ORIGINAL ARTICLE

Long-term regulation of local cytokine production following immunization in mice

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ABSTRACT

Vaccines based on pathogen components require adjuvants to enhance the antigen-specific adaptive immune response. Intramuscular injection of adjuvanted-vaccines induces inflammatory cytokines and inflammatory nodules at the injection site within 48 hr after injection (Vaccine 2014; 32: 3393-401). In the present study, long-term regulation of cytokine production was investigated at 3, 6, 24, and 48 hr, 5 and 7 days, and 2 and 4 weeks after immunization with human papilloma virus (HPV), diphtheria and tetanus toxoids combined with acellular pertussis (DTaP), Haemophilus influenzae type B (Hib), and pneumococcal conjugated (PCV) vaccines in mouse models. The second dose was given 4 weeks later, and cytokine profiles were investigated 2, 5, and 7 days after re-immunization. IL-1β, IL-6, granulocyte-colony stimulating factor (G-CSF), and MCP-1 were produced from 3 hr and peaked at 48 hr after immunization with Cervarix in mice. IL-4, MCP-1, and TNF-α peaked at 5 or 7 days after immunization with Gardasil. These cytokines decreased 7 days after immunization with Cervarix and Gardasil. After the second dose, similar responses were observed. Both vaccines induced neutrophil extracellular traps (NET) in inflammatory nodules. The peak amount of IL-1β, IL-6, G-CSF, and MCP-1 was observed on day 5 of immunization and that of IL-4 on days 5-7 of immunization with DTaP, but no increase in IL-6 and G-CSF was observed after re-immunization. A similar response was noted after immunization with PCV13. An inflammatory response is essential for the development of adaptive immunity through the production of inflammatory cytokines.

Key words    alum adjuvant, G-CSF, inflammatory cytokine, monophosphoryl lipid A (MPL).

All effective vaccines induce acquired immune responses of humoral antibodies with or without cellular immunity through the stimulation of innate immunity. Vaccine antigen components or adjuvants act as ligands to the receptors of innate immunity (1–5). The initiation of innate immunity involves two different patterns: pathogen-associated molecular patterns (PAMP) and damage-associated molecular patterns (DAMP). They modulate the development of acquired immune responses through cytokine production (6, 7). IFN-α/β or inflammatory cytokines, IL-1β, IL-6, and TNF-α, are produced and modulate cell-mediated immunity and antibody responses (1, 6, 8). TLR2 or 4 located on the cell surface recognizes the antigens located on the outer surface of the pathogens. TLR3, 7, 8, and 9 existing in endosomes act as general recognition

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List of Abbreviations: CRPS, complex regional pain syndrome; DAMP, damage-associated molecular patterns; DPT/IPV, DTaP combined with inactivated polio virus; DTaP, diphtheria and tetanus toxoids with acellular pertussis vaccine; G-CSF, granulocyte-colony stimulating factor; Hib, Haemophilus influenzae type B; HPV, human papilloma virus; JAK/STAT, Janus-activated kinase/signal transducer activator of transcription; JEV, Japanese encephalitis virus; KC, keratinocyte chemoattractant; MAPK, mitogen-activated protein kinase; MPL, monophosphoryl lipid A; MyD88, myeloid differentiation factor 88; NET, neutrophil extracellular traps; PAMP, pathogen-associated molecular pattern; PCV, pneumococcal conjugated vaccine; VLP, virus-like particle.
receptors, which result in the production of IFN-α/β and induce inflammatory cytokines through the activation of nuclear factor kappa B (NF-κB) by signal transduction (9–12). In several component vaccines, purified proteins are poorly immunogenic, and adjuvants are used. Aluminum salt (alum) has been used in many vaccines since 1930 (13, 14) and a new combined adjuvant, AS04, has been developed. AS04 is composed of MPL, a detoxified derivative of LPS, and alum (15–17).

In our previous study, inflammatory nodules were detected at the injection sites of alum-adjuvanted vaccines, DTaP, HPV (Cervarix and Gardasil), and PCV. However, vaccines without alum, Hib, JEV, and influenza virus vaccines did not provoke such a response (18). Cytokine profiles were investigated for 48 hr after vaccination with several vaccines in mouse models. AS04-adjuvanted HPV (Cervarix) induced higher levels of IL-1β, IL-6, KC, MIP-1, and G-CSF in muscle tissues than any other vaccines, but serum cytokine profiles were observed similar to those induced by the other vaccines. Vaccine-induced adverse reactions of febrile illness or local reactions occurred within 48 hr after immunization, and significantly higher levels of serum G-CSF were observed in vaccine recipients with febrile illness (19).

In the present study, cytokine profiles were investigated at 2, 5, and 7 days and 2 and 4 weeks after the first dose, and 2, 5, and 7 days after re-immunization for further understanding of the long-term regulation of local and systemic immunological responses after vaccination.

**MATERIALS AND METHODS**

**Vaccines**

All routine inactivated or component vaccines were examined. DPT/IPV (Biken, Osaka, Japan; Kaketsuken, Kumamoto, Japan), DTaP (Biken; Kitasato-Daichi-Sankyo Vaccine, Tokyo, Japan; Takeda Pharmaceuticals, Biken, Osaka, Japan), Hib (Sanofi Pasteur, Lyon, France), PCV13 (Pfizer, New York, NY, USA), 4-valent HPV (Gardasil; MSD, Kenilworth, NJ, USA), and 2-valent HPV (Cervarix; GSK, Wavre, Belgium) were purchased commercially. Different concentrations of alum adjuvant and MPL were used, as shown in Table 1. Cervarix contains 50 µg MPL together with 0.5 mg alum, Gardasil contains 0.225 mg alum, and PCV13 contains 0.125 mg alum. DTaP produced by Biken, Kaketsuken, Kitasato-Daichi-Sankyo Vaccine, and Takeda Pharmaceuticals contains different concentrations of alum at 0.08, <0.25, 0.15, and 0.1 mg, respectively. Hib contains no alum adjuvant.

**Study design**

Four-week-old BALB/c mice were purchased from Charles River Laboratories (Wilmington, MA, USA). All vaccines were given in a 100 µL volume through an i.m. route in the left quadriceps muscle in three mice for each vaccine (1/5 volume of human dose). Muscle tissues (approximately 30 mg) were obtained principally before and 3, 6, 24, and 48 hr, 5 and 7 days, and 2 and 4 weeks after immunization with Cervarix and Gardasil. Mice were re-immunized 4 weeks after a single injection at the same site and samples were obtained 2, 5, and 7 days after re-immunization. DTaP/IPV, 2-valent and 4-valent HPV, DTaP, Hib, and PCV vaccines were injected. As a control, muscle tissues of the opposite right quadriceps muscle (without injection) were obtained. Serum samples were also obtained using the same schedule, and cytokine profiles were examined. The study design was approved by the Committee of Animal Research of Kitasato Institute for Life Sciences.

**Cytokine production**

Muscle tissues weighing approximately 30 mg were dissected to examine the local production of cytokines.

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<th>Table 1. Adjuvants used for inactivated vaccines in the present study</th>
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<td><strong>Vaccines</strong></td>
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<td>HPV Cervarix</td>
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CRM197, non-toxic mutant of diphtheria toxin; FHA, filamentous hemagglutinin; PT, pertussis toxin; Str. pneumoniae, Streptococcus pneumoniae.
Tissue was cut into small pieces, and homogenized in 2 mL RPMI supplemented with 1% protease inhibitor (Nacalai Tesque, Kyoto, Japan) using Precellys Lysing Kits (BERTIN Corp., Rockville, MD, USA). The muscle homogenate was centrifuged, filtrated through a 0.45 μm filter, and stored at −80°C until assay. IL-1β, IL-2, IL-4, IL-6, IL-10, Eotaxin, G-CSF, KC, MCP-1, and TNF-α were measured using the Bio-Plex mouse cytokine panel (Bio-Plex; Bio-Rad Laboratories, Hercules, CA, USA) (19). Three mice were used for each vaccine group.

**Histological examination**

Quadriceps muscle tissues were fixed with 10% phosphate-buffered formalin and decalcified in PBS before embedding in paraffin. Muscle tissues were sliced at 5 μm and stained with hematoxylin and eosin (HE) using a conventional procedure (18). Double-stranded DNA was stained by Hoechst 33342 (Bio-Rad), which binds to the AT-rich region (20).

**RESULTS**

**Cytokine production at injection sites following immunization with HPV vaccines**

In the previous report, the cytokine profile at the local injection sites was investigated in the early phase until 48 hr following immunization with two HPV vaccines (Cervarix and Gardasil) (19). In the present study, quadriceps muscles were obtained immediately before, and 3, 6, 24, and 48 hr, 5 and 7 days, and 2 and 4 weeks after administration. The second dose of vaccines was given 4 weeks later, and tissue samples were obtained 2, 5, and 7 days after re-immunization. Results for IL-1β and IL-4 are shown in Figure 1. Peak IL-1β production was observed on day 2 of immunization with Cervarix, decreased to pre-immunization levels, and higher levels of IL-1β were observed 2 days after the re-immunization. No significant increase in IL-1β was observed 2 days after the first immunization and re-immunization with Gardasil. In contrast, local IL-4 production was induced by Gardasil 5–7 days after immunization, and then decreased, and a similar induction was observed on day 2 of re-immunization. Low levels of IL-4 were observed 7 days after immunization with Cervarix and on day 7 of re-immunization.

Kinetics of IL-6 and G-CSF are shown in Figure 2. IL-6 and G-CSF were induced by Cervarix 3–48 hr after immunization, then decreased to baseline levels, and peak production was observed 2 days after re-immunization. However, low levels of G-CSF were observed 5 days after immunization with Gardasil.

![Fig. 1. Local production of IL-1β and IL-4 after immunization with Cervarix and Gardasil.](image-url)

Kinetics of MCP-1 and TNF-α are shown in Figure 3. High levels of MCP-1 were detected 3 hr after immunization with Cervarix and 5 days after immunization with Gardasil. Higher levels of TNF-α were induced by Gardasil than by Cervarix.

Muscle tissues of the opposite side (right thigh) were obtained for the control, but no significant increase in cytokines was noted.

**Cytokine production at injection sites following immunization with DTaP, Hib, and PCV13 vaccines**

DTaP, Hib, and PCV13 have been used for vaccination of infants under 1 year of age in the pediatric
immunization program in developed countries including Japan. Different brands of DTaP vaccines and PCV vary in the concentration of alum, and Hib has no alum adjuvant (Table 1). From the results of the long-term cytokine profile in muscle tissues after immunization with HPV vaccines in the present study, the cytokine profile until 48 hr after immunization was similar to the previous report, but some cytokine profiles were different after 48 hr and re-immunization (19). In the following experiments, quadriceps muscles were obtained immediately before, and 2, 5, and 7 days, and 2 and 4 weeks after administration. The second doses of vaccines were given 4 weeks after the first dose and samples were obtained at 2, 5, and 7 days after re-immunization. Three mice were immunized for each group and cytokine levels are shown as the mean±SD. The lower panel shows the production of G-CSF. The black line (―) shows the production after immunization with Gardasil, and the gray line (―) shows that after immunization with Cervarix. The dotted line represents the cytokine levels in un-immunized right quadriceps muscle as a control. Vertical lines show the mean ±SD.

A high level of IL-4 was produced 5 days after immunization with DTaP (Kaketsuken, Takeda, and Kitasato), 7 days after immunization with DTaP (Biken), and low levels of IL-4 were produced 2–5 days after immunization with PCV13. No IL-4 was detected following immunization with Hib. After re-immunization, IL-4 was lower in comparison with that after the first immunization.

Production of IL-6 and G-CSF is shown in Figure 6. Peak IL-6 was observed 5 days after immunization with DTaP (Kaketsuken, Kitasato, and Takeda), but no significant IL-6 was produced after re-immunization. G-CSF production peaked on day 5 of immunization with DTaP (Kaketsuken, Kitasato, and Takeda) and PCV13.

Kinetics of MCP-1 and TNF-α production are shown in Figure 7. DTaP and PCV13 induced MCP-1 on day 2–5 of immunization, and similar responses were observed after re-immunization, but there was no response after giving Hib. No significant TNF-α production was observed, and TNF-α production was enhanced 5 days after re-immunization with PCV13.

**Histopathological findings immediately after immunization with HPV vaccines**

A high incidence of marked local pain and syncope was reported after the introduction of HPV vaccines. Histopathological findings after i.m. injection of Cervarix and Gardasil were previously reported. Cervarix and Gardasil
induced inflammatory nodules 3–6 hr after immunization and polymorphonuclear neutrophils with some eosinophils were infiltrated, as is shown in panels b and f of Figure 8 (18). Inflammatory nodules were examined by HE and Hoechst 33342 staining (Fig. 8). Linear and morphologically irregular stains (red arrows) were observed (Fig. 8 panels d, h). The morphologically irregular stains suggest the release of DNA from neutrophils, namely NET, although citrullinated histone H3 remains to be

Fig. 4. Serum cytokine profiles after immunization with Cervarix and Gardasil. Serum samples were obtained before and 3, 6, 24, and 48 hr, 5 and 7 days, and 2 and 4 weeks after immunization. The second dose was given 4 weeks after the first dose and samples were obtained at 2, 5, and 7 days after re-immunization. IL-1β, IL-4, IL-6, IL-10, G-CSF, KC, MCP-1, and TNF-α were measured. Three mice were immunized for each group and cytokine levels are shown as the mean ± SD. Vertical lines show mean ± SD.

Fig. 5. Local production of IL-1β and IL-4 after immunization with four brands of DTaP, Hib, and PCV13. Four brands of DTaP (Biken, Kaketsuken, Kitasato, and Takeda), Hib, and PCV13 were given. Upper panel shows the production of IL-1β in the injected muscle tissues obtained before and 2, 5, and 7 days, and 2 and 4 weeks after immunization, and the lower panel shows the production of IL-4. The second dose was given 4 weeks after the first dose and samples were obtained 2, 5, and 7 days after re-immunization. Three mice were immunized for each group and cytokine levels are shown as the mean ± SD.

Fig. 6. Local production of IL-6 and G-CSF after immunization with four brands of DTaP, Hib, and PCV13. Four brands of DTaP (Biken, Kaketsuken, Kitasato, and Takeda), Hib, and PCV13 were given. Upper panel shows the production of IL-6 in the injected muscle tissues obtained before and 2, 5, and 7 days, and 2 and 4 weeks after immunization, and the lower panel shows the production of G-CSF. The second dose was given 4 weeks after the first dose and samples were obtained at 2, 5, and 7 days after re-immunization. Three mice were immunized for each group and cytokine levels are shown as the mean ± SD.

Fig. 7. Local production of MCP-1 and TNF-α after immunization with four brands of DTaP, Hib, and PCV13. Four brands of DTaP (Biken, Kaketsuken, Kitasato, and Takeda), Hib, and PCV13 were given. Upper panel shows the production of MCP-1 in the injected muscle tissues obtained before and 2, 5, and 7 days, and 2 and 4 weeks after immunization, and the lower panel shows the production of TNF-α. The second dose was given 4 weeks after the first dose and samples were obtained at 2, 5, and 7 days after re-immunization. Three mice were immunized for each group and cytokine levels are shown as the mean ± SD.
proven. Many round-shaped spots (white arrows) were observed in inflammatory nodules after immunization with Gardasil (Fig. 8, panel h). They suggest rather robust nuclei in infiltrating inflammatory cells.

**DISCUSSION**

Some HPV vaccine recipients complained of serious adverse events with chronic pain and autonomic nervous disorders (21–23). This has caused controversy worldwide as there is no evidence of a causal relationship, but these symptoms were incidentally observed among young females with no history of HPV vaccination (24–26). However, HPV vaccines are still pending recommendation in Japan. The merits should be discussed considering their effectiveness (27). Regional local pain soon after immunization may be caused by vaccination. In a previous report, the local cytokine profile was investigated for Cervarix, Gardasil, PCV, Hib, DTaP, and JEV vaccines for 48 hr after immunization in mice (18). In the present study, we investigated the long-term regulation of local cytokine production. IL-1β, IL-6, MCP-1, and G-CSF were produced 3–48 hr after immunization with Cervarix, which was consistent with a previous report (18), and decreased to baseline levels 7 days after immunization. Peak production of IL-4, MCP-1, and TNF-α was observed 5–7 days after immunization with Gardasil, and similar responses were noted more promptly 2–5 days after re-immunization. Four different brands of DTaP, Hib, and PCV vaccines were also investigated. IL-1β, IL-6, G-CSF, and MCP-1 were detected 5 days after immunization with DTaP, and IL-4 was detected 5–7 days after immunization with different brands of DTaP. PCV13 induced IL-1β, IL-4, and G-CSF 5–7 days after immunization and TNF-α peaked 5 days after re-immunization. Hib did not induce significant cytokine production at the injection sites after immunization.

Alum, silica, uric acid, DNA, toxins, and crystal materials were also found to signal stimulation of the inflamasome as DAMP to produce inflammatory cytokines (12, 13). Different profiles of inflammatory cytokines might reflect the different concentration or composition of vaccine ingredients because DTaP (Kitasato and Kaketsuken) including alum promoted high cytokine levels but DTaP (Biken) with a lower concentration of alum and Hib without alum both led to low cytokine levels. AS04 was used as an adjuvant for Cervarix, and contains MPL and alum. MPL is a detoxified lipopolysaccharide originating from *Salmonella minnesota*, which acts as a ligand for

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**Fig. 8.** Hematoxylin eosin (HE) and Hoechst 33342 staining of inflammatory nodules 6 hr after immunization with Cervarix and Gardasil. Muscle tissues were obtained 6 hr after immunization with Cervarix and Gardasil. Panels (a) and (b) show HE staining of muscle tissues obtained after immunization with Cervarix, and panels (e) and (f) are those after Gardasil. Panels (c) and (d) show Hoechst 33342 staining of muscle tissues obtained after immunization with Cervarix, and panels (g) and (h) are those after Gardasil. Dotted areas of panels (a, c, e, g) are magnified in panels (b, d, f, h), respectively. White arrows indicate round-shaped spots of inflammatory cells in panel H. Red arrows indicate morphologically irregular stains suggesting NET in panels (d) and (h).
TLR4 (15, 16, 28, 29). IL-1β, IL-6, and TNF-α were produced when peripheral blood mononuclear cells (PBMC) or DC were stimulated with MPL or HPV VLP with MPL (16). TLR4 binds to myeloid differentiation factor 2 (MD2) to form the TLR4/MD2 complex, and receives signals from LPS in a CD14-dependent or CD14-independent way. In the CD14-independent reaction, LPS tightly binds to the TLR4/MD2 complex inducing TNF-α through the MyD88 signaling pathway and IFN-β through the TIR-domain-containing adaptor-inducing interferon-β (TRIF) pathway. In the CD14-dependent reaction, CD14 helps LPS bind to the TLR4/MD2 complex, which signals MyD88 to produce TNF-α and CD14 to transport the LPS-bound TLR4/MD2 complex into the endosome, where the TRIF pathway is activated to produce IFN-β. When MPL is used as a ligand instead of LPS, the CD14-dependent MyD88 pathway only is affected, which results in a weaker production of TNF-α (16, 30–32).

In the present study, G-CSF and inflammatory cytokines were produced 3 hr after immunization with Cervarix, probably because of the effects of MPL, and inflammatory nodules were observed at the injection sites consisting of neutrophils (18). G-CSF promotes the proliferation and differentiation of neutrophil precursors. Prominent accumulation of neutrophils was observed at the Cervarix injection sites compared with that of Gardasil, which was probably caused by greater induction of G-CSF. NET were typically observed in bacterial infections and include cellular DNA and histones (33, 34). This is consistent with the results reported by Munks et al. (35) showing that nodules following immunization with alum-adjuvanted vaccines contained the clotting protein fibrin and histones. DNA and histones released from neutrophils work as DAMP to stimulate innate immunity (36, 37). Inflammation causes the development of immunity and adverse reactions.

CRPS, fibromyalgia, and several disease entities with chronic pain were reported after bone fracture or surgery. Through a systematic review, the involvement of higher levels of inflammatory cytokines (IL-1β, IL-6, and IL-8) was reported, but their pathophysiological role in chronic pain has not been clearly elucidated (38). The study group also investigated the local expression of cytokines in skin punch biopsy, and gene expressions of IL-6 and IL-10 were higher in the painful group compared with that of painless neuropathies (39). There are no reports on CRPS after vaccination, but increased levels of inflammatory cytokine of IL-6 may be responsible for local pain. IL-6 has several wide-ranging biological functions. IL-6 was shown to contribute to nociceptor sensitization. A complex of IL-6 and the membrane IL-6 receptor on neural fibers is associated with signal transduction with gp130, inducing the subsequent intracellular signaling activation of JAK/STAT or MAPK (40). TNF-α and IL-1β are upstream of IL-6 signaling through the activation of NF-κB (40, 41).

In addition to IL-6, TNF-α, IL-1β, and G-CSF, IL-4 was produced after vaccination with different profiles for each vaccine. IL-4 modulates T-cell differentiation to CD4 helper Th2 cells (42). Gardasil induced low levels of G-CSF and IL-6, but high levels of IL-4 were observed 5–7 days after immunization. Three brands of DTaP also produced IL-4 as well as inflammatory cytokines. However, Cervarix produced basal levels of IL-4. It is possible that the Th1 response caused by MPL suppresses the production of IL-4, but the actual mechanism remains unknown.

In conclusion, high levels of inflammatory cytokines and G-CSF were detected within 7 days at the injection sites of alum- or AS04-adjuvanted vaccines, which are closely related to inflammatory nodules and probably to the acute onset of local pain, but decreased 7 days after immunization and afterward.

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DISCLOSURE

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REFERENCES

Cytokine production after immunization


