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Review

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Analysis of peripheral blood immune cells after prophylactic immunization with HPV-16/18 ASO4-adjuvanted vaccine

Ocena wybranych komórek układu odporności u kobiet poddanych immunizacji szczepionką przeciw HPV (typy 16 i 18) z adiuwantem ASO4

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Summary

Persistent infection with oncogenic types of human papillomavirus (HPV) is a causal factor for more than 99% of cervical cancers. Recently, prophylactic vaccines have been developed to prevent infections with cancer-associated HPV types (HPV16 and HPV18). The aim of this study was to analyze the changes in the immune system that occur within four weeks of the first dose of HPV-16/18 ASO4-adjuvanted vaccine. Assessment of the percentages of selected cell populations in peripheral blood of 20 healthy volunteers vaccinated with Cervarix was performed using flow cytometry. The analysis revealed an increase in the proportion of activated B and CD4+ T helper cells and an absence of significant differences in cytotoxic CD8+ T lymphocytes, indicating activation of the humoral response after vaccination, without a significant effect on cellular response. There were no significant changes in the NK cell population, and there was a reduction of the percentage of NKT-like cells, which may result from expiry of the primary response at the time of analysis. The presented results are preliminary, and in the context of the increasing use of the anti-HPV vaccine, it would be worth continuing the study in larger groups of patients and at earlier and later time points in combination with the measurement of specific anti-HPV16 and -HPV18 antibody levels. Such an assessment could therefore contribute not only to better understanding of the exact mechanism of action of the vaccine, but also to defining the immunological parameters that determine its effectiveness.

Keywords: **Cervical cancer • human papilloma virus (HPV) • prophylactic vaccine • immune cell response**

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Introduction

Epidemiological data on the incidence and mortality of cervical cancer indicate that this is one of the most important public health care problems in the world [2,24]. After breast cancer, it is the second most common cancer diagnosed in women globally. The prevalence rate is different in different countries. It is significantly lower where intensive programs are carried out aimed at both the early diagnosis and treatment of preinvasive cancer, as well as prevention, taking into account that cervical cancer has known etiology and risk factors with a persistent infection with oncogenic types of human papillomavirus (HPV) considered as the most important one [10.15]. HPV belongs to the family Papillomaviridae covering more than 200 types of viruses, which can be divided into groups with high and low oncogenic potential [6,7]. Oncogenic viruses include HPV 16, 18, 45, 31, 33, 52, 58, 35, 59, 56, 39, 51, 73, 68, 66. The most common are infections with HPV types 16 and 18 (the cause of 75% of cervical cancer). In 1996, the World Health Organization (WHO) considered the relationship between persistent infection with oncogenic HPV types and development of cervical intraepithelial neoplasia consequently leading to cancer as established and proven, and thus the cancer-causing virus types HPV 16 and 18 as human carcinogens [20]. The presence of HPV DNA in 99.7% of cases of cervical cancer confirms the link between infection and the development of cancer [3]. Recently, primary prevention of cervical cancer has become possible with the development of prophylactic HPV vaccines, based on the in vitro expression of papillomavirus major capsid protein L1 that forms papillomavirus-like particles (VLPs) [11,31]. There are now two licensed prophylactic HPV vaccines: bivalent, directed against oncogenic subtypes 16 and 18 (Cervarix from GlaxoSmithKline), and quadrivalent against subtypes 16 and 18 and non-oncogenic subtypes 6 and 11 (Silgard from Merck Sharp & Dohme Ltd). Both vaccines have been shown in randomized clinical trials to elicit neutralizing antibodies to papillomavirus in a titer sufficient to prevent infection with incorporated HPV types [12,13,21,23,30]. However, still little is known about the early immune response to the vaccine [8]. The aim of this study was to analyze the changes in the immune system that occur after four weeks of the first dose of Cervarix, by the evaluation of the following peripheral blood lymphocyte subsets: activated CD19+/CD25+ and CD19+/CD69+ B cells, activated CD3+/CD25+ and CD3+/CD69+ T cells, including CD4+ and CD8+ T cells, FOXP3+CD25+CD4 + regulatory T cells, NK and NKT-like cells. Additionally, we evaluated the correlations between the percentage of T regulatory cells prior to vaccination and the percentages of activated B, T, NK and NKT-like cells after vaccination as well as correlations between the percentage of T regulatory cells and the percentages of activated B, T, NK cells and NKT--like cells after vaccination.

Materials and methods

Peripheral blood (PB) specimens were obtained from 20 healthy women (median age 19.6 years; range: 13 to 30 years) before and four weeks after vaccine administration. All PB samples (10 ml) were collected into heparinized tubes and immediately processed. Mononuclear cells were separated by density gradient centrifugation on Gradisol L for 25 minutes at 400 x g at room temperature. Interphase cells were removed, washed twice and resuspended in phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+}.

In our study the standard immunophenotypic flow cytometric analysis included monoclonal antibodies (MoAbs): anti-CD3 FITC/CD19 PE anti-CD4 FITC/CD8 PE, anti-CD69 PE-Cy5, anti-CD25 PE-Cy5 and anti-CD3 FITC/HLA-DR PE. Cells were incubated for 20 min at room temperature (RT). CD25 and CD69 expression was analyzed within gated $CD3^+$, $CD19^+$, $CD4^+$, $CD8^+$ lymphocytes. The flow cytometric analysis also included monoclonal antibodies anti-CD3 FITC/$CD16^+$CD56 PE/CD45 PerCP (BD Biosciences) which permitted simultaneous assessment of T ($CD3^+$) lymphocytes and NK ($CD16^+CD56^+$) cells. During the analysis, the $CD3^+/CD16^+CD56^+$ population (NKT-like cells) was determined.

T regulatory cells were evaluated in the PB via analysis of the surface expression of CD4 and CD25 antigens, as well as intracellular expression of FoxP3 by flow cytometry. The percentage of $CD4^+/CD25^+/FoxP3^+$ T regulatory cells (Treg) among CD4+ lymphocytes was determined using the Human Treg Flow Kit (FOXP3 Alexa Fluor 488/CD4 PECy5/CD25 PE) from BioLegend according to the manufacturer's instructions.

The samples were analyzed by flow cytometry directly after preparation. For data acquisition and analysis, a FACSCalibur instrument (BD) with CellQuest software (BD) was used. For each analysis 10,000 events were acquired and analyzed. The percentage of positive cells was measured from a cut-off set using isotype-matched nonspecific control antibody.

We used Statistica 9 PL software for statistical procedures. Differences were considered statistically significant with a p-value £0.05.

Results

The median percentage of activated CD19+CD25+ B cells among CD19+ B cells after vaccination was 5.10%, and it was significantly higher as compared to the pro-

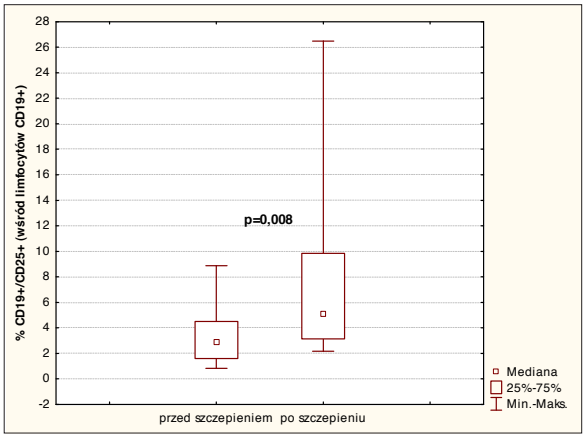
portion of these cells prior to the vaccination (median 2.88%) (Figure 1A). The median percentage of activated CD19+CD69+ B cells among CD19+ B cells was also significantly higher after the vaccination as compared to the pre-vaccination value (1.20% vs 0.31%) (Figure 1B).

The median percentage of activated CD4+CD25+ T helper cells among CD3+ T cells was 14.88% and was significantly higher compared to the pre-vaccination value (median 12.23%) (Figure 2).

The median percentage of FOXP3+CD25+CD4+ T regulatory cells among CD25+CD4+ T cells was significantly higher after vaccination as compared to the percentage of these cells before the vaccination (3.70% vs 2.88%) (Figure 3).

After vaccination, there was a significant correlation between the percentage of FOXP3+CD25+CD4+ regulatory T cells and the percentage of CD3+HLA-DR+ activated T cells (Figure 4).

The median percentage of CD3+CD16+CD56+ NKT-like cells after vaccination was 1.65% and was significantly



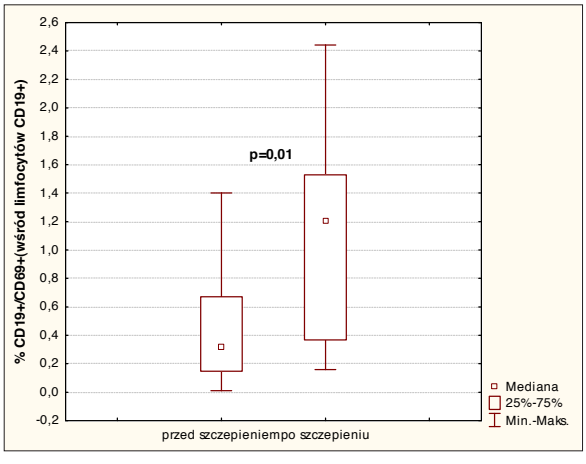


Fig. 1. Median percentage of activated CD19+CD25+ B cells (A) and CD19+CD69+ B cells (B) among CD19+ B cells before and after vaccination with prophylactic HPV vaccine (Wilcoxon test)

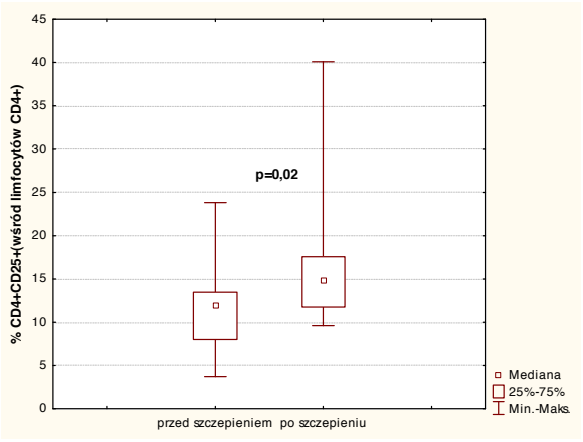


Fig. 2. Median percentage of activated CD4+CD25+ T helper cells among CD4+ T cells before and after vaccination with prophylactic HPV vaccine (Wilcoxon test)

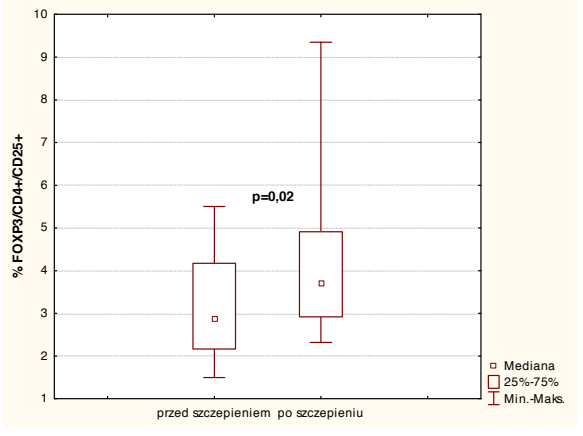


Fig. 3. Percentage of FOXP3+CD25+CD4+ regulatory T cells among CD25+CD4+ T cells before and after vaccination with prophylactic HPV vaccine (Wilcoxon test)

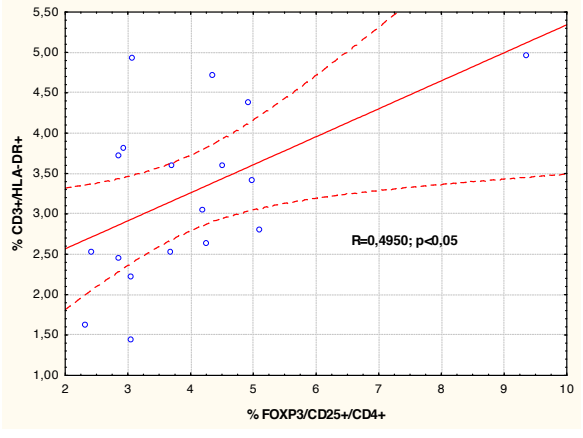


Fig. 4. The correlation between the percentage of FOXP3+CD25+CD4+ regulatory T cells and the percentage of CD3+HLA-DR+ activated T cells after vaccination with prophylactic HPV vaccine

lower as compared to the percentage of these cells before vaccination (median 2.56%) (Figure 5).

After vaccination, there was a significant negative correlation between the percentage of CD56+CD3-/CD16+ NK cells and the percentage of FOXP3+CD25+CD4+ T regulatory cells (Figure 6).

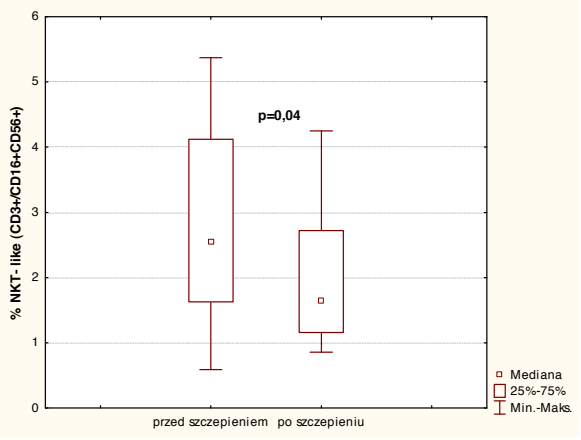


Fig. 5. Proportion of CD3+CD16+CD56+ NKT-like cells among CD45+ cells before and after vaccination with prophylactic HPV vaccine (Wilcoxon test)

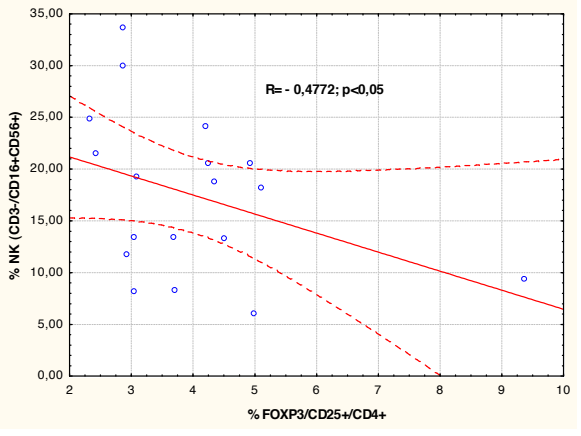


Fig. 6. Correlation between the percentage of CD56+CD3-/CD16+ NK cells and the percentage of FOXP3+CD25+CD4+ T regulatory cells after vaccination with prophylactic HPV vaccine

Discussion

In this study we analyzed selected populations of immune cells in the peripheral blood serum of women vaccinated with bivalent vaccine against human papillomavirus (Cervarix). Both cells involved in the innate and those involved in the adoptive response were evaluated. The antigens contained in the vaccine, VLPs, are highly immunogenic and generate high levels of neutralizing antibodies and long memory associated with immune B cells, in contrast to the response to natural HPV infection [1,4,5,29]. We evaluated the effect of vaccination on B cells by measuring the surface expression of markers of late and early activation, such as CD25 and CD69 respectively. The significant increase in the percentage of both populations of activated B cells, with expression of CD25 or CD69, that occurred four weeks after vaccination pointed directly to the stimulation of humoral immunity. The results of the next experiments further confirmed a significant effect of the vaccine in early humoral immunity. Analysis of T cell subpopulations showed a significant increase in the percentage of activated CD4+CD25+ T cells after vaccination as compared to pre-vaccination values. Since CD4+ helper T cells play a crucial role in the differentiation and maturation of B lymphocytes [9], their significant increase after administration of an anti-HPV vaccine seems to be directly related to the stimulation of the B cell response. However, there was no increase in the percentage of activated CD3+CD69+ T cells and CD8+ CD69+ cytotoxic T cells, suggesting no significant stimulation of the cellular response measured four weeks after the vaccination. In the course of natural infection, the specific T-cell response to the HPV antigen is very low, and induction of only very few clones of cytotoxic CD8+ T cells has been described [17,22]. After vaccination, a potential reason for the obtained results might be the effect of the adjuvant ASO4 included in the Cervarix vaccine, whose strong stimulating effect on the humoral Th2 response is associated with simultaneous suppression of Th1 effector cells [15] and cytotoxic CD8+ T cells through the induction of VLP specific IL-10 secreting CD4+ T cells [19,28]. One should also take into account that measurements in the present study were made at only one time point, which could not exclude the possibility of an earlier or, more likely, their subsequent activation. In the studies of Pinto et al., the authors observed a significant increase in proliferative properties of both CD4+ and CD8+ T cells and serum concentrations of Th1 and Th2 cytokines [25,26] two and seven months after the start of vaccination with the vaccine that included only HPV-16 L1 VLP without any adjuvant. In the next experiments, we noted a significant increase in the percentage of regulatory T cells after vaccination against HPV. The primary function of Treg cells is to inhibit the immune response [16], including the response against infectious agents. Induction of regulatory T cells occurring in the course of infection is considered to suppress excessive inflammation, thereby protecting the tissue from damage. Thus, a significant increase in the percentage of regulatory T-cells might be associated with induction of the immune response to the administration of the antigen in the form of L1 VLP, which could be further confirmed by a significant correlation between the percentage of T regulatory cells and the percentage of activated CD3+HLA-DR+ T cells observed after vaccination. In the last part of the experiments, we evaluated NK and NKT-like cells, populations involved in the primary immune response. In the natural course of HPV infection, there is no induction of a primary response similarly to the secondary response [27]. In contrast, after vaccination, high immunogenicity of the VLP particles contribute to the induction of primary immunity mechanisms, and subsequently, the

secondary immune response [18,32]. The lack of significant changes in the percentage of NK cells four weeks after vaccination was possibly related to the fact that the primary response occurs after the first contact with the antigen within 3-14 days and expires after a few weeks. Also, a negative correlation between the percentage of CD3-CD16+ CD56+ NK cells and the percentage of FOXP3+CD25+CD4+ T regulatory cells indicates the decrease in the primary response along with an increase in the secondary response of T cells. The percentage of NKT-like cells was even lower after vaccination as compared to values prior to vaccination, which was probably related to very fast activation of NKT-like cells after antigen stimulation (in this case VLP), followed by a return to baseline values or even their reduction.

In conclusion, evaluation of the immune response at four weeks after a prophylactic HPV vaccine revealed an increase in the proportion of activated B and CD4+ T helper cells and the absence of significant differences in the cytotoxic CD8+ T lymphocytes, indicating activation of the humoral response after vaccination, without a significant effect on the cellular response. The lack of significant changes in the NK cell population and the reduction of the percentage of NKT-like cells may result from expiry of the primary response at the time of analysis, four weeks after vaccination. Adequate assessment of this issue requires further analysis of the primary immune response of cells within a shorter time after vaccination. The presented results are preliminary, and in the context of the increasing use of the anti-HPV vaccine, it would be worth continuing the study in larger groups of patients and at earlier and later time points in combination with the measurement of neutralizing antibody levels. Indeed, it seems that despite the undoubtedly key role of antibodies in the protective action of the vaccine, one cannot rule out the importance of primary and cellular responses, and there are only very few publications concerning this subject. Such an assessment could therefore contribute not only to better understanding of the exact mechanism of action of the vaccine, but also to defining the immunological parameters that determine its effectiveness.

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The authors have no potential conflicts of interest to declare.