



Review

The Search for Novel Adjuvants for Early Life Vaccinations: Can “Danger” Motifs Show Us the Way?

JIRI KOVARIK* and CLAIRE-ANNE SIEGRIST

World Health Organization Collaborating Centre for Neonatal Vaccinology, Departments of Pathology and Pediatrics, University of Geneva Medical School, Geneva, Switzerland

Abstract. Potent but safe adjuvants are required to circumvent the many limitations of the newborn immune system to induce rapidly effective and long lasting immunity to subunit vaccines. By the use of pattern recognition receptors, antigen-presenting cells (APC) can very efficiently be activated by “danger” motifs expressed by various pathogens. APC activated by “danger” motifs, such as immunostimulatory sequences of bacterial DNA, can not only transmit the activation signal from the innate immunity to the adaptive compartment, but also shape the antigen-specific immune responses. Molecules or compounds expressing “danger” motifs could, therefore, be considered for use as adjuvants for subunit vaccines. In this review, the authors discuss the promises and potential drawbacks that such novel adjuvants could hold for their use in experimental and clinical early life vaccinations.

Key words: vaccines; adjuvants; newborns; infants.

Hurdles to the Induction of Efficient Vaccine Responses in Early Life

Neonates and young infants are highly susceptible to infections by bacterial and viral pathogens. Existing vaccination strategies, optimized for adult use, do not necessarily meet the requirements in this age group to induce protective immunity⁴⁹. Major components of innate immunity, such as neutrophils, the complement system and natural killer (NK) and lymphocyte-activated killer cell cytotoxicity, appear weakened in newborn infants^{36, 42}. Moreover, the limitations at the level of newborn monocytes/macrophages²⁷ and neonatal dendritic cells (DC)⁴¹ that have been reviewed previously could have a major impact on early life adaptive responses. They may explain the observed reduction in proliferative responses of neonatal T cells *in*

*vitro*²⁵. Neonatal and early life T cells do not seem to be intrinsically deficient, but their activation follows a different pattern when compared with adult T cells. In particular, neonatal T cell responses are characterized by reduced IFN- γ production when compared with adult responses, both in mice^{2, 3} and in humans^{34, 46}. In addition, after antigenic challenge newborn murine T cells show an increased IL-4 and IL-5 production compared with their adult counterparts^{7, 29}. This Th2 polarization of CD4 T cells goes along with an impaired induction of cytotoxic T lymphocyte cell (CTL) responses to protein, peptide and to some non-replicating live viral vaccine antigens in young mice^{7, 37}. The lack of Th1 and CTL responses could also be responsible for the increased susceptibility to intracellular pathogens, such as *Herpes simplex*, measles, RSV, *Mycobacterium tuberculosis* and *Listeria monocytogenes*, in early life.

* Correspondence to: Dr. Jiri Kovarik, Centre for Neonatal Vaccinology, C.M.U., 1, Rue Michel Servet, CH-1211 Geneva 4, Switzerland, fax: +41 22 702 57 46, e-mail:jiri.kovarik@medecine.unige.ch

The polarization of neonatal T cells towards the Th2 phenotype could be a consequence of a suboptimal activation of neonatal antigen-presenting cells (APC). This hypothesis is based on the observations in early life murine models²⁹ and human infants²⁰, where a limited production of IL-12 by neonatal APC and subsequent low IFN- γ vaccine responses to measles were found. Nevertheless, Th1 and CTL vaccine response can be induced in young mice by activating their APC through selected antigen delivery systems or adjuvants. Among these are strong Th1-driving synthetic adjuvants^{6, 19}, live replicating agents^{44, 50}, and DNA vaccines^{10, 37}. In contrast to the T cell responses, early life B cell responses show severe intrinsic deficiencies, such as reduced antibody production capacity, a limited diversity of the antibody repertoire and lower antibody affinities^{34, 45}. These characteristics, together with the apparent difficulties to respond to T-independent antigens (bacterial cell wall polysaccharides), point to an immaturity in the B cell compartment, which currently seems to be difficult to bypass, even by the use of strong immunostimulatory adjuvants. Infant antibody responses are furthermore marked by a strong predominance of IgG1 responses, whereas the generation of IgG2 antibodies remains weak during this period of life, irrespective of the antigen or delivery system used. However, this physiological IgG2 isotype deficiency could reflect the preferential induction of Th2 versus Th1 responses and can, at least in mice, be circumvented by strong Th1-driving adjuvants^{6, 29}.

APC are Activated by Detecting "Danger" Motifs

Although innate immunity has long been considered to generate rapid but bare defense mechanisms compared with the infinitely adaptable antigen-specific immune system, it is now widely accepted that innate immunity can largely determine the induction or nature of the adaptive response. Components of the innate immunity direct the adaptive immune responses to rapidly eliminate pathogens. However, the adaptive system itself signals back to the innate system to maintain the activation-state. APC represent the essential link between innate and adaptive immunity, since they use pattern recognition receptors (PRR). Using a limited number of germline-encoded PPR, APC can recognize a great variety of molecular structures associated with pathogens^{18, 26}. The engagement of PRR on the surface of APC signals the presence of pathogens to the defense system, and APC react by the expression of co-stimulatory molecules and the production of proinflamma-

tory cytokines leading subsequently to the activation of the adaptive immune system, including T and B lymphocytes³⁸. Cytokines play a major role in the control of CD4 T cell differentiation and they are largely induced upon innate immune recognition of pathogens, rather than during the course of an adaptive immune response¹. However, the activation of T lymphocytes requires a secondary signal (co-stimulation) provided by the same cells that present antigen to T cells. In the absence of such a secondary signal, the adaptive immune response is not activated, indicating that the antigens presented by the APC are either of self or of non-pathogen origin²⁶. Therefore, APC control not only the magnitude, but also the quality (i.e. Th1 versus Th2 polarization) of the immune response.

The Need for Novel Adjuvants for Subunit Vaccines

In vaccine research and development, safety considerations have largely lead to turning away from the live attenuated pathogens and whole inactivated organisms that were used in early vaccine preparations. Efforts have been made and are being made to replace them with newly developed recombinant protein or synthetic peptide subunit vaccines that generate fundamentally reduced reactivity after immunization. However, these formulations are also poorly immunogenic when administered without appropriate adjuvants. Imminent need exists, therefore, to combine these vaccine antigens with potent but safe adjuvants. Although a large variety of potent adjuvants have been developed in experimental models, most have been proved too toxic for routine clinical use. Particularly the vaccination of newborn and young infants demands an increased safety profile with minimal side effects³⁰. The only adjuvants currently approved for routine use by the US Food & Drug Administration are aluminum-based mineral salts (Alum). These appear to be safe, but poorly stimulate cell-mediated immunity and polarize immune responses towards the Th2 phenotype¹². The adsorption of protein and peptide antigens to Alum further enhances the observed Th2 preference of primed T cells of newborn infants²⁰ and mice⁷ when compared to adult immunization and, thus, renders the induction of early life Th1 and CTL responses even more difficult. Although neutralizing antibodies can be protective against many pathogens, Th1 and CTL responses are important in the protection and recovery from viruses and intracellular bacteria. Their induction

should, therefore, be considered as an important task for the development of new vaccine-adjuvants.

While it is still very difficult to define the exact mode of action of a particular adjuvant *in vivo*, the search for novel adjuvants seems to focus on molecules that can either directly stimulate innate immunity by mimicking “danger” motifs, or that are produced by the activated innate response. Until recently, apart from live attenuated viral and bacterial vaccines, only DNA vaccines were found able to induce Th1 and CTL responses in murine models of newborn immunization^{10, 37}. This unique capacity of DNA vaccines to stimulate the neonatal immune system were attributed, at least to an important part, to immunostimulatory motifs present on the backbone of the bacterial plasmids²⁸. Hopes were thus raised that these immunostimulatory motifs or other “danger” signals could be used for the development of a novel class of adjuvants for subunit vaccines leading to more protective, long-lived immunity, which is a particularly important issue in the neonatal period.

Activating APC by Mimicking Microbial “Danger” Motifs

CpG-motifs in bacterial DNA

Among other immunostimulatory bacterial products, (bacillus of Calmette and Guérin BCG; *M. bovis*) was shown to amplify immune reactions and was successfully applied to cause tumor regression in mice and humans⁶⁰. Interestingly, BCG, which is currently used to protect against infections with *M. tuberculosis*, is one of the few vaccine preparations that has the capacity to induce Th1 responses in early life to an extent similar to that in adult individuals³⁵. Recombinant BCG can also be used as a vector to deliver various vaccine antigens²¹. However, the relatively high reactogenicity of BCG remains a considerable limitation for its use as a vaccine vector in newborn vaccinations. The DNA-rich fraction was later identified as the active component of BCG that causes growth inhibition of tumors *in vivo* and that induces the production of IFN- α/β and IFN- γ in PBMC's and murine splenocytes and increases NK activity *in vitro*⁴⁸. Particular sequence motifs centered on a non-methylated CpG dinucleotide^{39, 58} were subsequently identified as the properties that distinguished immunostimulatory bacterial DNA from non-stimulatory vertebrate DNA. Synthetic CpG-rich oligodeoxynucleotides (ODN) with increased biological stability were constructed that proved capable to not only rapidly enhance transcription of IFN- α/β and

IL-12 from monocytes, but also to stimulate B cell proliferation and polyclonal IgM production *in vitro*³¹. Various CpG-rich ODN were shown to directly activate APC, NK and T cells, upregulating various co-stimulatory molecules (CD40, CD80 and CD86) and inducing production of proinflammatory cytokines (IL-6, IL-10, IL-12, IL-18, TNF- α , type I IFN's, and IFN- γ), resulting in a strong adjuvant effect on antigen specific immune responses *in vivo*, as reviewed recently by WAGNER⁵⁷. Particularly the strong Th1-driving capacity of CpG-rich ODN administered with antigens is of potential interest for its use as an adjuvant for vaccines.

As shown previously for adult mice¹³, early life Th2 responses to peptide and protein vaccine-antigens were efficiently converted into Th1-type responses with CpG-ODN in several murine immunization models^{11, 29}. These results could indicate that at least a part of the success of DNA vaccines to induce Th1 and CTL responses in early life immunizations could be directly attributed to the presence of CpG-motifs on the antigen-encoding plasmids²⁸. However, despite the induction of strong T cell responses in young mice, B cell responses could not be driven to adult-like levels, although IgG2a titers were clearly increased²⁹. Furthermore, IgG antibody responses to pure T cell-independent type 2 antigens, such as pneumococcal polysaccharides, could not be enhanced by CpG-ODN either in adult or 2-week-old mice (KOVARIK et al., submitted). Thus, CpG-ODN do not have the capacity to completely correct the early life B cell deficiency, despite the activation of neonatal murine B cells *in vitro*³¹. These results suggest that the CpG-ODN Th1-activating effect is largely mediated by APC²⁸ and NK cells⁵.

In terms of safety, CpG-ODN perform much better than could have been anticipated. The risk of chromosomal integration of the foreign DNA is largely reduced with CpG-ODN, compared with plasmid DNA vaccines. In spite of their strong stimulatory activity on B cells, high affinity anti-DNA antibodies were not detectable in mice treated with pure CpG-rich DNA⁴⁰, but were seen when the DNA was conjugated to protein²². However, when CpG-ODN were administered to adult mice in very high doses, the excessive immune stimulation resulted in toxicity and death, presumably by releasing high quantities of TNF- α ⁵¹. In our hands, toxic side effects in young mice, best reflected by suboptimal weight gain, were only observed when high doses (>20 μ g) of CpG-ODN were administered (KOVARIK et al., unpublished). They completely disappeared when the doses were reduced to non-toxic levels, still preserving their immunostimulatory activity²⁹. A major concern for the clinical use of CpG-ODN as adjuvants remains

their potential risk to induce Th1-driven autoimmune reactions. Controversial results on this issue have been reported in rodent studies, since the exacerbation of organ-specific autoimmunity has been observed in some experimental models^{4, 53}, but not in others⁹. Ongoing and future studies with primates are necessary to appropriately address these safety issues.

Toll-like receptors (TLR)

All Gram-negative bacteria share the structure of lipopolysaccharide (LPS), which is recognized by the signaling-incompetent plasma membrane CD14⁵⁴. Bound to CD14, LPS is subsequently capable of interacting with the signaling-competent TLR4 expressed on APC⁵⁹. LPS-mediated signaling leads to the secretion of TNF- α and IL-12 and to the expression of co-stimulatory molecules by macrophages and DC, and to the production of IFN- γ by NK cells^{8, 55}. The extreme potency of LPS to activate innate immunity is highlighted by its severe toxicity, leading to septic shock¹⁵. The overproduction of proinflammatory cytokines by LPS-activated APC has been identified to be the key event in this multifactorial condition, and hampers its use for clinical purposes. Nevertheless, the LPS-derived molecule monophosphoryl lipid A (MPL) is now being used as a vaccine adjuvant in clinical trials. MPL triggers cells, in a way similar to LPS, to produce proinflammatory cytokines such as IFN- γ and, thus, drive Th1-differentiation, apparently without generating serious toxicity in human adults²⁴. The safety profile of this adjuvant has now to be evaluated for its use in infant vaccinations. Preliminary experiments in murine models of early life immunization, however, suggest that the Th1-driving capacity of MPL may be significantly lower in early life than in adults (SIEGRIST et al., unpublished). Also, the Th1-driving capacity of MPL may not be sufficient even in adults for certain conditions. Indeed, immunization with a recombinant *Plasmodium falciparum* malaria vaccine (RTS-S) only protected adult volunteers against a sporozoite challenge when administered in a QS21-MPL-Alum formulation, and not when administered in MPL-Alum alone²³.

Mannose receptors

The family of multilectin mannose receptors contains molecules that share common structural characteristics, such as mannose receptor (MR), phospholipase A2 receptor, DEC 205 and C-type lectin receptor⁵². The MRs recognize a wide range of Gram-

-negative and Gram-positive bacteria, yeast, parasites and mycobacteria. MRs are expressed on tissue macrophages as well as on DC¹⁷. The innate immune defense relies on MRs for antigen uptake for the subsequent killing of the pathogens. Some observations, however, suggest that the MR (but not DEC205) could be a signal-transducing receptor that triggers a variety of immune responses, including the secretion of the proinflammatory cytokines TNF- α , IL-1 β , IL-6, GM-CSF and IL-12^{47, 58}. Although MRs have recently been shown to be implicated in the uptake of antibody/antigen complexes by macrophages and DC¹⁶, their precise role in the activation of the adaptive immune response has yet to be demonstrated.

Complement receptors

Components of the innate immunity seem not only able to control the development of the T cell immunity, but also to influence B cell responses. B cells express CD21 (complement receptor type 2), the receptor for the C3d protein of the complement system. By using CD19 as a signal-transducing moiety, the engagement of both CD21 and mIg/BCR can lead to a synergistic activation of the B cell. By the chemical attachment of C3d to the model antigen HEL, the threshold dose for immunizing mice could be reduced by a factor of 10 000¹⁴. But coupling of C3 fragments to antigens not only directly stimulates B cells, but also enhances uptake of antigen by APC and its processing to T cells, as shown recently with the HEL-C3b complex⁵⁶. Since the reduced levels of CD21 expression and low complement activity observed in infants could represent potential limitations to this approach, it would be of interest to assess whether coupling of C3 fragments to vaccine antigens would result in enhancement of early life antibody responses⁴³.

Concluding Remarks

We can so far only postulate that one or the other approach to activate neonatal APC by "danger" signals could result in the development of an adjuvant for use in clinical vaccinations in general and in neonatal immunizations in particular. Indeed, preliminary results obtained in murine models of early life immunization using CpG-ODN indicate that adult-like T cell responses to subunit vaccines, including the generation of Th1 and CTL, can be induced in young mice. This is most likely achieved under conditions where neonatal APC are optimally activated to transmit appropriate activa-

Table 1. Activation of the immune system after *in vivo* CpG-ODN adjuvantation

Mode	Target	Effect	Consequences
Direct	APC	co-stimulation cytokines	upregulation of CD40, CD80, CD86 induction of IL-12, TNF- α , IFN- γ , IL-6, GM-CSF
Direct	NK cells	cytokines	expression of IFN- γ , increased lytic activity
Indirect	T cells	activation	Th1 differentiation, induction of CTL
Indirect	B cells	activation	antibody production, induction of IgG2a

tion signals to the adaptive immune system, by the expression of various co-stimulatory molecules and proinflammatory cytokines (Table 1). Potential targets for novel adjuvants, could therefore, include molecules expressing “danger” motifs as well as the immunostimulatory products of activated APC. Thus, “danger” motifs might well point out the way to novel classes of adjuvants with promising characteristics for infant vaccination.

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