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Original Article

The Role of Levamisole and HIV-1 Nef-p24 Fusion Protein in *IL-4* Gene Expression for Evaluating Humoral Immune Response

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Abstract

Introduction: Acquired immunodeficiency syndrome (AIDS) is one of the most common infectious diseases in the world. It is transmitted via the Human Immunodeficiency Virus (HIV-1). So, a HIV-1 vaccine should be effective in the prevention of virus infection and induces immune responses. The main aim of this study was to evaluate the humoral immune induction through measuring the expression of interleukin4 (IL-4) in response to levamisole, as an adjuvant, with the HIV-1 Nef-p24 fusion protein as an immunogenic sequence.

Materials and Methods: In this study, 56 BalB/c female mice, aged 6 to 8 weeks were divided into 4 groups. There were 14 mice in each group. Prime and Booster injections were arranged in these groups receiving PBS, levamisole, Nef-p24, and Nef-p24 along with levamisole (Nef-p24/levamisole). All injections were performed peritoneally.

Results: Real-time PCR results showed that *IL-4* transcripts level increased significantly ($P < 0.05$) in boostered groups, receiving levamisole, Nef-p24, and Nef-p24/levamisole compared to primed groups. However, the results of ELISA revealed the enhancement in IL-4 expression in levamisole primed groups in comparison to Nef-p24/levamisole boostered group.

Conclusion: The results of the present study showed that the HIV-1 Nef-p24 fusion protein and levamisole could be considered as effective candidates as to increase the expression of IL-4 which may stimulate the humoral immune response.

Keywords: ELISA, HIV-1 Nef-p24 fusion protein, Immune response, IL-4, Levamisole, Real time

1. Introduction

Acquired immunodeficiency syndrome (AIDS), one of the most common infectious diseases in human history, is caused by Human Immunodeficiency Virus (HIV) [1]. The World Health Organization estimates that about 36.7 million people are living with HIV in 2015 and 1.1 million people lost their lives for that cause [2]. UNAIDS (United Nations Programme on HIV/AID) has adopted determined targets which are to identify HIV-infected population, and to provide access to Antiretroviral Therapy (ART) for infected the population and viral suppression for the population using ART [3]. Also Highly Activated Antiretroviral Virus medication (HAART) has developed quickly; transforming the HIV-1 into a controllable chronic infection [4]. While global commitment to manage the HIV/AIDS epidemic has increased considerably in recent years, the virus continues to spread with alarming and increasing speed. During the past century,

vaccination has turned to be the most effective medical method in reducing the death rate caused by viral factors [5, 6]. Although a vast range of alternative potential vaccination is available for avoidance and therapy, development of HIV vaccination strategy is still considered to be a serious scientific challenge [7]. On the other hand, the development of a vaccine for HIV has proved to be very difficult, partly because of the complex nature of the virus [8]. HIV is highly adaptable because of its high mutation rate and there are multiple strains belonging to a number of different clades [9], so it is probable that a multi-component vaccine comprising several proteins or peptides will be required to invoke broad and powerful immunity. Subunit vaccines offer the advantage of targeting specific epitopes that lie within conserved areas of the virus. Two potential antigens, the 24-kDa capsid protein p24 and the 31-kDa regulatory protein Nef, are the focus of this study. P24 sequence with 159-173 amino acids plays an important role in HIV control through inducing appropriate cellular immune response. Nef sequence protein with 102-107 amino acids as a regulator protein plays a vital role in virus replication and pathogenicity. According to the available studies, it is expected that a combination of these two proteins acts as a strong immunogenic sequence in designing recombinant HIV vaccines. [10-16]. Moreover, Interleukin 4 (IL-4) is a cytokine that participates in the regulation of the immune system at multiple levels [13]. The ability of cytokines to influence HIV-1 propagation has been studied extensively. Levamisole is a synthetic, orally active agent that has anthelmintic and immunomodulatory properties. Adjuvants are chemical or biological compounds which induce a non-specific immune system against antigen/s that are injected along with the compound. Reducing the adverse effects of vaccination and stimulating a specific type of immunity leads to a vast and novel development of adjuvants [11]. Adding an adjuvant into a vaccine antigen shows several advantages such as dose reduction and faster response induction [12]. The purpose of current research is to evaluate the IL-4 gene expression after immunization through Nef-p24 immunogenic peptide sequences along with levamisole adjuvant.

2. Materials and Methods

2.1 Study Design

This study was a true experimental research in which the posttest-only control group design was selected. All experiments were done in summer of 2018 in Genetics department of Tehran medical Sciences, Islamic Azad University, Iran.

2.2 Preparing Adjuvant and Recombinant HIV-1 Nef-p24 Fusion Protein

The considered recombinant fusion protein for this research consisted of Nef immunogenic epitope and p24 from HIV-1 virus. The sequences of these proteins were found in PDB site according to a similar study [13]. PDB ID for NEF and p24 were 2 NEF and 4 XFX respectively. By putting together the sequences, the following final sequence was obtained:

HSQRRQDILDLWIYHTVEEKAFSPEVI PMFS

The recombinant protein with a length of 31 amino acids and 95% purity was purchased from Takapouzist Company, eventually. Levamisole adjuvant with 99% purity was purchased from Pursina Pharmaceutical Company. To prepare the immunogenic sequence with the candidate adjuvant, recombinant HIV-1 Nef-p24 fusion protein was mixed with levamisole adjuvant. The mixture was shaken for an hour on a shaker. For every 200 µL of levamisole, 2µgr of candidate recombinant protein was applied [17, 18].

2.3 Classification and Laboratory Animal Treatment

Fifty-six BalB/c female mice were purchased from Karaj Pasteur Institute. They were kept in an animal laboratory in 36°C and a suitable air conditioned area. Health, having the age between 6-8 weeks and equal weight of 25gr for mice were considered as study inclusion criteria. Mice which were not injected correctly or had failed blood sampling, were excluded from the experiment.

Considered mice were classified into four main groups for the study. Each group consisted of 14 mice. The first group received Nef-p24 immunogenic sequence with adjuvant (Nef-p24/levamisole). The second group received Nef-p24 immunogenic sequence without adjuvant. The third and fourth group received only adjuvant and PBS, respectively. All the mice were put inside the separate cages. The final volume of injection and vaccination dose were 50µL and 20µg, respectively.

Injection schedule took place in two shifts for each mice. Fourteen days after prime injection, booster injection was done for half of the mice population using peritoneum hypodermic injection. 14 days after the first and the second injection, the mice were gone under an operation to evaluate the immune response, respectively. They first were comatose using chloroform. To obtain more peripheral blood, a sampling syringe was used to collect blood from animal`s heart.

2.4 ELISA

Enzyme-Linked Immunosorbent Assay (ELISA) is a commonly used analytical biochemistry assay. ELISA is done based on the antibody level produced in virus-infected persons. Here, the method of Sandwich ELISA was applied to analyze IL-4 protein expression, using anti IL-4 antibodies labeled with biotin. The purified sample used in ELISA was treated based on the Bio Assay kit`s instructions. At final stage, sample OD was read at 450 nm [13].

2.5 Real Time PCR

RNA was extracted from peripheral blood using a RNX PLUS kit (Sina Gene Company). The extraction was exactly done according to the kit protocol and samples OD were measured using a Nano drop device. Then, cDNA was synthesize by 10 µL of RNA using Easy cDNA Synthesis Kit. The protocol was followed precisely.

To analyze *IL-4* gene expression level, first *IL-4* and *GAPDH* (as a positive control) gene mRNA sequences were obtained from NCBI. Then, the intended primer was designed based on the required standards using Primer3 software. To identify the homolog parts with intended products, certain primers were searched in BLAST and NCBI to have the product of specific target gene. Designed primers were purchased from Sina Clone Company (Table 1). For Real Time PCR procedure, primers and cDNA were mixed based on the kit's procedure. Bioneer device was applied for Real-Time PCR and the intended schedule was regulated according to Table 2.

Table 1. *GAPDH* and *IL-4* primers sequence

	Length	Sequence
Interlokin-4 FWR	20	AACGAGGTCACAGGAGAAGG
Interlokin-4 REV	20	TCTGCAGCTCCATGAGAACA
GAPDH FWR	20	GAAGGTGAAGGTCGGAGTGA
GAPDH REV	20	AATGAAGGGGTCATTGATGG

Table2. Real-Time PCR reaction temperature and scheduling

Steps	Temperature	Time
Denaturation and enzyme activation	94	10'
Step1: Denaturation	94	1'
Step2: Annealing	56	20"
Step3: Extension & fluorescence acquiring	72	10"
Melting curve analysis	95-67	1 s/degree

2.6 Statistical Analysis

The general trial method of the study was done in 7 phases. Data were examined using the SPSS software ver. 24. Analysis of variance (ANOVA) and Tukey's tests ($P \leq 0.05$) of mean comparison were applied to show statistical differences between means. Gene expression analysis was done based on sample`s OD and Ct, obtained from the Real-Time PCR.

3. Results

Real Time PCR

To ensure the accuracy of the primers, a PCR was done and one specified band was observed (Figure 1A). The amplification plot of *IL-4* and *GAPDH* is shown in Figure 1B. In addition the specificity of the primers used in Real-Time PCR was determined through melting curve analysis (Figure 1C).

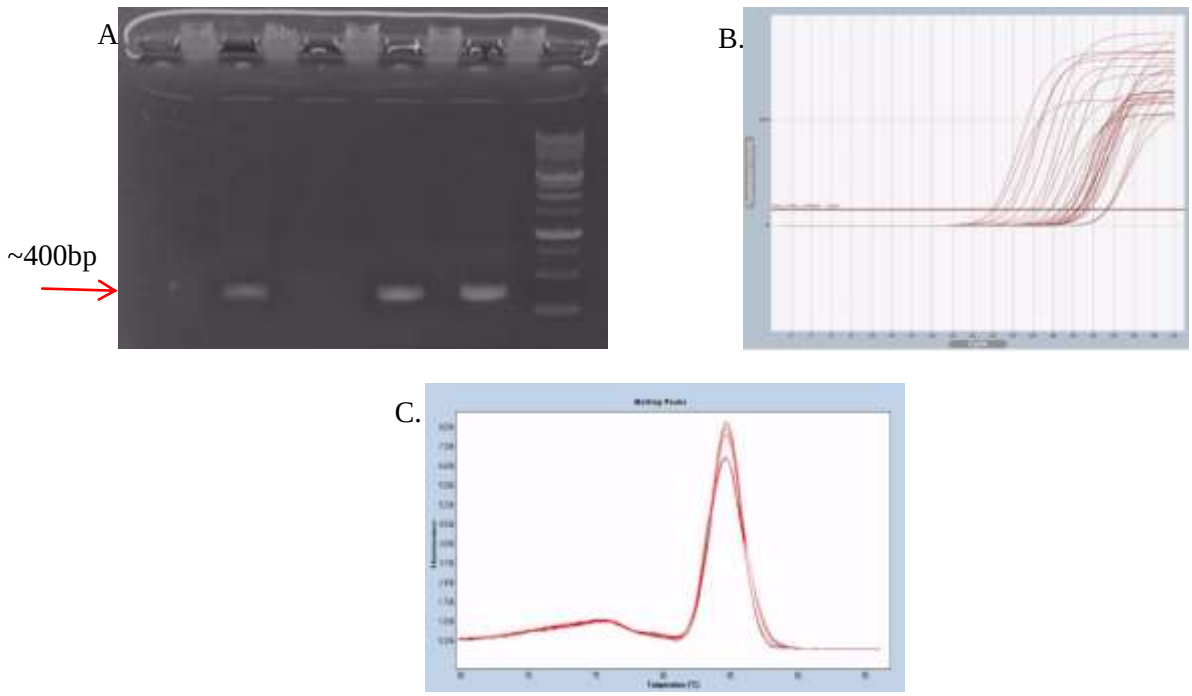

Figure1. Real time PCR results. A. PCR gel electrophoresis of IL-4 gene expression. B. IL-4 and GAPDH amplification plot. C Melting curve analysis of IL-4 gene.

Expression of IL-4 Gene Transcripts

A significant increase in *IL-4* transcripts level was observed in all boostered groups in comparison with primed groups (Figure 2). A remarkable difference was shown in transcription of *IL-4* in the groups receiving levamisole, Nef-p24 and Nef-p24/levamisole in their second round of

injection. In addition, among the boostered groups, *IL-4* transcripts level reached the highest level in the Nef-p24 group (Figure 2).

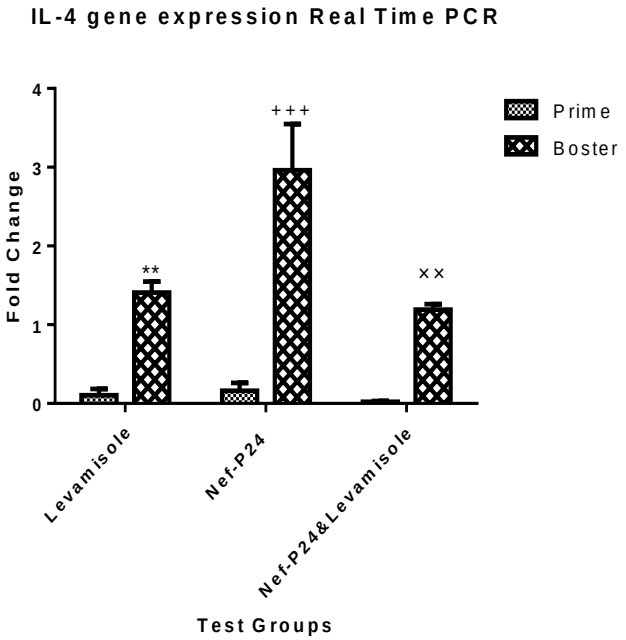

Figure 2. Comparison of IL-4 gene transcripts expression between primed and boostered groups receiving levamisole, Nef-p24 and Nef-p24/levamisole in BalB/c female mice based on the control group.
All data are presented as the means ± SD with n = 3. **, +++ and ×× show significant difference at $P \leq 0.05$.

ELISA

Data analysis showed the significant difference in IL-4 expression in the first injected (primed) mice; between group receiving levamisole (OD=0.48) and control group (OD=0.97) (Figure 3). In fact, IL-4 protein expression decreased remarkably in group receiving levamisole in comparison to the control ($P<0.05$) (×). There was no significant difference in IL-4 expression between groups receiving Nef-P24/levamisole (OD=0.95), and Nef-p24 (OD=0.81) in the primed injection.

Data comparison between prime and booster injected mice showed a significant increase in IL-4 expression level in the primed control, compared to boostered group receiving levamisole (OD=0.71) ($P<0.001$) (+++). Furthermore, a notable enhancement in IL-4 expression was observed in primed group receiving levamisole in comparison with boostered receiving Nef-p24/levamisole (OD=0.16) ($P<0.001$) (***).

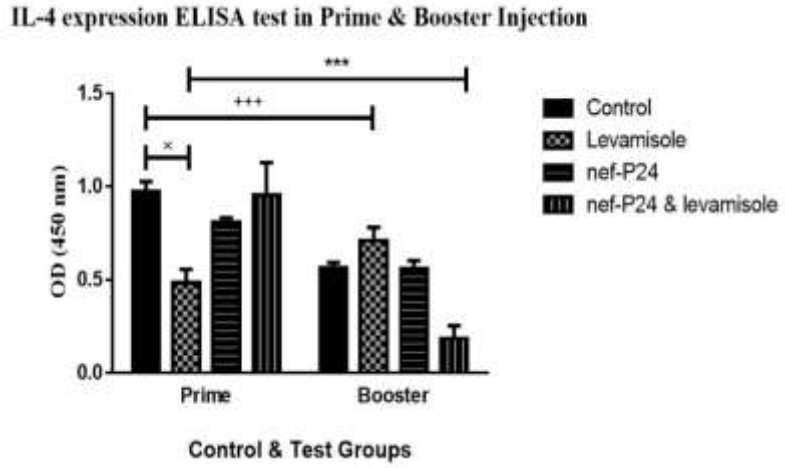

Figure 3. Comparison of IL-4 expression level by ELIZA between primed and boostered groups receiving levamisole, Nef-p24 and Nef-p24/levamisole in BalB/c female mice based on the control group. All data are presented as the means ± SD with n = 3. ×, and +++ and *** show significant difference at P≤ 0.05 and P<0.001, respectively.

4. Discussion

Since HIV viral factor has been discovered, several amino acid sequences have been proposed as candidate vaccines. In the present study, recombinant HIV-1 Nef-p24 fusion protein was used along with levamisole adjuvant to assess the immune response in mice by *IL-4* gene expression. Therefore, Real-Time PCR and ELISA test was applied to evaluate the immune response. The results of Real Time PCR showed a significant enhancement in *IL-4* transcripts expression in boostered groups receiving levamisole, Nef-p24 and Nef-p24/levamisole, compared to primed groups. However, the results of ELISA cleared the increase in IL-4 expression in levamisole primed groups in comparison to Nef-p24/levamisole boostered group. It was shown that IL-4 as a cytokine takes part in the immune system regulation at multiple levels [13].

In the present study, the Nef-p24fusion protein was used as an immunogenic sequence. Investigating of the immune response produced against each of these proteins alone indicated that they could induce both cellular and humoral immune responses [13]. In a study accomplished by Gavioli et al. (2008), Tat protein along with complete Freund`s adjuvant was used in the first stage and incomplete Freund`s adjuvant was used at booster stage. Results showed that the adjuvanted fusion peptide induced cytokines interferon gamma (IFN-γ) and IL-4 in the Th1 pattern and increased level of cellular immunity [19]. Another research was done on surveying Gag, Pol and Nef protein in combination with equal amount of adjuvant and without any adjuvant. The improved cellular immune response was evaluated through IFN-γ level induction by T-CD4 and T-CD8 cells. [20]. Moreover, Kang et al. (2018) studied ENV protein along with gp140 adjuvant. Enhanced Humoral immunity was evaluated using ELISA test for measuring IgG and IgA [21]. Mahdavi et al. (2010) conducted a trial on immunized BALB/c mice with a pure recombinant peptide Gag p24-Nef. Results displayed a significant increase in humoral immunity level by inducing IgG2a antibody, and in cellular immunity by cytokines IFN-γ and IL-4 [13]. In addition, specified immune responses were stimulated in mice when co-immunizing with LIGHT (member of TNF family) expression plasmids and HIV-1 nef DNA vaccine plasmids. It was suggested that Nef and LIGHT can increase the humoral immunity level [22].

Also, a research on effectiveness of p24, Nef, and p17 as a fusion protein along with AS01B adjuvant was done. Results

illustrated an increase in both humoral and cellular immunity. In this trial, INF-γ was measured using ELISA test [23]. It was shown that Nef-p24 is a candidate as a constituent of a vaccine provided by oral boosting, following subcutaneous priming by injection of Nef and/or p24 [24]. Moreover, Nef peptide and 1MVA vaccine showed a strong response in immune system. It was proved that Nef-p24 has a high level of immunization. In this research, IFN-γ, was measured using ELISA [25]. In a study a nef expressing DNA was injected to mice and GM-CSF cytokine was used to increase the immune response. Results represented a severe immune response towards the Nef through raised level of *IL-2* and *IFN-γ* [26].

It has been noticed that the ratio of IgG2a/IgG1 would increase in the adjuvanted Nef-p24 immunization group, which verified the Th1 profile of immune response in this group [13, 27, 28]. Structural proteins, such as p24, are good candidates for vaccine components because of their high conservation [14]. Nef, is an indispensable early post-infection regulatory protein. Researches in non-human primates have shown that vaccine induced cytotoxic T-lymphocyte responses against early proteins, such as Nef, provide a degree of protection against pathogenic virus challenges [14, 18].

On the other hand, in this research levamisole was studied as the first adjuvant in HIV-1 studies stimulating humoral immunity system. In some researches levamisole was used as an adjuvant along with DNA vaccine and hepatitis B vaccine to increase the immunity level in tumor cells and patients suffering AIDS (50mg twice a day), respectively [17, 29]. However, Cazella et al. (2009) conducted a trial in which application of levamisole (6.0 mg/kg of live weight) in rabies-primed vaccines did not affect the humoral immune system [30]. In another research levamisole and hepatitis B vaccine were applied in oral form. An enhancement of immunity response in the group receiving levamisole was obtained. In this study, antibody titer was used to measure the immunity level [31]. A research done on Salmon showed a different result of levamisole. Although levamisole adjuvanted vaccine induced immune response in fish, this response was still less than that of the group with no adjuvant [32]. These results indicate that levamisole as an adjuvant has a restricted range of efficacy. It was shown that levamisole triggers dendritic cells by binding to Toll-like receptor- (TLR-) 2. This adjuvant induces Th1 immune response and stimulates production of IL-12 [17].

In the present study, since there was a limitation to investigate different doses of Nef-p24 and levamisole, it is suggested to assess their impact on immune system to find their optimum concentrations. Moreover, it is necessary to analyze humoral immune responses to Nef-p24 and levamisole, by expression of other involved cytokines. On the other hand, evaluation of cellular immune responses to Nef-p24 immunogenic sequence and levamisole along with the presented results might introduce them as a new vaccine candidate for HIV-1.

5. Conclusion

The results of the present study showed that HIV-1 Nef-p24 fusion protein and levamisole adjuvant, enhanced IL-4 expression in the injected mice. In fact, our results as well as previous studies revealed that Nef-p24 along with levamisole could be considered as effective candidates to induce the humoral immune response by increasing the expression of IL-4. It seems that further investigations focused at other aspects of immune responses are needed.

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Conflict of interest

The authors declare no conflict of interest.

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