific IgG and secretory IgA are found locally in the cervical mucosa, at low levels however [22-24].

The protective level of anti-HPV antibodies and the duration of immunity induced by natural incident infection are not known, but only 50–60% of women develop serum antibodies to HPV after natural infection [25].

Although HPV infection can lead to seroconversion, antibody levels after natural infection are unlikely to be sufficient for long-term protection. The local innate immune response is attenuated and cannot control or eradicate the virus, the infection may become persistent, or seropositive individuals can be reinfected with the same type of HPV [26]. Therefore a direct correlation between natural antibody levels and protection can not be assumed [27].

Need for a Vaccine

To provide strong and long-term protection against incident and persistent infections with HPV and associated precancerous lesions, a prophylactic HPV vaccination needs to improve on natural immunity. In addition for such a virus that is widely spread in the population and that is acquired early through first sexual contacts by adolescents and young women, it is important to vaccinate as early as possible, preferably before sexual debut. Moreover, as infection can occur throughout a woman's sexually active life, it is important to protect women from re-infections or new infections throughout their lifetime. Thus the aim of HPV vaccination is to induce a strong immune response that will last as long as possible.

CHALLENGES IN DEVELOPING AN HPV VACCINE

The Antigen Selection

Several efforts towards antigens to be used in an HPV vaccine have been made for both prophylactic and therapeutic use. The selected approach for prophylactic HPV vaccines is based on L1 virus like particles (VLPs).

VLP Approach

The L1 major capsid protein is present at the surface of the virus and expressed in upper layers of the epithelium at the end of infection process. VLPs consisting just of the major capsid protein L1 alone, were shown to be morphologically and antigenically similar to natural papillomavirions [28-30]. VLPs are produced via genetic engineering techniques and the expressed L1 proteins self-assemble to form highly immunogenic empty viral capsids [31].

Species specific L1 VLP vaccination induced short term protective immunity against homologous papillomavirus challenge and subsequent lesion development in three different animal model systems. Systemic vaccination with canine oral papillomavirus L1 VLPs in dogs demonstrated the ability to protect against papillomavirus challenge infection via the mucosal route. Antibody-mediated protection following vaccination was proven in passive transfer experiments in several animal models [32-37].

These preclinical observations demonstrated the potential of a vaccine based on L1 VLP to induce protection and to neutralise the virus before entering the epithelial cells, thus reducing the incidence of infections as well as the burden of diseases.

Another important finding was antibody-mediated protection and the ability of neutralizing antibodies to block the virus before entering the cells.

The Need for a High and Sustained Antibody Level

A prophylactic HPV vaccine needs to provide high levels of antibodies that will be able to prevent the virus from en-

tering the cells at the site of infection and will induce, as all vaccines, a systemic immune response. In the particular case of HPV, the systemic immune response has to act locally at the level of the cervical mucosa where the virus enters and stays. As mentioned before, it seems that a high level of specific antibodies in the cervical mucosa at the time of HPV exposure is key for protection. HPV can be counteracted in vaccinated women by local IgG, the main immunoglobulin present in the female genital tract. These neutralizing antibodies are not produced locally and have to migrate via transudation or exudation from serum to the cervical mucus. It has previously been shown that vaccine-induced IgG transudates from the serum across the endo-cervical and squamo-columnar surfaces and could therefore play a prominent role in local immunity in the cervicovaginal mucus [38]. In addition to this local protective effect, the systemic presence of vaccine-induced neutralizing antibodies is thought to prevent a new infectious event at the same or a distant site through an exudation process due to microtrauma by neutralizing viral particles released by actively infected cells. Moreover, these neutralizing antibodies may bind to virus particles and prevent them from progressing to the transformation zone [39]. The protective level of anti-HPV antibodies after vaccination is not known and antibody levels elicited after natural infection may not be reliably protective. Some seropositive individuals had a lower probability to be infected while others were re-infected with the same type of HPV [40]. However, it is obvious that an HPV vaccine must improve upon nature and provide very high antibody levels at the mucosal site.

Another obvious need for an HPV vaccine is the induction of a sustained neutralising antibody level in order to ensure continuous antibody presence at the site of infection in case of a future encounter with HPV. This requirement is very specific for the HPV virus, since another encounter with the same HPV type may not necessarily result in a boost of the antibody level. The HPV virus stays local and may therefore remain invisible to the pool of memory cells due to its absence of systemic exposure during the early infection stage. It is not known today if the anamnestic response, e.g. response to wild virus exposure after vaccination, takes place for the HPV virus. The immune response elicited by the HPV vaccine must therefore allow for long-term protection through a sustained systemic antibody response active at the site of primary infection.

The Role of B Cell Memory

A sustained specific antibody production reflects the generation of long-lived plasma cells as well as the induction of memory B cells, needed to regenerate the pool of antibody-secreting cells. A positive correlation between the frequency of antigen-specific memory B cells and antigen-specific serum antibody levels has been demonstrated recently for vaccines against tetanus toxoid [41], smallpox [42], and hepatitis B (HBV) [43]. Based on these data it is important that an HPV vaccine has the ability to enhance significantly the level and persistence of serum antibodies and at the same time the generation of cellular immunological memory, in particular the induction of memory B cells.

The need for high and sustained antibody response together with the importance of generating higher frequency of memory B cells implies that in the vaccine formulation special attention must be given not only to the selection of the antigens that provides the specificity of the immune response, but as well to the adjuvant selection that is responsible of the amplification and quality of the immune response. To understand the importance of memory B cells and the crucial role of adjuvants in vaccine formulation we have to introduce first some key concepts of the immune response to pathogens that have been unveiled the recent years.

IMMUNOLOGICAL MECHANISMS

How the Immune System Recognizes a Pathogen?

Pathogens which passed the external physical and chemical barriers are encountered by innate and adaptive immune responses.

The innate immune system provides early and non-specific protection against a large variety of pathogens. Local cell death and/or injury at the site of infection, results in local inflammation, which is the principal marker of innate immune response.

Phagocytic cells like neutrophils, dendritic cells (DCs), and macrophages are recruited and soluble mediators such as cytokines are released. DCs have a special role, as they serve as pivotal point between innate and adaptive immune responses. These cells take up and process microbial antigens, migrate to secondary lymphoid organs, the draining lymph nodes (DLN) and are further refined to fully-functional APCs which interact with T helper lymphocytes that are part of adaptive immune system.

Adaptive immunity generates pathogen-specific effector cell responses and immunological memory, allowing a more rapid and vigorous response in case of repeated encountering of the same pathogen. T helper lymphocytes play two different roles in the adaptive immune system. They promote the differentiation of cytotoxic T lymphocytes, a class of effector T cells which migrate to the infected site to kill pathogen-infected cells. Some of the T helper lymphocytes and cytotoxic T lymphocytes become memory T cells and are part of the T cell memory pool. The other function of T helper lymphocytes is the activation of the humoral arm of the adaptive immune system, resulting in the production of pathogen-specific antibodies.

Activation by T helper lymphocytes induces the differentiation of B lymphocytes into plasma cells. These cells secrete pathogen-specific antibodies targeted to the extracellular pathogen and marking it for attack and destruction by phagocytic cells. The antibodies may also bind to the viral capsid proteins, thereby preventing the virus to enter the host cells. Some of the plasma cells are transformed into long-lived plasma cells to guarantee a continuous secretion of antibodies to maintain a minimum level of long-term protection after the first contact with the pathogen. A fraction of B cells differentiates into memory B lymphocytes which are part of the humoral memory pool, which ensures a rapid and amplified antibody response in case of a repeated encountering of the same pathogen.

The Innate Immune System as a Strategic Target to Improve Pathogen Recognition and Specific Immune Response

Cells of the innate immune system detect pathogens through a limited set of germ-line encoded receptors. These innate immune receptors recognize a series of conserved molecular structures expressed by pathogens, the PAMPs. These pathogen-derived molecules generally represent complex molecules that are very specific for a set of pathogens [44]. TLRs represent a set of immune pattern recognition receptors able to alert the immune system immediately after infection with a pathogen. They play an important role as pivotal components between innate and adaptive immunity and are able to scent out many pathogens ranging from viruses to parasites. The first characterized TLR was shown to be responsible for anti-fungal responses in the adult Drosophila fly [45] and 10 human equivalents involved in pathogen recognition are identified to date [46]. TLRs can be classified into different groups based on their localization and the type of PAMPs they recognize. TLRs 1, 2, 4, 5 and 6 are principally expressed on the cell surface, where they recognize mostly bacterial products, while TLRs 3, 7, 8 and 9 are localized into intracellular compartments and recognize mostly viral products and nucleic acids.

Another family of "pathogen-sensing molecules" able to react to intracellular pathogen-derived structures has been identified recently, the "inflammasome" complex which is expressed in the cytoplasm. More than 20 members of this nucleotide-binding oligomerization domain (NOD) related family are known [47]. These molecules have the ability to sense cellular damage, even in the absence of a microbial trigger. Extracellular nucleotides, alteration in cellular ion content, or lysosomal damage seem to activate components of this intracellular sensing machinery, ultimately leading to the processing and release of inflammatory cytokines [48]. These natural ligands, also referred to as danger associated molecular patterns (DAMP), often represent intracellular constituents such as adenosin-triphosphate (ATP) or uric acid, which are released upon cell lysis caused by infection or trauma [49].

The recognition of the important role of innate immune cells in regulating the adaptive response to pathogens has helped vaccine manufacturers to uncover the mechanisms by which adjuvants exert their immunostimulatory properties. Depending on their mechanism of action, they are able to attract, stimulate and activate innate immune cells, and together with the antigens they induce maturation of these cells into APCs. The careful selection of adjuvants or adjuvant combination can better tailor the adaptive immune response to induce the expected protective immune response. For example, the discovery of TLRs and recognition of the link between innate and adaptive immunity, has laid the foundation for subsequent development of a series of novel adjuvants or immunoenhancers. Immunoenhancing substances exert their adjuvant functions typically through direct stimulation of innate cells such as monocytes, macrophages, and dendritic cells. The concept of Adjuvant System has also been introduced to leverage more than one adjuvant effect. Fig. (**1**).

Adjuvant Systems

One approach to tailor vaccines with effective immune responses adapted to both the pathogen and target population is the combination of antigens with more than one adjuvant, the Adjuvant Systems. The rational of this approach is the fine-tuning of innate immune responses and its subsequent effect on the adaptive responses. This strategy allows a more differentiated activation of APCs, thus influencing the subsequent adaptive pathways and inducing a more robust immune response.

AS04 is one of these new generation adjuvants now licensed for use in humans and consists of MPL adsorbed onto a particulate form of aluminium salt [50]. The idea behind is to leverage the combined effect the two immunoenhancers, MPL and aluminium salt. AS04 is currently used in two licensed vaccines for HBV and HPV, a third vaccine against herpes simplex (HSV) is currently in phase III clinical trials.

Aluminium Salts Adjuvants and the Innate Immune System

Aluminium salts were used as adjuvants for more than 80 years, without knowing the exact mechanism of action, based on the empirical observation that their inclusion in vaccine formulation enhanced the immune response to antigens. Even today, their mechanism of action is not fully understood. Due to its particulate nature, aluminium salt is considered to act as a depot for vaccine antigen components which enhances antigen uptake by APCs [51-53]. Recent studies demonstrated that the antigens absorbed by the aluminium salts and thereby presented as a particulate multivalent form, are more efficiently internalized by APCs [54]. Aluminium salts enhance mainly Th2-driven antibody responses having little or no effect on Th1-type responses, which are instrumental for protection against many pathogens [51]. For that reason they are not the optimal choice for several challenging vaccines currently under development where a Th1-type of immune response is needed [55].

Recently, Aluminium salts have been found to activate the inflammasome, an intracellular multiprotein platform required for the recruitment and activation of caspase-1 and the subsequent processing of proform of cytokines such as IL-1β or IL-18. Although they have been shown to stimulate Nlrp3 a member of the NOD-like family [56-58] and a key component of this signalling platform, the role of the inflammasome in mediating the adjuvant properties of aluminium salts is still a matter of debate [59].

MPL Adjuvant and the Innate Immune System

3-O-desacyl-4′-monophosphoryl lipid A (MPL) is one of the recently developed adjuvants Fig. (**2**).

MPL is a detoxified derivative of the lipopolysaccharide (LPS) isolated from the Gram negative bacterium *Salmonella minnesota* R595 strain [60-62]. LPS has been found to function as a specific agonist of TLR4 [63,64] and MPL interacts as expected via TLR4 [65-67]. Several immunogenicity studies in mice, guinea pigs, monkeys and humans have shown its effectiveness to improve specific antibody and cellular immune responses [68,69]. MPL activates TLR4 pathway, resulting in an enhanced production of cytokines leading to the maturation and

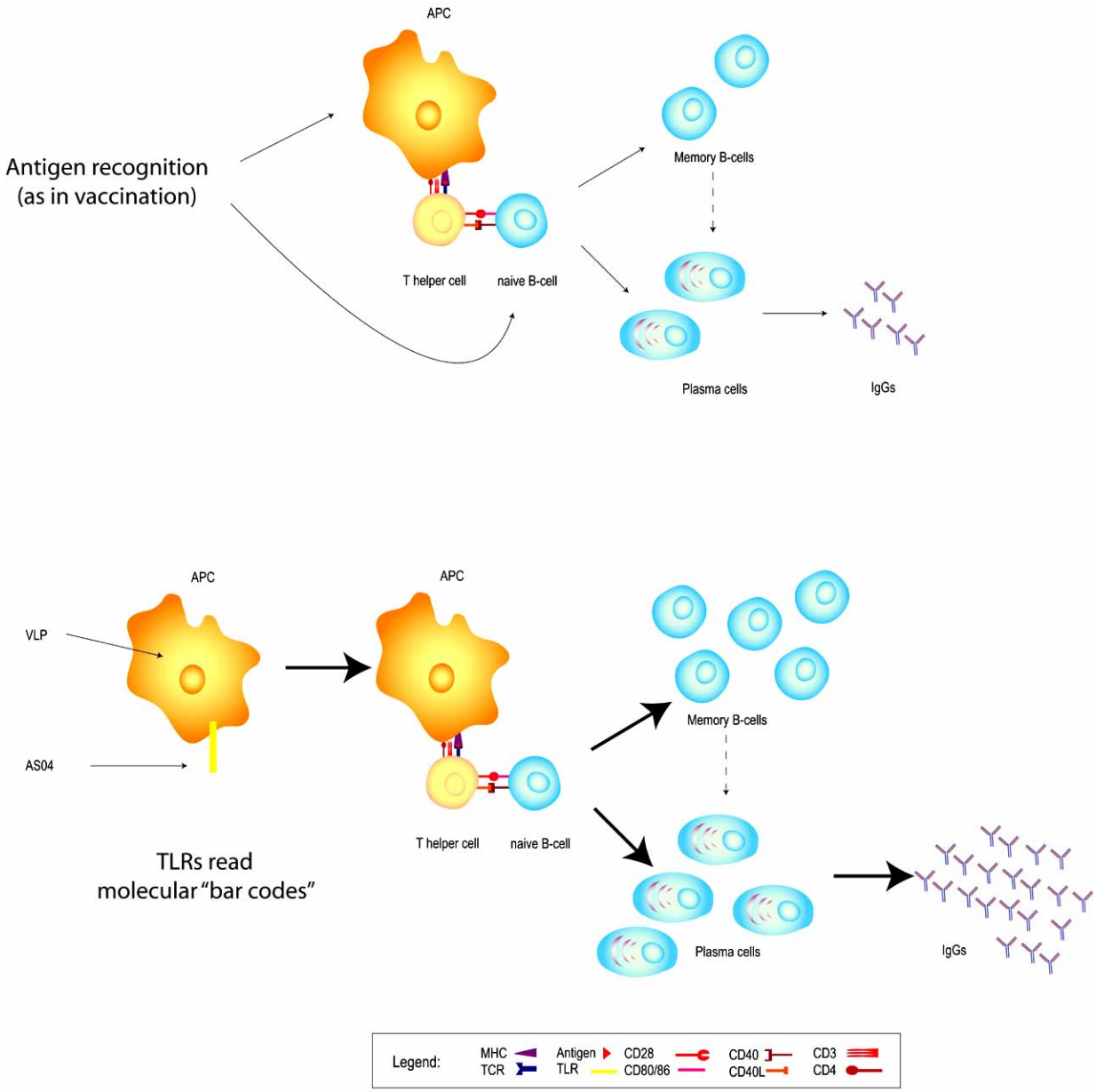


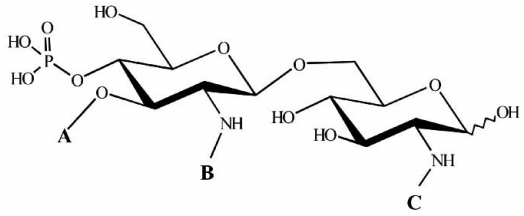
Fig. (1). The antigen presenting cell (APC) is the key element for immune response. DCs present antigen to naive T cells and depending on the nature of co-stimulating signals and secreted cytokines, the transition of naive T cell to maturated T helper cells is initiated. Depending on the nature of the stimulating signals, the activated B cells can mature to plasma cells or memory B cells. Some antigens are able to stimulate directly B cell proliferation through the B cell receptor (BCR). The interaction between antigen and BCR induces maturation to a plasma cell, which produces antigen-specific antibodies.
The use of Adjuvant Systems like AS04 amplifies the specific information provided by VLPs to the immune system. More memory B cells, Plasma cells and antibodies can be generated.

leading to the maturation and migration of APCs to the lymph nodes.

TLR4 stimulation can contribute to the activation of the innate immune response, by activating NF-κB transcriptional activity and the subsequent expression of pro-inflammatory cytokines, such as TNF-α and IL-6 [70]. These cytokines can in turn enhance the adaptive immune response by stimulating the maturation of APCs while repressing the tolerance response through the inhibition of regulatory T cell activity [71]. MPL is generally reported to promote IFN-γ production by antigen-specific CD4+ T cells, therefore skewing the immune response towards a Th1 profile [61].

Mechanism of Action AS04

Recently the mechanism of action of AS04 has been investigated in mice *in vivo* and on human cells *in vitro*. The activation of innate responses is mainly elicited by MPL, whereas aluminium salt is likely to restrict and prolong the MPL effect at the injection site. It has been found that the adjuvant activity of AS04 is only seen when both components of AS04 and HPV antigen are administered spatially and temporally at the same intramuscular site. AS04 induces transiently a local NF-κB activity and cytokine release, which leads to an increased number of APCs and monocytes



Number of Fatty Acids	Position		
	A	B	C
6	$C_{14}(OC_{14})$	$C_{14}(OC_{12})$	$C_{14}(OC_{16})$
5	$C_{14}(OC_{14})$	$C_{14}(OC_{12})$	$C_{14}OH$
	$C_{14}OH$	$C_{14}(OC_{12})$	$C_{14}(OC_{16})$
	ΔC_{14}	$C_{14}(OC_{12})$	$C_{14}(OC_{16})$
4	$C_{14}OH$	$C_{14}(OC_{12})$	$C_{14}OH$
	Δ-C_{14}	$C_{14}(OC_{12})$	$C_{14}OH$
	H	$C_{14}(OC_{12})$	$C_{14}(OC_{16})$
3	H	$C_{14}(OC_{12})$	$C_{14}OH$

$C_{14}(OC_{16})$ = 3-(R)-hexadecanoyloxytetradecanoyl
$C_{14}(OC_{14})$ = 3-(R)-tetradecanoyloxytetradecanoyl
$C_{14}(OC_{12})$ = 3-(R)-dodecanoyloxytetradecanoyl
$C_{14}OH$ = 3-(R)-hydroxytetradecanoyl
Δ-C_{14} = tetradecanoyl

Fig. (2). Major 3-O-desacyl monophosphoryl lipid A congeners in MPL. Congener species all contain the same backbone consisting of a β-1',6-linked disaccharide of 2-deoxy-2-aminoglucose, phosphorylated at the 4' position, but contain variable numbers and types of fatty acyl groups at the 2, 2' and 3' positions. The 1, 3, 4 and 6'positions of the backbone are unsubstituted in all monophosphoryl lipid A species present in MPL. The 2, 2' and 3' positions may be substituted with tetradecanoic, 3-(R)-hydroxytetradecanoic, or 3-(R)-acyloxytetradecanoic acids, depending on the position, so that the total number of fatty acyl groups varies from three to six. (Previously published in [55]).

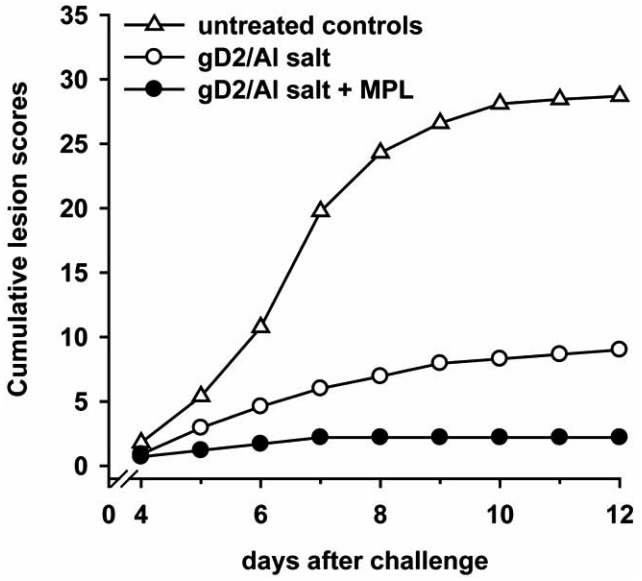


Fig. (3). Assessment of vaccine efficacy against HSV-2 by *in vivo* virus challenge assay. Groups of 12 female Hartley guinea pigs (200-250g) were immunized at days 0 and 28 by the subcutaneous route with HSV type 2 glycoprotein D (gD2, 5 μg) formulated in aluminium salt (0.5 mg equivalents Al^{3+}) or aluminium salt plus 50 μg MPL. Injections were given in a 0.5 ml dose. In order to compare the protective immunity induced by both gD2 formulations, all guinea pigs were challenged intravaginally 29 days after the last immunization with 10^6 pfu of HSV-2 strain MS. After challenge, guinea pigs were monitored daily for clinical signs of acute disease (days 4 to 12 postchallenge). The severity of each lesion observed was scored on a scale of 1-16 with 0 for animals with no lesions, 0.5-l for vaginal lesions, and 2, 4, 8, or 16 for external skin lesions. Cumulative lesion scores (days 4-12) were calculated from the mean daily scores. (Previously published in [55]).

in the DLN close to the injection site. These APCs interact in the DLN with antigen-specific T lymphocytes, trigger the induction of adaptive immune responses, and initiate the generation of T helper cells and production of antibodies by B cells. The experiments on human cells revealed that AS04 stimulates APCs, but not T or B cells directly. Moreover, there was no evidence of induction of IFNα, a cytokine which may be involved in the development of autoimmune diseases. AS04 combines the immunostimulatory properties of MPL and of aluminium hydroxide without altering their intrinsic qualities and no systemic immune responses are initiated [72].

When an antigen is combined with AS04, the adjuvant is able to recruit and activate the innate immune cells at the site of injection. The local effect is transient for few hours or days, however it is able to activate a cascade of events where more DCs loaded with the antigen and other innate immune cells are activated and migrate to the DLN enhancing the APC-antigen-specific T cells interaction and subsequently inducing high and sustained antibody response and high frequency of memory cells.

PROOF OF CONCEPT STUDIES WITH AS04 ADJUVANTATION

Considering the individual properties of aluminium salts and MPL as adjuvants, researchers at GSK Biologicals undertook a series of animal and clinical studies to evaluate the immunostimulatory properties of AS04.

Preclinical Studies with AS04 as Adjuvant

The adjuvant capacity of AS04 has been evaluated during the development of several candidate vaccines, including HBV, HSV and HPV. Studies in mice showed that a recombinant yeast-derived HBV surface antigen (HBsAg) adjuvanted with aluminium salt and MPL was able to induce an overall increase in antibody titres compared to the classical adjuvantation with aluminium alone, both in young and elderly animals.

An HSV vaccine formulated with AS04 was tested for its ability to induce a protective immune response against HSV in preclinical efficacy studies in the guinea pig model Fig. (**3**).

The results indicated that the formulation with AS04 improved the prophylactic efficacy according to the lower-to-non lesion index observed after challenge compared to the aluminium based formulation [55].

The immunogenicity of both AS04 and aluminium adjuvanted HPV vaccine was investigated in mice and monkeys, and AS04 formulation showed significantly higher titres of HPV-specific antibodies than aluminium salt formulation Fig. (**4**).

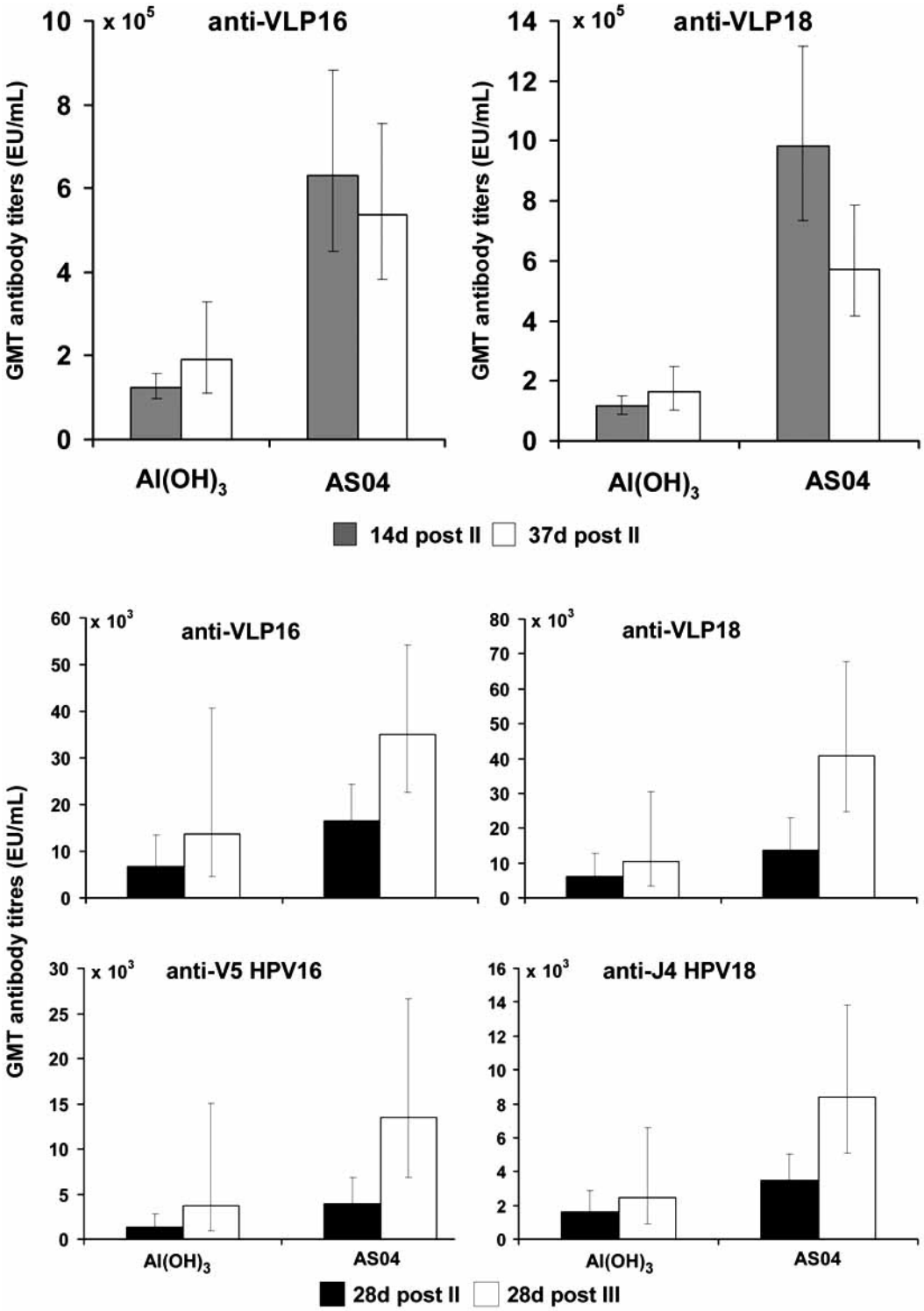


Fig. (4). (a) AS04 Adjuvant System induces a higher antibody response to HPV-16/18 L1 VLP antigens in mice. Mice (n = 12 per group) were vaccinated with the combination of HPV-16 and HPV-18 L1 VLPs adjuvanted with MPL adsorbed to aluminium salt (AS04) or with aluminium salt alone. Following two intra-muscular injections (0 and 21 days), serum samples collected 14- and 37-day post II GMT: Geometric mean antibody titres; CI: Confidence interval were assayed for anti-HPV-16 or HPV-18 L1 VLP antibody response by ELISA. Results are expressed as ELISA Units/ml (GMT±95% CI). (Previously published in [73]).

(b) AS04 Adjuvant System induces a higher antibody response to HPV-16/18 L1 VLP antigens in monkeys. Monkeys (n = 5 per group) were vaccinated with the combination of HPV-16 and HPV-18 L1 VLPs adjuvanted with MPL adsorbed to aluminium salt (AS04) or with aluminium salt alone. Animals received three intra-muscular injections (0, 28 and 84 days), and serum samples at 28-day post II and III were assayed for anti- HPV-16 and HPV-18 L1 VLP antibodies by ELISA. Antibodies specific for V5/J4 neutralising/conformational GMT: Geometric mean antibody titres; CI: Confidence interval epitopes of HPV-16 and HPV-18, respectively, were assayed using an inhibition ELISA. Results are expressed as ELISA Units/ml (GMT±95% CI). (Previously published in [73]).

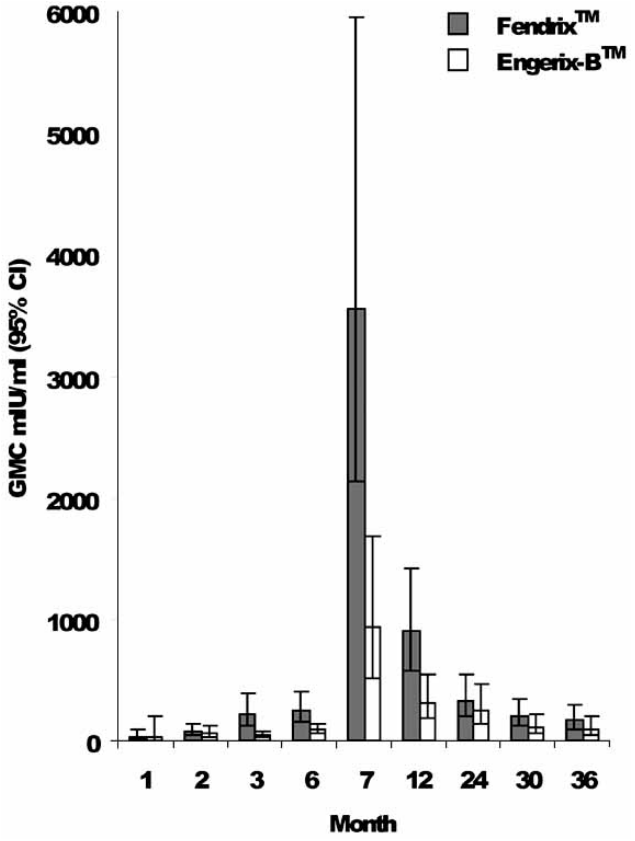


Fig. (5). GMCs and 95% CIs during and after vaccination with Fendrix™ or a double dose of Engerix-B™, both applying the 0-/1-/2-/6-month schedule in preheamodialysis and haemodialysis patients. GMC: Geometric mean concentration; CI: Confidence interval (Adapted from [78]; data from [77]).

In order to evaluate the quality of the humoral response, specific HPV neutralizing antibodies were analyzed in monkeys. The formulation with AS04 induced higher titres of neutralizing antibodies compared to the aluminium salt formulation [73].

Local and systemic safety of formulations containing MPL and an aluminium salt was evaluated in rabbits and rats. There was no evidence of systemic toxicity and symptoms were limited to the injection site but did not extend to the local lymph nodes. Pre- and postnatal preclinical development studies in rats have shown that repeated administration of a dose equivalent to 30 times the human dose per unit body weight did not induce treatment-related effects on mothers or their offsprings. A pharmacology study investigating cardiovascular and respiratory functions revealed no treatment-related effects of AS04-containing vaccines at doses up to 60 times the human dose per unit body weight. Altogether, the preclinical safety data demonstrated that AS04 alone or AS04-formulated vaccines are safe and well tolerated [55].

Previous Experience with AS04-Adjuvanted HBV and HSV Vaccines

Although HBV vaccines adjuvanted with aluminium salts have demonstrated their efficacy against HBV diseases, the immune response to vaccination is impaired in selected patient populations. For example, patients with end-stage kidney disease (ESRD) have a higher risk for developing chronic HBV infection [74,75]. A novel HBV vaccine formulated with AS04 as adjuvant was developed to improve the magnitude and the kinetics of the antibody response in patients on haemodialysis. This new vaccine (*Fendrix*™) showed in several clinical trials its ability to induce higher antibody titres with longer persistence and enhanced cell-mediated immunity responses in pre-haemodialysis and haemodialysis patients compared to a vaccine adjuvanted with aluminium alone (*Engerix-B*™) Fig. (**5**).

The differences in humoral immune responses between the AS04-adjuvanted vaccine and standard vaccine persisted for the follow-up period of the studies, up to 36 months. Furthermore, significantly fewer subjects primed with the AS04-adjuvanted vaccine needed a booster dose as a consequence of anti-HBs antibodies falling below the level of 10 IU/L that is considered protective [76-78].

The pre-clinical data of an AS04 adjuvanted HSV vaccine was confirmed in several clinical studies. Evaluation of this vaccine showed a significant protection of 73% against the disease in HSV-1 and HSV-2 seronegative women. Vaccination elicited both binding and neutralizing antibodies against HSV, as well as a cellular response evidenced by lymphoproliferation and IFN-γ secretion. The protective mechanism of this formulation is most likely based upon the concomitant induction of an effector cell mediated immune response by MPL and a potent virus-neutralizing response [79].

All the preclinical and clinical results for the HBV and HSV vaccines adjuvanted with AS04, indicated that this Adjuvant System is also a good candidate for an HPV vaccine aimed to provide a rapid and long-term protection through induction of high and sustained specific neutralizing antibodies.

AS04-ADJUVANTED HPV VACCINE

The AS04-adjuvanted HPV vaccine revealed a superior, and long lasting antibody response compared to the same antigen adjuvanted with aluminium hydroxide alone Fig. (**6**).

Moreover, AS04 elicited an increased frequency (2.2–5.2-fold) of HPV-16 and HPV-18 L1 VLP-specific memory B cells when compared with aluminium salt formulations [73] Fig. (**7**).

The AS04-adjuvanted prophylactic cervical cancer vaccine, *Cervarix*™, elicits high and sustained antibody responses together with high and sustained efficacy for up to 6.4 years against HPV-16 and HPV-18 related infections and precancerous lesions [80]. Further analysis at 7.3 years demonstrated that levels of total antibodies as measured by ELISA, were at least 13-fold higher (for HPV-16) and 11-fold higher for (HPV-18) when compared to the levels of natural infection [81]. Overall, the observations demonstrate the induction of consistently higher humoral and memory B cell responses for the AS04 formulation compared to the classical aluminium salt formulation.

A study in women aged 15 to 55 years showed 100% seroconversion for both HPV-16 and HPV-18 1 month after

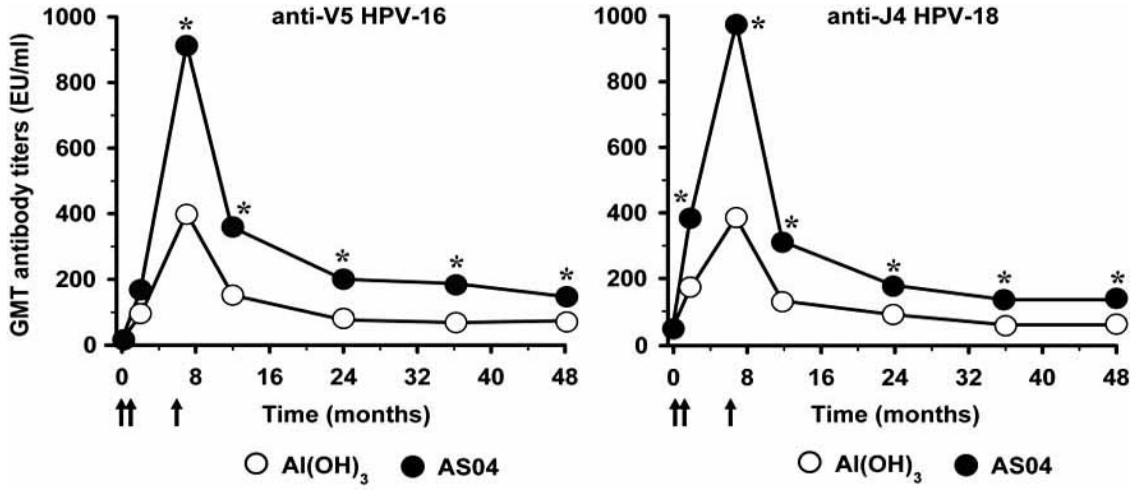


Fig. (6). AS04 Adjuvant System induces a higher and longer lasting antibody response to HPV16/18 L1 VLP antigens in humans. In two separate clinical trials, human subjects were vaccinated with HPV-16 and HPV-18 L1 VLPs adjuvanted with AS04 or with aluminium salt alone. V5/J4 specific antibody responses were evaluated by ELISA at several time points and expressed as geometric mean titres (GMT) in ELISA units/mL. Significant differences ($p<0.05$) between the antibody titres of the AS04 and the aluminium salt group are indicated by asterisks (n= 9–19 subjects for the aluminium salt group, n = 21–37 subjects for the AS04 group). Arrows indicate vaccination time points. (Previously published in [73]).

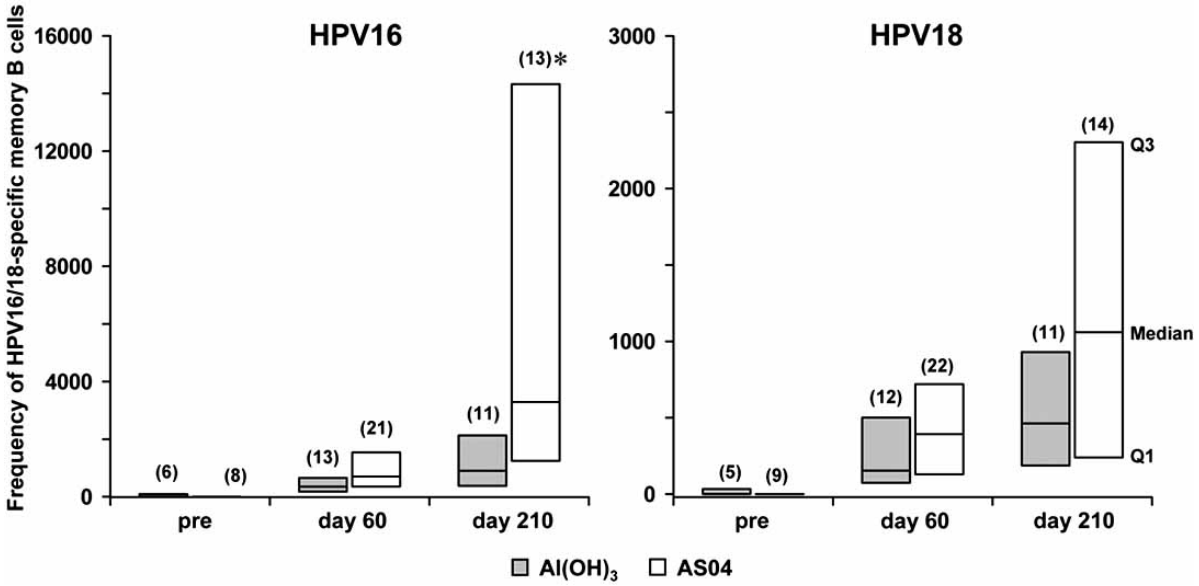


Fig. (7). Frequency of HPV-16- and HPV-18-specific memory B cells in humans. Subjects from two separate clinical trials were vaccinated with HPV-16 and HVP-18 L1 VLPs adjuvanted with AS04 or aluminium salt alone. Memory B cell responses directed against HPV-16 or HPV-18 L1 VLPs were quantified by ELISPOT at two time points post-vaccination. Results are represented as the frequency of HPV-16 or HPV-18-specific memory B cells per 10^6 PBMCs. The number of subjects is given in parenthesis. Asterisk represents significant difference between the aluminium and AS04 group ($p < 0.05$). (Previously published in [73]).

the third vaccine dose. In all age groups, a strong correlation was observed between serum and cervicovaginal secretions (CVS) antibody titres for both HPV types 16 and 18, indicating a passive antibody transfer via transudation and exudation from the serum to the cervical mucosa [82].

In a broad Phase III study in women aged 15 to 25 years (enrolment without screening for HPV infections) vaccination confirmed high efficacy up to 98% against CIN2 or worse (CIN2+) related to HPV types 16 and 18. The overall vaccine efficacy to CIN2+, irrespective of HPV type, was 70.2% [95% CI: 54.7, 80.9] for HPV naive cohort representing young adolescents before sexual activities [83]. Cross-protection was seen against CIN2+ with a 92% [CI: protection was seen against CIN2+ with a 92% [CI: 66.0, 99.2; 2 cases in the vaccine group vs 25 cases in the control group] vaccine efficacy in the case of HPV-31, 52% [95% CI: -2.9, 78.9; 12 cases in the vaccine group vs 25 cases in the control group] for HPV-33, 100% [95% CI: -67.8, 100; 0 case in the vaccine group vs 4 cases in the control group] for HPV-45. For all 3 types, statistical significance was achieved for persistent infection endpoints [83].

These results were an important step in vaccine development as one of the requirements for an effective HPV vaccine was the presence of vaccine-induced neutralizing antibodies at the site of HPV infection to prevent virus particles

from infecting the transformation zone where cervical cancers usually develop. The data on CVS confirmed that the HPV vaccine is capable to induce high neutralising antibody levels at the site of infection [20,73] and that these antibodies are not produced locally, but transudate or exudate from serum to the cervical mucus [27].

The added value of AS04 adjuvantation in inducing a stronger immune response has been observed in the comparison with another licensed HPV vaccine. *Cervarix*™ contains L1 VLPs for HPV types 16 and 18 designed for the prevention of related infection and precancerous lesions. The second vaccine, *Gardasil*®, contains L1 VLPs for HPV types 6, 11, 16 and 18 designed for the prevention of precancerous lesions caused by HPV-16/18 and genital warts caused by HPV-6/11. *Cervarix*™ was shown to provide higher serum anti-HPV-16 and -18 neutralizing antibody titres and higher circulating HPV-16 and -18 specific memory B cell frequencies compared to *Gardasil*®, a vaccine formulated with amorphous aluminium hydroxyphosphate sulfate salt [84].

SAFETY PROFILE OF HPV/AS04 VACCINE

More than 33.000 girls and women have received at least one dose of AS04-adjuvanted HPV vaccine, and the results of safety analysis have demonstrated that the AS04-adjuvanted HPV vaccine is generally well tolerated with a satisfactory safety profile. An integrated safety summary of 11 clinical studies showed that rates of solicited local symptoms were higher in the adjuvanted vaccine group than in the control groups [85]. Pain at the injection site was the most frequently reported local symptom. Most frequently reported general symptoms were headache, fatigue and myalgia. All solicited symptoms were mild to moderate in intensity and short-lived. No clinically relevant differences between AS04-adjuvanted vaccine group and control group were observed for unsolicited symptoms, serious adverse events, medically significant conditions, or the new onset of autoimmune diseases [85]. Autoimmune diseases are more and more under discussion in the context with vaccination, but immune-mediated conditions are not rare and they are among those events that might be mistaken for vaccine related adverse events [86].

A large integrated analysis including all randomized and controlled studies with registered and candidate vaccines containing AS04 as adjuvant with more than 68.000 participants was performed and no enhanced risk of autoimmune disease with AS04-adjuvanted vaccines was revealed. Overall the reporting rate of autoimmune diseases was low with an observed event rate of approximately 0.5% for both study groups [87]. These results are in line with other reports concluding that there is no evidence for a causal association between autoimmune diseases and most vaccines [88,89]. More than 9 million doses have been distributed to date and post-marketing surveillance has not reported changes to the safety profile of *Cervarix*™.

CONCLUSION

The development of new vaccines has highlighted the need for new strategies to enhance and guide the immune response for effective and long lasting protection. In particular, vaccines based on soluble recombinant antigens typically require adjuvants in order to enhance an antigen specific adaptive immune response, i.e. a T cell and antibody response. Recent advances in immunology and the better understanding of the innate and adaptive immune system interactions has provided new insights on how to design new vaccines using appropriate selection of antigens and new adjuvants adapted to the desired immune response. APCs play a key role towards a specific adaptive immune response, and adjuvants such as MPL interact with APCs through specific receptors.

Previous pre-clinical and clinical experience with AS04 in HBV and HSV vaccines has demonstrated the added value of the Adjuvant System and its superiority compared to aluminium-based vaccines. The AS04 Adjuvant System has been selected for a HPV vaccine formulation to induce high and sustained antibody levels, and high frequency of memory cells.

HPV represents a demanding challenge for vaccine development as the virus remains local, hides from the immune system, and fails thus to induce a reliable long-lasting protection upon natural infection. The target population for an HPV vaccine are young girls and women, and they are at risk of infection throughout their sexual active life. An effective vaccine has therefore to induce a long-term protection, preferably with reduced or no need for booster shots.

Several clinical studies have demonstrated that the AS04-adjuvanted HPV vaccine enhances the immune response and provides a high efficacy against infection and pre-cancerous lesions in combination with good tolerability and an acceptable safety profile.

On the basis of improved understanding of the immune system, it is now possible to design vaccines containing the appropriate match of antigens and adjuvants to respond to the needs of challenging diseases, as shown here for HPV. This approach will allow in the future, the development of effective vaccines against other remaining challenges in immunization.

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CONFLICT OF INTEREST

Nathalie Garçon declares she is employee of GSK Biologicals. Oberdan Leo is a consultant for GSK but was not directly involved in the development of the vaccines referred to in this manuscript.

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TRADEMARKS

Fendrix, Engerix-B, Cervarix are trademarks of the GlaxoSmithKline group of companies. Gardasil is a registered trademark of Merck & Co, Inc.

REFERENCES

[1] Kawai T, Akira S. Signaling to NF-kappaB by Toll-like receptors. Trends Mol Med 2007; 13(11): 460-9.

[2] Beutler B, Jiang Z, Georgel P, *et al*. Genetic analysis of host resistance: Toll-like receptor signaling and immunity at large. Annu Rev Immunol 2006; 24: 353-89.

[3] Takeda K, Akira S. Toll-like receptors in innate immunity. Int Immunol 2005; 17(1): 1-14.

[4] McKee AS, Munks MW, Marrack P. How do adjuvants work? Important considerations for new generation adjuvants. Immunity 2007; 27(5): 687-90.

[5] Glenny AT, Buttle GAH, Stevens MF. Rate of disappearance of diphtheria toxoid injected into rabbits and guinea pigs: toxoid precipitated with alum. J Pathol 1931; 34: 267-75.

[6] Ramon G. Procédés pour accroître la production des antitoxines. Ann Inst Pasteur 1926; 40: 1-10.

[7] Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. CA Cancer J Clin 2005; 55(2): 74-108.

[8] Kjaer SK, van den Brule AJC, Bock JE, *et al*. Human papillomavirus--the most significant risk determinant of cervical intraepithelial neoplasia. Int J Cancer 1996; 65(5): 601-6.

[9] Walboomers JMM, Jacobs MV, Manos MM, *et al*. Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. J Pathol 1999; 189(1): 12-9.

[10] de Villiers EM, Fauquet C, Broker TR, Bernard HU, Zur Hausen H. Classification of papillomaviruses. Virology 2004; 324(1): 17-27.

[11] Stanley MA, Pett MR, Coleman N. HPV: from infection to cancer. Biochem Soc Trans 2007; 35(Pt 6): 1456-60.

[12] Bosch FX, Lorincz A, Muñoz N, Meijer CJLM, Shah KV. The causal relation between human papillomavirus and cervical cancer. J Clin Pathol 2002; 55(4): 244-65.

[13] Cogliano V, Baan R, Straif K, Grosse Y, Secretan B, El Ghissassi F. Carcinogenicity of human papillomaviruses. Lancet Oncol 2005; 6(4): 204.

[14] Muñoz N, Bosch FX, Castellsagué X, *et al*. Against which human papillomavirus types shall we vaccinate and screen? The international perspective. Int J Cancer 2004; 111(2): 278-85.

[15] Baseman JG, Koutsky LA. The epidemiology of human papillomavirus infections. J Clin Virol 2005; 32(Suppl 1): S16-S24.

[16] Trottier H, Mahmud S, Lindsay L, Wieting S, Jenkins D, Franco EL. Risk of cervical lesions following incident human papillomavirus infection in young women without evidence of prior exposure. 24th International Papillomavirus Conference and Clinical Workshop, Beijing, China, Nov 3-9, 2007.

[17] Muñoz N, Castellsagué X, Berrington de González A, Gissmann L. Chapter 1: HPV in the etiology of human cancer. Vaccine 2006; 24(Suppl 3): S3-1-S3/10.

[18] Stoler MH, Rhodes CR, Whitbeck A, Wolinsky SM, Chow LT, Broker TR. Human papillomavirus type 16 and 18 gene expression in cervical neoplasias. Hum Pathol 1992; 23(2): 117-28.

[19] de Gruijl TD, Bontkes HJ, Walboomers JMM, *et al*. Differential T helper cell responses to human papillomavirus type 16 E7 related to viral clearance or persistence in patients with cervical neoplasia: a longitudinal study. Cancer Res 1998; 58(8): 1700-6.

[20] Stanley M, Lowy DR, Frazer I. Chapter 12: Prophylactic HPV vaccines: Underlying mechanisms. Vaccine 2006; 24(Suppl 3): S3-106-S3/113.

[21] van der Burg SH, Piersma SJ, de Jong A, *et al*. Association of cervical cancer with the presence of $CD4^+$ regulatory T cells specific for human papillomavirus antigens. Proc Natl Acad Sci USA 2007; 104(29): 12087-92.

[22] Bontkes HJ, de Gruijl TD, Walboomers JMM, *et al*. Immune responses against human papillomavirus (HPV) type 16 virus-like particles in a cohort study of women with cervical intraepithelial neoplasia. II. Systemic but not local IgA responses correlate with clearance of HPV-16. J Gen Virol 1999; 80(Pt 2): 409-17.

[23] Passmore J-AS, Marais DJ, Sampson C, *et al*. Cervicovaginal, oral, and serum IgG and IgA responses to human papillomavirus type 16 in women with cervical intraepithelial neoplasia. J Med Virol 2007; 79(9): 1375-80.

[24] Rocha-Zavaleta L, Pereira-Suarez AL, Yescas G, Cruz-Mimiaga RM, Garcia-Carranca A, Cruz-Talonia F. Mucosal IgG and IgA responses to human papillomavirus type 16 capsid proteins in HPV16-infected women without visible pathology. Viral Immunol 2003; 16(2): 159-68.

[25] Carter JJ, Koutsky LA, Hughes JP, *et al*. Comparison of human papillomavirus types 16, 18, and 6 capsid antibody responses following incident infection. J Infect Dis 2000; 181(6): 1911-9.

[26] Einstein MH, Schiller JT, Viscidi RP, *et al*. Clinician's guide to human papillomavirus immunology: knowns and unknowns. Lancet Infect Dis 2009; 9(6): 347-56.

[27] Schwarz TF, Leo O. Immune response to human papillomavirus after prophylactic vaccination with AS04-adjuvanted HPV-16/18 vaccine: improving upon nature. Gynecol Oncol 2008; 110(3 Suppl 1): S1-10.

[28] Christensen ND, Hopfl R, DiAngelo SL, *et al*. Assembled baculovirus-expressed human papillomavirus type 11 L1 capsid protein virus-like particles are recognized by neutralizing monoclonal antibodies and induce high titres of neutralizing antibodies. J Gen Virol 1994; 75 (Pt 9): 2271-6.

[29] Hagensee ME, Olson NH, Baker TS, Galloway DA. Three-dimensional structure of vaccinia virus-produced human papillomavirus type 1 capsids. J Virol 1994; 68(7): 4503-5.

[30] Rose RC, Reichman RC, Bonnez W. Human papillomavirus (HPV) type 11 recombinant virus-like particles induce the formation of neutralizing antibodies and detect HPV-specific antibodies in human sera. J Gen Virol 1994; 75(Pt 8): 2075-9.

[31] Kirnbauer R, Booy F, Cheng N, Lowy DR, Schiller JT. Papillomavirus L1 major capsid protein self-assembles into virus-like particles that are highly immunogenic. Proc Natl Acad Sci USA 1992; 89(24): 12180-4.

[32] Breitburd F, Kirnbauer R, Hubbert NL, *et al*. Immunization with viruslike particles from cottontail rabbit papillomavirus (CRPV) can protect against experimental CRPV infection. J Virol 1995; 69(6): 3959-63.

[33] Christensen ND, Reed CA, Cladel NM, Han R, Kreider JW. Immunization with viruslike particles induces long-term protection of rabbits against challenge with cottontail rabbit papillomavirus. J Virol 1996; 70(2): 960-5.

[34] Jansen KU, Rosolowsky M, Schultz LD, *et al*. Vaccination with yeast-expressed cottontail rabbit papillomavirus (CRPV) virus-like particles protects rabbits from CRPV-induced papilloma formation. Vaccine 1995; 13(16): 1509-14.

[35] Kirnbauer R, Chandrachud LM, O'Neil BW, *et al*. Virus-like particles of bovine papillomavirus type 4 in prophylactic and therapeutic immunization. Virology 1996; 219(1): 37-44.

[36] Lin Y-L, Borenstein LA, Selvakumar R, Ahmed R, Wettstein FO. Effective vaccination against papilloma development by immunization with L1 or L2 structural protein of cottontail rabbit papillomavirus. Virology 1992; 187(2): 612-9.

[37] Suzich JA, Ghim S-J, Palmer-Hill FJ, *et al*. Systemic immunization with papillomavirus L1 protein completely prevents the development of viral mucosal papillomas. Proc Natl Acad Sci USA 1995; 92(25): 11553-7.

[38] Nardelli-Haefliger D, Wirthner D, Schiller JT, *et al*. Specific antibody levels at the cervix during the menstrual cycle of women vaccinated with human papillomavirus 16 virus-like particles. J Natl Cancer Inst 2003; 95(15): 1128-37.

[39] Schiller JT, Davies P. Delivering on the promise: HPV vaccines and cervical cancer. Nat Rev Microbiol 2004; 2(4): 343-7.

[40] Viscidi RP, Schiffman M, Hildesheim A, *et al*. Seroreactivity to human papillomavirus (HPV) types 16, 18, or 31 and risk of subsequent HPV infection: results from a population-based study in Costa Rica. Cancer Epidemiol Biomarkers Prev 2004; 13(2): 324-7.

[41] Bernasconi NL, Traggiai E, Lanzavecchia A. Maintenance of serological memory by polyclonal activation of human memory B cells. Science 2002; 298(5601): 2199-202.

[42] Traggiai E, Puzone R, Lanzavecchia A. Antigen dependent and independent mechanisms that sustain serum antibody levels. Vaccine 2003; 21(Suppl 2): S2-35-S2/37.

[43] Banatvala J, Van Damme P, Oehen S. Lifelong protection against hepatitis B: the role of vaccine immunogenicity in immune memory. Vaccine 2001; 19(7-8): 877-85.

[44] Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. Cell 2006; 124(4): 783-801.

[45] Lemaitre B, Nicolas E, Michaut L, Reichhart JM, Hoffmann JA. The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. Cell 1996; 86(6): 973-83.

[46] Medzhitov R, Preston-Hurlburt P, Janeway CA, Jr. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. Nature 1997; 388(6640): 394-7.

[47] Meylan E, Tschopp J, Karin M. Intracellular pattern recognition receptors in the host response. Nature 2006; 442(7098): 39-44.
[48] Martinon F, Burns K, Tschopp J. The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. Mol Cell 2002; 10(2): 417-26.
[49] Shi Y, Evans JE, Rock KL. Molecular identification of a danger signal that alerts the immune system to dying cells. Nature 2003; 425(6957): 516-21.
[50] Garçon N, Chomez P, Van Mechelen M. GlaxoSmithKline Adjuvant Systems in vaccines: concepts, achievements and perspectives. Expert Rev Vaccines 2007; 6(5): 723-39.
[51] Brewer JM. (How) do aluminium adjuvants work? Immunol Lett 2006; 102(1): 10-5.
[52] HogenEsch H. Mechanisms of stimulation of the immune response by aluminum adjuvants. Vaccine 2002; 20 Suppl 3: S34-S39.
[53] Lindblad EB. Aluminium adjuvants-in retrospect and prospect. Vaccine 2004; 22(27-28): 3658-68.
[54] Morefield GL, Sokolovska A, Jiang D, HogenEsch H, Robinson JP, Hem SL. Role of aluminum-containing adjuvants in antigen internalization by dendritic cells *in vitro*. Vaccine 2005; 23(13): 1588-95.
[55] Garçon N, Van Mechelen M, Wettendorff M. Development and evaluation of AS04, a novel and improved immunological adjuvant system containing MPL and aluminium salt. In: Schijns V, O'Hagan D, editors. Immunopotentiators in Modern Vaccines. London Elsevier Academic Press, 2006; pp. 161-77.
[56] Eisenbarth SC, Colegio OR, O'Connor W, Sutterwala FS, Flavell RA. Crucial role for the Nalp3 inflammasome in the immunostimulatory properties of aluminium adjuvants. Nature 2008; 453(7198): 1122-6.
[57] Lambrecht BN, Kool M, Willart MAM, Hammad H. Mechanism of action of clinically approved adjuvants. Curr Opinion Immunol 2009; 21(1): 23-9.
[58] Li H, Nookala S, Re F. Aluminum hydroxide adjuvants activate caspase-1 and induce IL-1beta and IL-18 release. J Immunol 2007; 178(8): 5271-6.
[59] Marrack P, McKee AS, Munks MW. Towards an understanding of the adjuvant action of aluminium. Nat Rev Immunol 2009; 9(4): 287-93.
[60] Baldridge JR, McGowan P, Evans JT, *et al*. Taking a Toll on human disease: Toll-like receptor 4 agonists as vaccine adjuvants and monotherapeutic agents. Expert Opin Biol Ther 2004; 4(7): 1129-38.
[61] Casella CR, Mitchell TC. Putting endotoxin to work for us: monophosphoryl lipid A as a safe and effective vaccine adjuvant. Cell Mol Life Sci 2008; 65(20): 3231-40.
[62] Qureshi N, Takayama K, Ribi E. Purification and structural determination of nontoxic lipid A obtained from the lipopolysaccharide of Salmonella typhimurium. J Biol Chem 1982; 257(19): 11808-15.
[63] Hirschfeld M, Ma Y, Weis JH, Vogel SN, Weis JJ. Cutting edge: repurification of lipopolysaccharide eliminates signaling through both human and murine toll-like receptor 2. J Immunol 2000; 165(2): 618-22.
[64] Tapping RI, Akashi S, Miyake K, Godowski PJ, Tobias PS. Toll-like receptor 4, but not toll-like receptor 2, is a signaling receptor for *Escherichia* and *Salmonella* lipopolysaccharides. J Immunol 2000; 165(10): 5780-7.
[65] Evans JT, Cluff CW, Johnson DA, Lacy MJ, Persing DH, Baldridge JR. Enhancement of antigen-specific immunity via the TLR4 ligands MPL adjuvant and Ribi.529. Expert Rev Vaccines 2003; 2(2): 219-29.
[66] Martin M, Michalek SM, Katz J. Role of innate immune factors in the adjuvant activity of monophosphoryl lipid A. Infect Immun 2003; 71(5): 2498-507.
[67] Tiberio L, Fletcher L, Eldridge JH, Duncan DD. Host factors impacting the innate response in humans to the candidate adjuvants RC529 and monophosphoryl lipid A. Vaccine 2004; 22(11-12): 1515-23.
[68] Tomai MA, Johnson AG. T cell and interferon-gamma involvement in the adjuvant action of a detoxified endotoxin. J Biol Response Mod 1989; 8(6): 625-43.
[69] Ulrich JT, Myers KR. Monophosphoryl lipid A as an adjuvant. Past experiences and new directions. Pharm Biotechnol 1995; 6: 495-524.
[70] Iwasaki A, Medzhitov R. Toll-like receptor control of the adaptive immune responses. Nat Immunol 2004; 5(10): 987-95.
[71] Pasare C, Medzhitov R. Toll pathway-dependent blockade of CD4+CD25+ T cell-mediated suppression by dendritic cells. Science 2003; 299(5609): 1033-6.
[72] Didierlaurent AM, Morel S, Lockman L, *et al*. AS04, an aluminum Salt- and TLR-4 agonist-based adjuvant system, induces a transient localized innate immune response leading to enhanced adaptive immunity. J Immunol 2009; 183: 6186-97.
[73] Giannini SL, Hanon E, Moris P, *et al*. Enhanced humoral and memory B cellular immunity using HPV16/18 L1 VLP vaccine formulated with the MPL/aluminium salt combination (AS04) compared to aluminium salt only. Vaccine 2006; 24(33-34): 5937-49.
[74] Girndt M, Köhler H. Hepatitis B virus infection in hemodialysis patients. Semin Nephrol 2002; 22(4): 340-50.
[75] Zacks SL, Fried MW. Hepatitis B and C and renal failure. Infect Dis Clin North Am 2001; 15(3): 877-99.
[76] Beran J. Safety and immunogenicity of a new hepatitis B vaccine for the protection of patients with renal insufficiency including pre-haemodialysis and haemodialysis patients. Expert Opin Biol Ther 2008; 8(2): 235-47.
[77] Kong NCT, Beran J, Kee SA, *et al*. Immunogenicity and safety of an adjuvanted hepatitis B vaccine in pre-hemodialysis and hemodialysis patients. Kidney Int 2005; 68(5): 2298-303.
[78] Kundi M. New hepatitis B vaccine formulated with an improved adjuvant system. Expert Rev Vaccines 2007; 6(2): 133-40.
[79] Stanberry LR, Spruance SL, Cunningham AL, *et al*. Glycoprotein-D-adjuvant vaccine to prevent genital herpes. N Engl J Med 2002; 347(21): 1652-61.
[80] The GlaxoSmithKline Vaccine HPV-007 Study Group. Sustained efficacy and immunogenicity of the HPV-16/18 AS04-adjuvanted vaccine: analysis of a randomised placebo-controlled trial up to 6.4 years. Lancet 2009; 374(9706): 1975-85.
[81] De Carvalho N, Roteli-Martins C, Teixeira J, *et al*. Immunogenicity and safety of HPV-16/18 AS04-adjuvanted vaccine up to 7.3y. Abstract presented at: 25th International Papillomavirus Conference and Clinical Workshop,Malmö, Sweden, 8-14 May, 2009.
[82] Schwarz TF, Spaczynski M, Schneider A, *et al*. Immunogenicity and tolerability of an HPV-16/18 AS04-adjuvanted prophylactic cervical cancer vaccine in women aged 15-55 years. Vaccine 2009; 27(4): 581-7.
[83] Paavonen J, Naud P, Salmeron J, *et al*. Efficacy of human papillomavirus (HPV)-16/18 AS04-adjuvanted vaccine against cervical infection and precancer caused by oncogenic HPV types (PATRICIA): final analysis of a double-blind, randomised study in young women. Lancet 2009; 374(9686): 301-14.
[84] Einstein MH, Baron M, Levin MJ, *et al*. Comparison of the immunogenicity and safety of *Cervarix*™ and *Gardasil*® human papillomavirus (HPV) cervical cancer vaccines in healthy women aged 18-45 years. Hum Vaccin 2009; 5(10): 705-19.
[85] Descamps D, Hardt K, Spiessens B, *et al*. Safety of human papillomavirus (HPV)-16/18 AS04-adjuvanted vaccine for cervical cancer prevention: A pooled analysis of 11 clinical trials. Hum Vaccin 2009; 5(5); 332-40.
[86] Siegrist CA, Lewis EM, Eskola J, Evans SJ, Black SB. Human papilloma virus immunization in adolescent and young adults: a cohort study to illustrate what events might be mistaken for adverse reactions. Pediatr Infect Dis J 2007; 26(11): 979-84.
[87] Verstraeten T, Descamps D, David MP, *et al*. Analysis of adverse events of potential autoimmune aetiology in a large integrated safety database of AS04 adjuvanted vaccines. Vaccine 2008; 26(51): 6630-8.
[88] Schattner A. Consequence or coincidence? The occurrence, pathogenesis and significance of autoimmune manifestations after viral vaccines. Vaccine 2005; 23(30): 3876-86.
[89] Wraith DC, Goldman M, Lambert P-H. Vaccination and autoimmune disease: what is the evidence? Lancet 2003; 362(9396): 1659-66.

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