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ГЕНЕТИЧЕСКИЕ МАРКЕРЫ ИММУННОГО ОТВЕТА НА АНТИГЕНЫ YERSINIA PESTIS F1 И V МИКРОКАПСУЛИРОВАННОЙ ЧУМНОЙ ВАКЦИНЫ

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РЕЗЮМЕ: Эффективность иммунного ответа на вакцину зависит от активации антиген-презентирующих клеток и антиген-специфических Т- и В-лимфоцитов, индукции клеток памяти, регуляторных клеток и секретирующих антитела плазматических клеток, а также от длительности пребывания антигена в организме. Сила иммунного ответа главным образом зависит от генов, задействованных в этом процессе. Настоящая работа была посвящена изучению полиморфизма генов, который у части людей может быть причиной неудовлетворительного иммунного ответа на молекулярную микрокапсулированную чумную вакцину (ММЧВ). После двух последовательных вакцинаций среди 9 добровольцев у 67 % зафиксированы титры специфических антител не меньше порогового уровня. Однако у 33 % добровольцев не найдено специфических антител ни к V, ни к F1 (Caf1) антигену по истечении 90 суток после последней вакцинации. Мы исследовали 20 одиночных нуклеотидных полиморфизмов (ОНП) для 14 генов, ответственных за иммунный ответ, с целью найти связь между конкретным аллелем и способностью формировать антитела к антигенам ММЧВ. Гомозиготность гена IL1β по варианту дикого типа C-3953, либо гомозиготность по мутантному аллелю T-3953 делали возможной продукцию анти-F1 IgG, в то время как гетерозиготы оставались серонегативными. Гетерозиготы по гену TLR9 в положении 2848 (A2848G) также реагировали на F1. Среди реагировавших на антиген V (LcrV), продукция IgG наблюдалась лишь у носителей мутантного аллеля IL4 C-589T, у носителей мутантного аллеля C-174G гена IL6, как гомо- так и гетерозиготных, и не наблюдалась у гомозигот по дикому типу аллеля. Отсутствие мутантного аллеля C-819T гена IL10 также было связано с нереагирующим фенотипом. Наши результаты позволяют предположить, что потенциально нереагирующие на ММЧВ могут быть опознаны по ОНП в генах IL1β (C-3953 T), IL4 (C-589T), IL6 (C-174G), IL10 (C-819T) и TLR9 (A2848G). Индивидов с нереагирующим генотипом следует включить в программу клинических испытаний новых универсально эффективных чумных вакцин.

КЛЮЧЕВЫЕ СЛОВА: чумная вакцина; иммунный ответ; полиморфизм генов; цитокины; Толл-подобные рецепторы.

GENETIC MARKERS OF IMMUNE RESPONSE TO *YERSINIA PESTIS* F1 AND V ANTIGENS-LOADED MICROSPHERES VACCINE AGAINST PLAGUE

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ABSTRACT: An efficient immune response to a vaccine depends on activation of antigen-presenting cells and antigen-specific T and B lymphocytes, induction of memory cells, regulatory cells and antibody-secreting plasma cells, as well as on the persistence of the antigen in the body. The strength of immune response largely depends on the genes participating in this process. This study was focused on the gene polymorphism that could be responsible for insufficient immune response to a plague vaccine (molecular microencapsulated plague vaccine, MMPV) in a proportion of humans. After two subsequent vaccinations of the nine human volunteers, 67 % of them developed specific antibody titers equal to or exceeding the threshold level. However, 33 % of the subjects did not develop specific antibodies to either V, or F1 (Caf1) antigen during 90 days of observation post vaccination. We have investigated 20 single nucleotide polymorphisms (SNP) in 14 of immune response genes in an attempt to find an association between a particular allele and the ability to develop antibodies to MMPV antigens. Homozygosity for the IL1β gene wild type allele variant C-3953, or homozygosity for a mutated allele T-3953 allowed to produce anti-F1 IgG, whereas heterozygotes remained seronegative. Heterozygotes for the TLR9 gene at position 2848 (A2848G) were also responders to F1. Among responders to the V (LcrV) antigen, IgG production was observed only in carriers of mutant IL4 allele C-589T and in carriers of mutant allele of IL6 gene C-174G, either homozygotes or heterozygotes, and was not observed in homozygotes of the wild type allele. The lack of a mutant allele of IL10 gene C-819T was also associated with a non-responder phenotype. Our data suggest that potential non-responders to the MMPV may be identified by SNP in the IL1β (C-3953 T), IL4 (C-589T), IL6 (C-174G), IL10 (C-819T), and TLR9 (A2848G) genes. Individuals with non-responder genotype should be included in the clinical trials assessing novel, universally effective plague vaccines.

KEYWORDS: plague vaccine; immune response; gene polymorphism; cytokines; toll-like receptors.

INTRODUCTION

The causative agent of plague, *Yersinia pestis*, was the cause of three pandemics, which claimed millions of human lives. Plague is an infection affecting annually more than 3000 people [3]. The live plague vaccine used in the former Soviet Union stimulates intense immunity and protects from lethality caused by infection with *Y. pestis* [14], but it is associated with local and systemic adverse reactions of various severity in 5–29 % of persons with a normal immune status [44, 45, 53]. Therefore, a search for a better live plague vaccine superior to the existing one in immunogenicity, yet having less side effects, is very important [20–23, 41, 43, 55]. Another approach is to design a subunit (molecular) vaccine based on a few known protective antigens: such vaccines are aimed to minimize immune responses to non-specific components.

An example of such an antigen is a capsular protein F1 (Caf1) — a multifunctional factor of pathogenicity of *Y. pestis*. Serologic diagnosis of plague is based on detection of anti-F1 antibodies, and this antigen is the most important component of plague vaccines. Its important role in induction of a robust immune response in humans has been demonstrated [2]. The V antigen (LcrV) is another multifunctional factor of pathogenicity and an important protective antigen of *Y. pestis* [49]. It inhibits chemotaxis of neutrophils and production of pro-inflammatory cytokines [35, 52].

A vaccine is supposed to activate many different mechanisms of immune response [7, 19]. However, although vaccines induce an immune response in the majority of individuals, a proportion of them may respond weakly or remain non-responsive [4, 5, 10, 33]. For instance, induction of a protective antibody response to HIV vaccine depends on particular alleles of HLA class II. The immune response is controlled by various other genes, among which — genes of pattern recognition receptors (PRRs) and genes of cytokines. PRRs are represented by several families which recognize macromolecules characteristic for bacteria and viruses, as well as endogenous molecules that become visible to the immune system as a result of tissue damage. Toll-like receptors (TLR) are most well studied PRRs. Binding of TLRs by their ligands results in activation of nuclear factor-κB (NF-κB) with subsequent transcription of genes of pro-inflammatory cytokines [16].

Genes for PPRs and cytokines have a notable polymorphism in their regulatory loci that influences on expression of the encoded protein and may affect its function. The most common type of mutation of these genes is a single nucleotide polymorphism (SNP) which often leads to amino acid substitution that may influence the immune response [8, 9].

The aim of this pilot study was to investigate a possible role of SNP in heterogeneity of specific humoral immune response to the molecular microencapsulated plague vaccine (MMPV) in normal human volunteers.

MATERIALS AND METHODS

Human subjects

All volunteers signed an informed content to participate in this study. The protocol of the study PKI-00–01/2014 was approved

by the local Ethical Committee and was in agreement with Helsinki Declaration (2013). The Protocol was in compliance with the requirements of Good Clinical Practice (GCP) and with the regulations of the State Committee on Sanitary and Epidemiology Surveillance of the Russian Federation of 31.10.1996 No. 33) on the basis of permission No. 190 of the Ministry of Health of the Russian Federation regarding clinical trials of MMPV (16.04.2015). A total of 9 individuals at the age of 38–54 years (median — 44 years), six males and three females participated in the vaccination protocol. Control placebo-treated group consisted of 20 age- and sex-matched healthy volunteers. Immunologic reactivity to the antigens was estimated at the time of screening (three days before the first vaccination) and up to 90 days (or 69 days after the second administration of MMPV).

Vaccine

This study was performed as a part of clinical trial for medical application of MMPV, a lyophilized preparation consisting of two recombinant antigens: F1 (17.7 kDa), and V (37 kDa) both at the dose 25–30 μg per injection dose. The vaccine was given subcutaneously twice at an interval of 21 days. The vaccine was manufactured according to the requirements for Good manufacturing Practice (GMP).

Laboratory tests

Genetic studies were based on the DNA obtained from peripheral blood leukocytes as described [24]. The single nucleotide polymorphism of immune response genes was carried out using allele — specific primers (NPF LyTech, Moscow) and electrophoretic detection of PCR products in agarose gel. Twenty polymorphic sites of 14 genes were amplified: interleukin (*IL*) *–1β* ((T-31C), (T-511C), (G-1473C), (C-3953T); *IL2* (T-330G); *IL4* (C-589T); *IL6* (C-174G); *IL10* (C-819T, G-1082A); *IL17* (G-197A); *TNF* (G-308A); receptors *CD14* (C-159T), *FCGR2A* (G-166A), *TLR2* (G-753A), *TLR3* (G-421A), *TLR4* (G-299A), *TLR4* (C-399T), *TLR6* (C-249 E), *TLR9* (T-1237C, A2848G). The respective SNP are recorded in the NCBI dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/index.html) and have the frequency of minor alleles from 1.0 to 47 %.

For serological analysis, blood was taken from the cubital vein in the morning and serum separated. Levels of specific IgG antibodies to the F1 and the V antigens of *Y. pestis* were determined by ELISA. For that, microtiter plates (Greiner Bio-One, Austria) were coated with 100 ng/well of Caf1 or V protein in 0.1 M sodium bicarbonate buffer (pH 9.6) overnight at 4 °C. Non-specific binding was blocked with 3 % gelatin from cold water fish skin (Sigma) in 0.01 M PBS, pH 7.2. Test sera were added using 2.5-fold serial dilutions in 0.01 M PBS buffer containing 0.05 % Tween-20 (PBST) and incubated for 2 h at 37 °C. After four washes with 0.01M PBST, 100 μl of sheep anti-mouse IgG conjugated to horseradish peroxidase (GE Healthcare) at the dilution of 1:4000 was added for 1.5 h at 37 °C. The plates were washed with PBST, and 100 μl of 0.01 % o-phenylendiamine-H_2O_2 was added to each well. The reaction was stopped by the addition of 100 μl of 1 M H_2SO_4 per well, and OD was read at 450 nm using EVOLIS Twin Plus System (BIO-RAD, USA). The titer of antibodies was estimated as the maximum dilution of serum giving an OD reading that exceeded the background by 0.1. Titer 1:100 was considered as the diagnostic threshold, that is, all individuals who demonstrated positive signal in serum diluted up to 1:100 were considered negative.

Statistical analysis

Titers of specific antibodies were compared between genotype groups for each SNP studied and the differences were analyzed by non-parametric analysis of variance (ANOVA) using GraphPad Prism V software. $P<0.05$ was considered significant. Correlation between the antibody titer and the number of alleles associated with a responder phenotype was performed using linear regression analysis.

RESULTS

Six of nine vaccinated individuals (67 %) developed IgG antibodies to F1 and/or to V antigen (IgG-F1 and IgG-V respectively), and three (33 %) remained seronegative. Five volunteers (55 %) responded to F1 with diagnostic titers > 1:100 (group 1), while 4 others (44 %) remained non-responders. Analysis of SNP showed that serological responders were among individuals with heterozygosity in *TLR9* (AG), *IL6* (CT), and *IL10* (CG) genes. On the other hand, the responders were homozygous for the wild type *IL1β* allele (CC), and for the wild type *IL4* allele (CC). Carriers of the mutant allele of *TLR9* gene (GG) and heterozygotes for the *IL1β* gene (CT) appear to be potential non-responders (Figure 1 A-E). Linear regression analysis revealed a positive correlation between the number of responder alleles in an individual and the IgG titer to F1 (Fig. 1F).

Four of nine vaccinated individuals (44 %) developed antibodies to the V antigen, whereas five remained seronegative (55 %). Analysis of SNP revealed that heterozygosity in all the genes studied does not make individuals non-responsive to the antigen. Homozygosity for mutant alleles in *IL1β* and *IL4* genes (TT), and *IL6* gene (GG) was found in responders. On the other hand, homozygosity for a wild type *IL4* allele (CC) was associated with non-responsiveness to the V antigen (Fig. 2 A-E). Again, linear regression analysis revealed a positive correlation between the number of responder alleles in an individual and the IgG titer to F1 (Fig. 2F).

The analysis of genotypes of *IL1β* (T-31C, G-1473C, T-511C), *IL2* (T-330G), *IL10* (G-1082A), *IL17* (G-197A), *TNF* (G-308A), *CD14* (C-159T), *FCGR2A* (His166Arg), *TLR2* (G-753A), *TLR3* (G-421A), *TLR4* (G-299A), *TLR4* (C-399T), *TLR6* (C-249 E), *TLR9* (T-1237C) did not reveal an association of polymorphism with antibody responses to MMPV (data not shown).

DISCUSSION

Efficiency of novel plague vaccines is a matter of an outmost importance, since the disease is associated with high mortality rates. However, a proportion of vaccinated individuals does

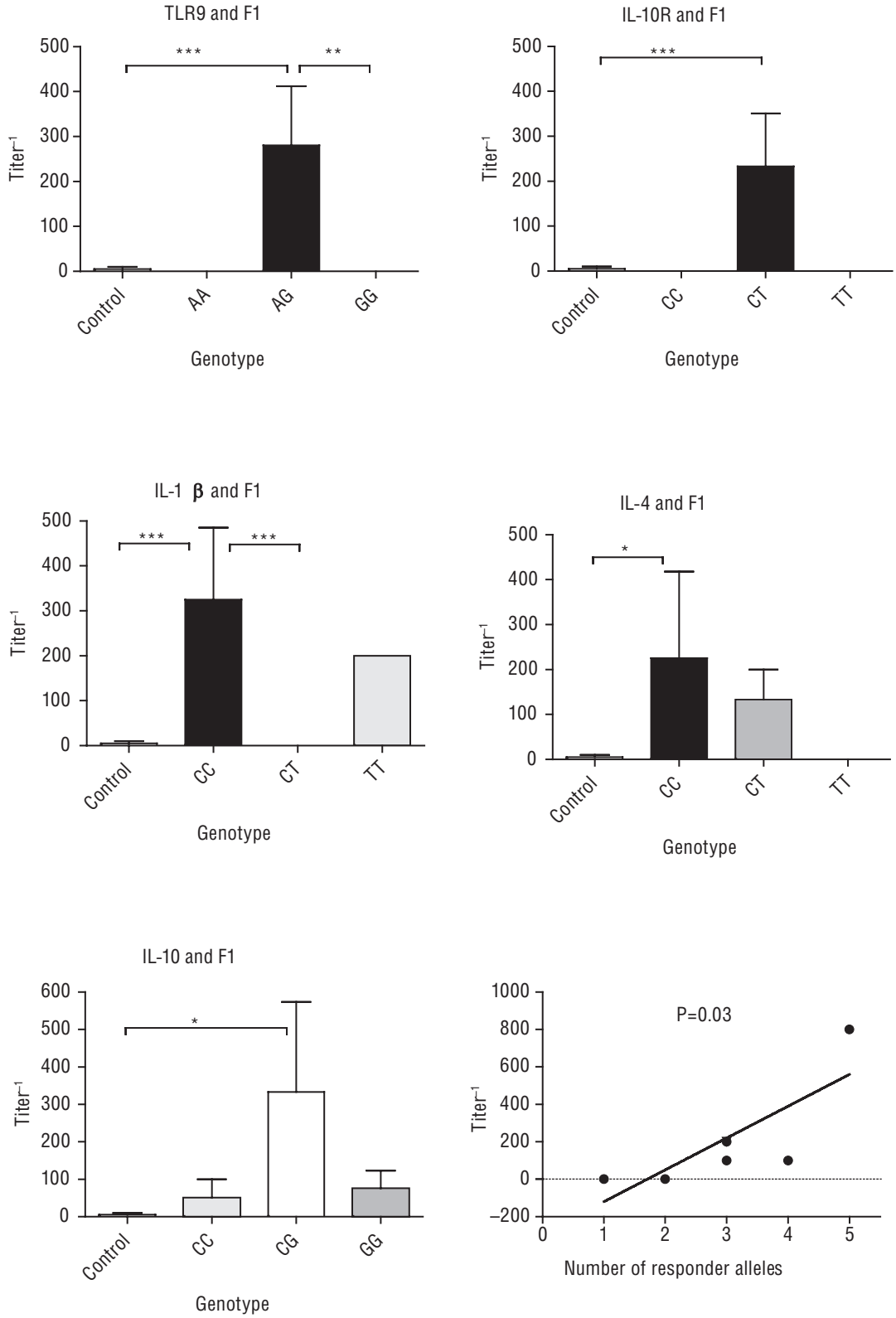

Fig. 1. Effect of SNP on antibody titers to F1 antigen 6 weeks after the second vaccination. (A) *TLR9*; (B) *IL10*; (C) *IL1β* (D) *IL4*; (E) *IL6*. IgG titers were compared by ANOVA. *P<0.05; **P<0.01; ***P<0.001. (F) Correlation of the anti-F1 IgG titer with the number of permissive alleles (alleles that permit IgG response) in individual subjects (P=0.03)

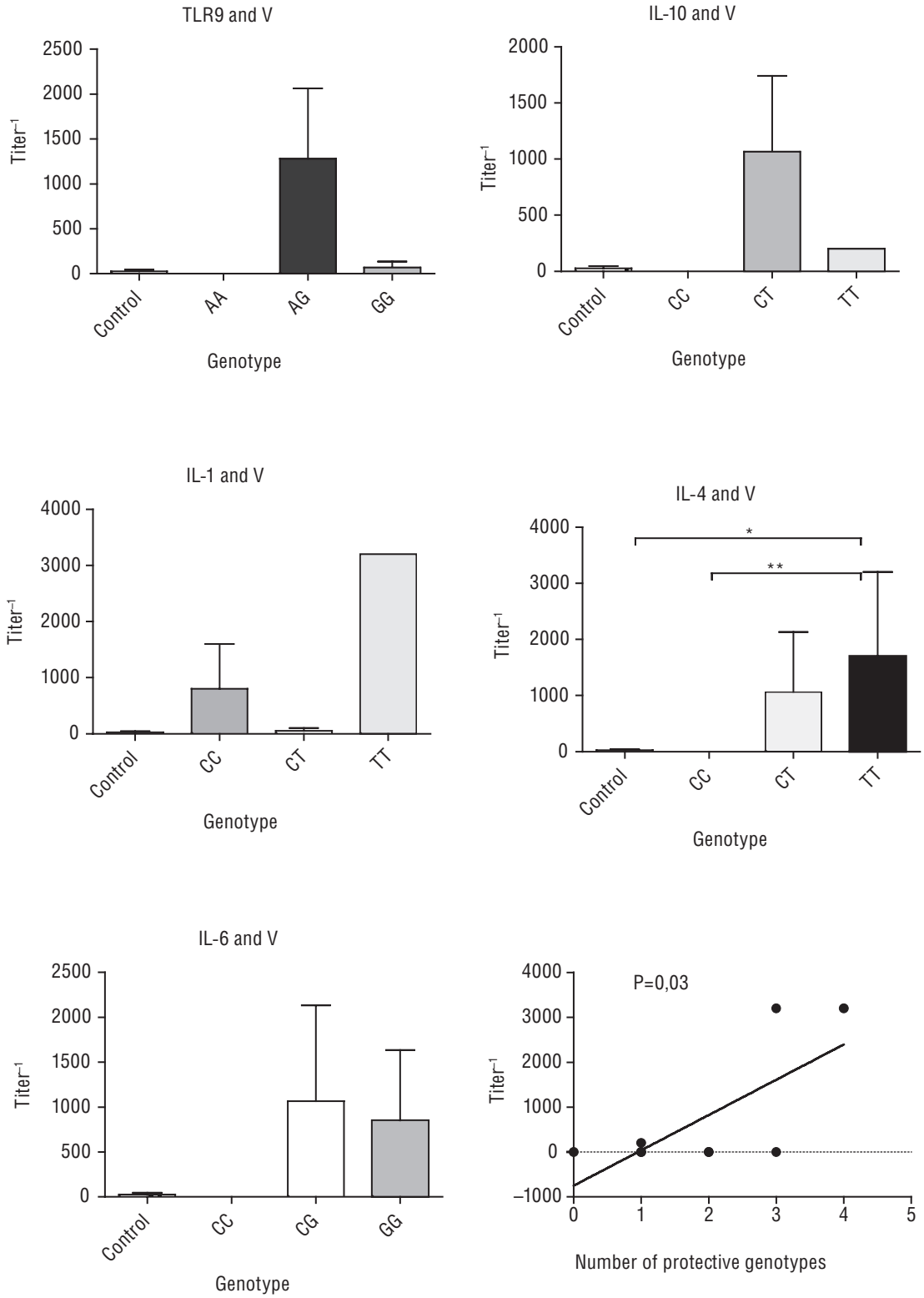

Fig. 2. Effect of SNP on antibody titers to V antigen 6 weeks after the second vaccination. (A) *TLR9*; (B) *IL10*; (C) *IL1β* (D) *IL4*; (E) *IL6*. IgG titers were compared by ANOVA. *P<0.05; **P<0.01. (F) Correlation of the anti-V IgG titer with the number of permissive alleles (alleles that permit IgG response) in individual subjects (P=0.03)

not respond to one or both F1 and V antigens, therefore, identification of such non-responders on the basis of genetic or other markers could help to avoid unnecessary vaccination, or could promote creation of an alternative vaccine. In this small pilot study we investigated SNP of a number of genes that are known for their role in immune responses. We found that five genes are involved in the strength of specific humoral immune response to MMPV, namely *IL1β, IL4, IL6, IL10*, and *TLR9*, and this involvement was associated with SNP at specific positions.

Cytokines are involved in intercellular cooperation and regulation of the immune response. They are produced mostly by activated leukocytes [1, 6, 42]. Some of them play a key role in maintaining of balance between cellular (Th1) and humoral (Th2) immune responses, where Th1 cells provide defense against intracellular pathogens, while Th2 cells — protection against extracellular agents [28]. Genes encoding cytokines and their receptors are characterized by allelic polymorphism, which may have an impact on the function of their respective products [12]. There are more than seven million SNP with a frequency of the minor allele within 5–10 %. Some of these SNP are synonymous, but others affect expression or function of the gene product. Therefore, functionally different alleles can define the risk of a poor immune response [42].

IL-1β is a pro-inflammatory cytokine synthesized mostly by the activated macrophages, keratinocytes, fibroblasts, and stimulated B-lymphocytes. The *IL1β* gene is a part of the cluster of genes located on the long arm of chromosome 2. It consists of 22 exons and nine introns. SNP of the *IL1β* gene at positions –511, —31 and +3953 are well documented [11]. *IL1β* gene wild type allele has C at the position +3953, and a mutant allele has T [30]. This mutation is associated with an excess in IL-1β production [12]. According to our data, the presence of both normal and mutant alleles in homozygosity correlates with humoral immune response to both F1 and V antigens (Fig. 1C and 2C). On the contrary, the heterozygous state was associated with a low or no response to both vaccine components. It has been shown that the structure of highly polymorphic regions of *IL1β* gene affects specific immune response to malaria [26]. A disadvantage of heterozygotes observed in our study is difficult to explain and requires further elucidation.

TLR9 is a receptor capable of prokaryotic DNA recognition due to the presence of non-methylated CpG motifs [13]. TLR9 expressing germinal center plasma cells participate in differentiation of T lymphocytes towards Th1 or Th2 subpopulations [40, 50]. Polymorphism of *TLR9* gene may influence immune response [34, 46]. Some haplotypes of *TLR9* gene are associated with a decreased level of the gene expression [48], which may result in a reduced production of immunoglobulins [29]. In our work, the heterozygotes for *TLR9* gene at the position-2848 were responders to the vaccine, whereas homozygotes for either wild type, or mutant allele were non-responders.

The *IL4* gene is located in a cluster of genes (together with *IL3, IL5, IL13* and *CSF2*) on the long arm of chromosome 5 and contains several polymorphic sites, some of which participate in regulation of IL-4 production. This locus controls Th2 responses [27]. There is evidence that certain haplotypes of *IL4* gene in the position — 590C/T up-regulate its transcription and result in hyper-production of IL-4 [17, 47]. It has been reported that SNP at this gene position influences production of antibodies to malaria [47]. Thus, *IL4* haplotype may be important for subsequent generation of Th2 cells producing various amounts of IL-4 [36]. Bartova et al. [15] have shown that *IL4* polymorphism may be important for IL-6 production.

The *IL6* gene is located on the short arm of chromosome 7 and consists of five exons and four introns. The polymorphic locus of C-174G the regulatory area of the gene is the most studied one [11]. Our data demonstrate that the presence of mutant allele (C-174G) is associated with a responder phenotype, whereas the wild type allele is not. Effect of *IL6* polymorphism on antibody response has been demonstrated. Patel *et al.* demonstrated that SNP at position 174 modulates IL-6 production in response to RCV so that in carriers of the wild type allele IL-6 secretion is inhibited. Authors concluded that RSV, perhaps, has the ability to suppress *IL6* gene transcription and the mutant allele provides an escape mechanism from this inhibition, but this phenomenon requires further studies [38]. Poland et al. showed that *IL6* polymorphism were associated with a more efficient synthesis of antibodies to hemagglutinin in response to inactivated vaccine against seasonal influenza [39].

IL-6 inhibits production of TNF and IL-1 and stimulates secretion of IL-10 [17]. IL-10 is an anti-inflammatory cytokine which inhibits synthesis of Th1 of cytokines, expression of HLA class II antigens on macrophages, increases the number of IL-4 producing T-cells and enhances survival and proliferation of B cells, and hence — antibody production. The *IL10* gene is located on the long arm of chromosome 1 and contains five exons. The most studied SNPs of this gene are G-1082A, C-819T and C-592A [29]. We found that the absence of the mutant allele at gene position 819 was associated with the responder phenotype to both antigens. Polymorphism of *IL10* gene acts at the level of gene transcription and subsequent protein biosynthesis [11]. It has been reported that polymorphism at the position 1082 of *IL10* gene was associated with variability of humoral immune response to EBV [25].

The strategy of creating anti-plague vaccines was focused mostly on induction of protective antibodies specific to *Y. pestis* targets [3, 44, 45, 49]. The important role of humoral immune response has been demonstrated by passive transfer of immunity with sera from recovered or vaccinated animals, and also by monoclonal antibodies, specific to F1 and V [51]. Unfortunately, the majority of these and relevant studies have an experimental character and their conclusions are based on mouse models [32, 37, 51, 54].

Anti-plague immunity in humans also depends on both humoral and cellular immune responses to other antigens (for example, Yop) which are not a part of these subunit vaccines [31, 54]. Participation of specific CD4+ and CD8 + T-lymphocytes [25, 38], and subpopulations of Th17 and Th1 cells that may attenuate

or enhance vaccines [32] should be taken into consideration for creation of better vaccine in the future.

Our study pores light on the possibility that genetic pre-screening may allow for selection of human individuals that would likely respond to the F1/V subunit MMPV vaccination by mounting a specific humoral protective immunity. Humans that fall into the group of non-responders would benefit from an alternative method of protection, including an alternative vaccine, to be designed yet. If our findings hold true in a study of a larger cohort, we could recommend genotyping candidate individuals for SNP in genes of *IL1β* (C-3953T), *IL4* (C-589T), *IL6* (C-174G), *IL10* (C-819T), and *TLR9* (A2848G). In addition, we would suggest that individuals with non-responder genotype should be included in the future clinical trials assessing novel, universally effective plague vaccines.

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