

Adjuvant Effect of Dendritic Cells Activator Imiquimod in Genetic Immunization with HIV-1 p55 Gag

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Received date: 07-Dec-2022, Manuscript No. JIDIT-22-82135;

Editor assigned date: 09-Dec-2022, PreQC No. JIDIT-22-82135 (PQ);

Reviewed date: 23-Dec-2022, QC No. JIDIT-22-82135;

Revised date: 30-Dec-2022, Manuscript No. JIDIT-22-82135 (R);

Published date: 10-Jan-2023, DOI: 10.4172/2329-9541.1000330.

Abstract

Dendritic Cells (DC) are important antigen-presenting cells that have abilities to induce and maintain T-cell immunity, or attenuate it during hyperimmunization. Additional activation of DCs may be useful for vaccination purposes. Imiquimod is known to be a specific agonist of the Toll-Like Receptors (TLR7), which are located mainly on DCs. To study the effect of DC stimulation on the effectiveness of an HIV-1 p55 gag DNA vaccine in mice model, we employed 25, 50 and 100 nM of imiquimod as an adjuvant. Subsequently, western blot analysis was used to quantify p55 protein production after the immunization. To characterize T-cells immune response, both the frequency of IFN- γ -secreting cells and IFN- γ and IL-4 production were measured, *via* an ELISpot assay and ELISA, respectively. Low concentrations of imiquimod were found to effectively stimulate Gag production and the magnitude of the T-cell immune response, whereas higher concentrations reduced vaccination effects. Our results show that the adjuvant effects of imiquimod depend on concentration. The use of imiquimod may be helpful to study DC to T cell communication, including possible induction of immunotolerance.

Keywords: Imiquimod; Dendritic cells; TLR7; p55 Gag; T-Cells; Cellular immunity

Introduction

Human Immunodeficiency Virus (HIV) is an enveloped RNA virus responsible for Acquired Immunodeficiency Syndrome (AIDS). HIV continues to be a major global public health problem, and according to UNAIDS to date has claimed more than 36.3 million lives. In 2020, one million people died from HIV-related causes worldwide (World Health Organization).

Currently, despite adverse effects caused by infection, the introduction of highly effective antiretroviral therapy, known as Combined Antiretroviral Therapy (cART), has drastically improved the quality of life and life expectancy of people living with HIV [1]. Although cART reduces the viral load significantly, this approach doesn't cure the disease completely. Unfortunately, after the removal of constant anti-retroviral therapy the HIV viral load comes back. The eradication of this pandemic would require novel antiretroviral combinations capable of a complete "sterilizing cure" of the infection or the introduction of a pre-exposure HIV vaccine, which could potentially prevent infection and/or help control the viral load after infection. On the other hand, the development of post-exposure vaccines could potentially improve immune response in patients that have already been infected. An effective immune response to HIV is possible because it exists in "elite controllers", which are people who live with HIV for many years without any immune system damage and with very low viral loads by maintaining strong virus-specific T cell-mediated immune responses.

Without vaccination, some blood cells act as reservoirs for HIV-1 and 'dose' T cells with the virus over extended periods, eventually reducing the immune response. It is known that the cells responsible for HIV viral latency are mainly CD4 T cells and myeloid dendritic cells [2,3]. Thus, a number of post-exposure vaccine clinical trials, where DCs are exposed *ex vivo* with HIV antigens and then re-introduced into the HIV-positive individual to elicit a protective immune response have been reported [4]. Intrigued by this approach, we decided to try another tactic then DC cells became pre-activated *in-vivo* before HIV vaccination using specific adjuvants that preferentially activate DC.

One of the substances with a property to modulate DC is imiquimod. It is an imidazoquinoline amine with immune-response modifier properties that has shown adjuvant effectiveness in herpes simplex virus therapy, which is essentially a post-exposure vaccine [5]. Topical imiquimod alone was used against non-melanoma skin neoplasia and has been shown to have broad anticancer properties [6-9]. It has also been used as an adjuvant and has been shown to elicit humoral and cellular responses in melanoma patients immunized with anti-cancer peptides [10,11]. Adjuvant effect of topical imiquimod for HIV vaccine was also shown in a mouse model while the imiquimode/montanide mix was used to boost adenovirus ChAdV63. HIVconsv vaccine in macaques [12,13]. Resiquimod, an imiquimod analog, was used as an adjuvant to an HIV-1 gag DNA vaccine in mouse models and was reported to enhance IFN- γ production, increase antibody titers, and promote a strong Th1 response in comparison to HIV-1 gag-based genetic immunization alone [14]. As an adjuvant, imiquimod induces

the expression of several Th1 cytokines including IFN- α , IL-12 mainly produced by DCs, and IFN- γ through the activation of the Toll-Like Receptor-7 (TLR7) through the MyD88 pathway [15,16]. Most interestingly, imiquimod can specifically pre-activate DCs, inducing local DC maturation, because expression of TLR7 is predominant in human and murine dendritic cells [17]. The selective activation of TLR7 for the modulation of adaptive immune responses to vaccines is one of our research interests.

Genetic immunization with vaccines is an approach considered to elicit both humoral and cell-mediated immune responses against HIV. DNA vaccines usually use small circular plasmids. While both DNA and RNA vaccines are cost-effective and are under development, RNA vaccines are unstable at room temperature. Therefore, DNA vaccines are more promising for immunization, especially in settings with low resources. Genetic vaccines are generally safe since their nucleic material codes only a few proteins, reducing the risks associated with the presence of complete viral particles. Antigens generated after DNA vaccination contain the same post-translational modifications produced after a real infection because the host produces the viral proteins. DNA vaccines have been tested safely in clinical trials without adverse events however, DNA vaccines do not induce strong immune responses in large animals/humans [18,19]. For this reason, DNA vaccination requires a vaccine adjuvant capable of promoting a stronger immune response. Conventional vaccine adjuvants including alum particles and MF59 emulsions have been shown to improve the immunogenicity of DNA vaccines when mixed with plasmids, stimulating cytokine production [20,21]. Some adjuvants can pre-activate particular immune cells making them especially responsive to the vaccination. CpG oligonucleotides that activate TLR9 have been shown to significantly increase the T-cellular response when used as adjuvants for DNA vaccines [22].

Human immunodeficiency virus type-1 (HIV-1) encodes polypeptides called Gag, which are important during the late phase of the HIV-1 infection when Gag proteins are transported to the Plasma Membrane (PM) for virion assembly. All Gag proteins are cleaved from a precursor protein called p55. In this study, we tested the production of immunogenic p55 protein as a result of HIV-1 gag DNA vaccination accompanied with different doses of imiquimod (TLR7 activator) as an adjuvant in BALB/c mice. We hypothesized that the use of an imiquimod-adjuvanted DNA vaccine will induce considerable production of HIV-1 p55 Gag protein in contrast to genetic immunization alone.

Materials and Methods

Animal handling

Four to six week old female BALB/c mice were purchased from Charles River (Wilmington, MA, USA). All animal experiments were performed according to the National Institute of Health guidelines (Bethesda, MD, USA). The protocol was also approved by the Universidad Central del Caribe Institutional Care and Use Committee (IACUC) (Approval #041-2014-1100PHA). After vaccination, mice were anesthetized by intraperitoneal injection with a mixture of ketamine and xylazine, and euthanized *via* cervical dislocation prior to analysis (Figure 1). This method is consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association.

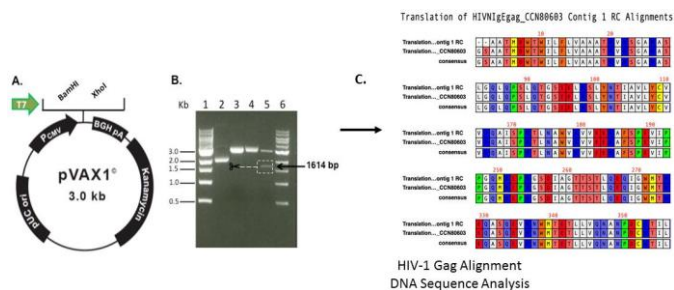


Figure 1: (A) Schematic representation of the vector and the determination of their genetic integrity before and after immunization. (B) Enzymatic digestion of cloned vaccinia constructs on pVax vector with BamHI and XhoI. Lanes: 1-1 Kb DNA ladder; 2-pVax uncut; 3-pVax cut; 4-HIV-1 Gag uncut; 5-HIV-1 Gag cut; 6-1 Kb DNA ladder. HIV-1 Gag DNA sequence analysis with PCR. Abbreviations: pUC origin–insert from high-copy-number plasmid pUC allowing high expression, Pcmv–Promoter from Cytomegalovirus (CMV), BGH pA–Bovine Growth Hormone (BGH) polyadenylation termination sequence for protein expression in eukaryotic cells, T7–bacteriophage T7 RNA polymerase control system, Kanamycin–Kanamycin resistance cassette.

Immunizations schedule

Adult female BALB/c (15-20g) mice were used (n=28 animals divided into groups of 4 mice). Vaccines were formulated at a 100 μ g/ μ L concentration of DNA in PBS (pH 7.4) plus imiquimod and were administered by intramuscular injection to the quadriceps muscle of both hind limbs, three times at two-week intervals. Three doses of imiquimod (25nM, 50nM, and 100nM) were assessed (Invivogen, San Diego, CA). The doses were administered in a total volume of 100 μ L (50 μ L per limb). To eliminate any anti-Gag response induced by the backbone effect, the control mice were injected with pVax1, and the mice in the naive group were injected with PBS (Figure 2). On day 35, animals were sacrificed and tissue (spleen and muscle from their back limbs) were collected for further analysis.

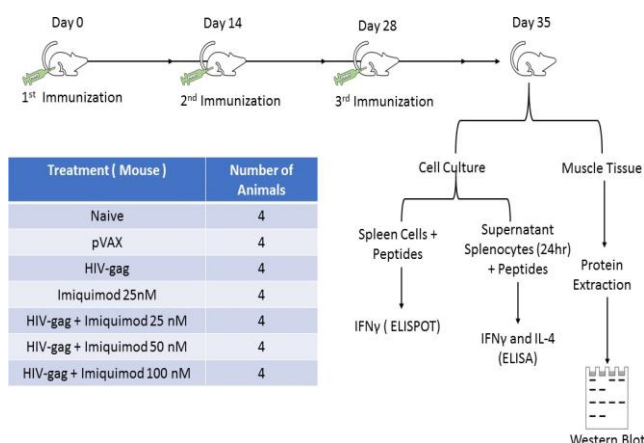


Figure 2: The vaccine formulation consisted of recombinant HIV-1 Gag DNA plasmid mixed with serial doses of the adjuvant (imiquimod). Vaccines were administered *via* intramuscular immunizations every two weeks, to identify the optimal concentration of imiquimod. After immunization, mice were sacrificed and tissue samples were collected.

DNA plasmids

We used a modified chimera plasmid pVax1 vector vaccine construct (both the construct and the original pVax1 (parent plasmid without Gag) were gifts from Dr. Miguel Otero), which includes the mammal-RNA-optimized truncated HIV-1 p55-gag gene DNA. It encodes a secreted form of the HIV-1 p55 protein, an IgE secretion sequence, and a Kozak motif to enhance Gag secretion and expression (Figure 1). Before the immunization, we transformed the p55 construct in *E. coli* Top 10 (Invitrogen, Grand Island, NY, USA) for expansion and purified the p55-Gag-HIV plasmid using the Qiagen Plasmid Giga Kit (Germantown, MD, USA).

Evaluation of cellular immune responses

To characterize the T-cell immune IFN- γ response, the protocol used for cytokine specific murine enzyme-linked immunospot (ELISPOT) assays was followed as indicated in the literature [23,24]. The synthetic peptides used in this study were derived from the sequence of the Gag protein and synthesized as 3-mer overlapped 15-mer amino acid peptides by JPT Peptide Technologies (Berlin, Germany). A mix was prepared as an equal weight peptide pool, diluted to a peptide concentration of 0.4 mg/mL in sodium carbonate buffer (0.05M, pH 9.5–9.6), and stored at -20° C.

High-Protein Binding IP 96-well Multiscreen TM plates (Millipore, Bedford, MA, USA) were coated with anti-mouse IFN- γ antibody (R&D Systems, Minneapolis, MN, USA) by overnight incubation at 4°C. The plates were washed and blocked with 1% BSA. Then, 2×10^5 spleen cells were added to each well in complete medium, and stimulated overnight with pooled peptides (JPT Peptide Technologies, Berlin, Germany) at 37°C, 5% CO₂. Concanavalin A (Con A, 5 mg/mL; Sigma-Aldrich, St. Louis MO, USA) and media were used as positive and negative controls, respectively. After 24 hours of stimulation, the plates were washed and incubated overnight at 4°C in the presence of biotinylated anti-mouse IFN- γ antibody. The next day, plates were washed and streptavidin-alkaline phosphatase was added to each well followed by two-hour incubation at room temperature. The plates were washed again and 5-Bromo-4-Chloro-3' Indolylphosphate p-Toluidine Salt (BCIP) and Nitro Blue Tetrazololium Chloride (NBT)(R and D Systems, Minneapolis, MN) was added to each well for 30 minutes at room temperature. Subsequently, plates were rinsed with distilled water and dried at room temperature. Spots were quantified by an automated ELISPOT reader system (CTL analyzers, Cleveland OH, USA) with the ImmunoSpot software. The mean number of spots from triplicate wells was adjusted to 1×10^6 splenocytes. Antigen-specific responses to IFN- γ were obtained after subtracting the number of spots formed in the wells containing the control medium from the spots formed in response to the peptide pool. ELISPOT data are expressed as mean \pm standard error of the mean.

Evaluation of T-cell-specific cytokine profile

T-cell-specific cytokine profiles were determined by ELISA, using the commercially available Quantikine Mouse IFN- γ and IL-4 immunoassays, following the manufacturer's protocol (R&D Systems, Minneapolis, MN, USA). Briefly, the mouse splenocytes supernatant samples collected from cultured splenocytes after 24 hours were incubated in a 96-well plate at 2×10^5 cells/well in complete medium, and stimulated overnight with pooled peptides at 37°C, 5% CO₂. Concanavalin A (Con A, 5 mg/mL; Sigma-Aldrich, St. Louis MO, USA) and media were used as positive and negative controls, respectively.

Then, 50 μ L of each sample supernatant was transferred to another plate and incubated with 50 μ L of Assay Diluent for 2 hours at room temperature. After washing, 100 μ L of Mouse IFN- γ or IL-4 conjugate were added to each well, incubated for 2 hours at room temperature, and washed again. Then, 100 μ L of Substrate Solution was added to each well and incubated for 30 min at room temperature. After stopping the reaction, the optical density of each well was determined by a microplate reader Wallac 1420 Victor 2 Microplate Reader (PerkinElmer Inc., Waltham, MA, USA) at 450 nm. The concentration of each sample was obtained after correlating with a standard curve.

Determining protein HIVp55 vector expression using western blot assay

The muscle tissue was collected in radioimmunoprecipitation assay buffer containing 1% Igepal CA-630, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS (Invitrogen), 50 mM Tris (BioRad) pH 8.0 and a cocktail of protease inhibitors. Insoluble cell debris was removed by centrifugation for 5 min at 12,000 rpm, 4°C. The concentration of soluble protein in the supernatant was determined using the Bradford reagent (Bradford MM., 1976). For Western-blot assays, 40 μ g of protein were separated on a 12% SDS-PAGE, followed by blotting onto PVDF membrane and staining the membrane with India Ink (Beckon Dickinson and Company) for visualization of the protein size ladder (SDS-PAGE standards, broad range, BIO-RAD, Hercules, CA). The PVDF membrane was destained and the nonspecific-binding sites of the membrane were blocked by treatment with TBS-T containing 5% w/v nonfat dry milk for one hour. The PVDF membrane was incubated overnight with primary anti-HIVp55 antibodies (Company Abcam Cat#ab63917) at 4°C. The next day, secondary antibodies conjugated with horseradish peroxidase were added, and immunolabeling was detected by enhanced chemiluminescence using the ECL reagent (GE Healthcare, Piscataway, NJ) visualized using ChemiDoc XRS reader powered by Image Lab software (BIO-RAD, Hercules, CA). Data shown in Figure 3 are representative of four independent experiments.

Statistical analysis

A descriptive analysis of the variables (frequencies, percentages, and central tendency measures, as well as variability measures) was performed. Normality criteria were evaluated to select the correct parametric or non-parametric test using the Shapiro-Wilk estimator. The differences between the results (mean values) were probed using the k independent samples ordinary ANOVA test with Levene's homogeneity of variances test. The pairwise comparisons were made using Dunnett's adjustment. The statistical analysis was performed with SPSS 23.0 (Chicago, IL). The overall significance level (α) was set at 0.001.

Results

DNA plasmids integrity analysis

To check the functional integrity of the recombinant plasmid, the HIV-1 Gag constructs that were extracted from the transformed *E. coli* top 10 bacteria were enzymatically digested with BamHI type II and XhoI restriction enzymes. Restriction enzyme analysis with BamHI and XhoI, followed by southern blotting were used to confirm the presence of the p55 Gag gene, which encodes the antigenic protein in the plasmid (Figure 1 A,B). A 1,614 base pair band corresponds to HIV-1 p55 Gag (Figure 1 B, lane 5). The band was cut and sent for

sequencing (Figure 1 B and C, see also supporting material) to confirm genetic integrity (Avance Biosciences Inc. TX, USA).

Expression of p55 Gag precursor protein

To determine the overall effect of adjuvant concentrations on HIV-1 Gag precursor (p55) protein expression, we detected this protein in mouse muscle after the immunization. Expression of HIV p55 protein was confirmed by Western Blotting using anti-HIV p55 antibody (Abcam, Cambridge, UK, Cat# ab63917). Non-immunized naive mice were used as negative controls. The estimated molecular weight of the used HIV-1 Gag protein is ~55kDa according to the Bioinformatics Software Mac Vector (Cary, NC, USA). All Gag plus imiquimod samples were positive, as shown in Figure 3. The positive control confirmed protein expression in muscle samples from immunized mice. Interestingly, Low concentrations of imiquimod injected simultaneously with the DNA vaccine enhanced Gag precursor protein production ($p < 0.001$), but decreased protein expression at higher concentrations ($p = 0.013$).

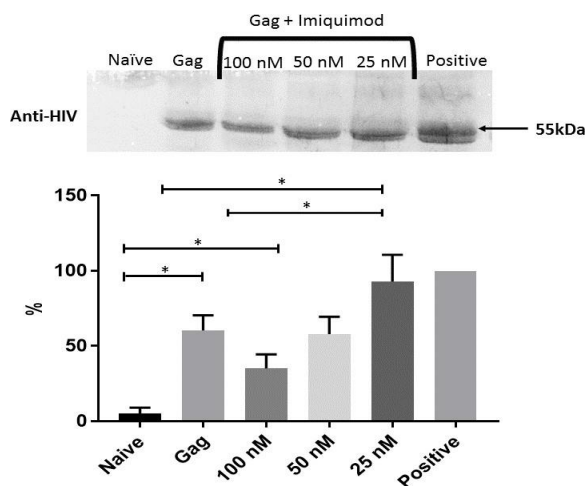


Figure 3: Expression of HIV-1 Gag protein after intramuscular immunization. Antigen expression was confirmed by western blot analysis. Four mice per group were immunized three times, *via* intramuscular injection. Data shown on the western blot (upper panel) are representative of 4 independent experiments. Data on the graph (lower panel) was normalized as a percentage (%) against positive control. * $p < 0.001$.

Evaluation of immune response

After vaccinating BALB/c mice three times every two weeks in Figure 2 the cellular immune response was examined by ELISpot analysis using the splenocytes pools collected from each group of mice ($n=4$) one week after the third immunization. The ELISpot analysis shows an increase in IFN- γ producing cells in mice treated with HIV-1 Gag plus imiquimod. Higher production of IFN- γ producing cells was observed in the group immunized with HIV-1 gag plus imiquimod 25 nM, with a significant increase observed when compared to naive mice, and mice injected with pVax and Gag. These data demonstrate the immunomodulatory ability of imiquimod adjuvant to significantly enhance the cellular immune response specific for the Gag antigen by stimulating the amount of IFN- γ producing splenocytes (Figure 4).

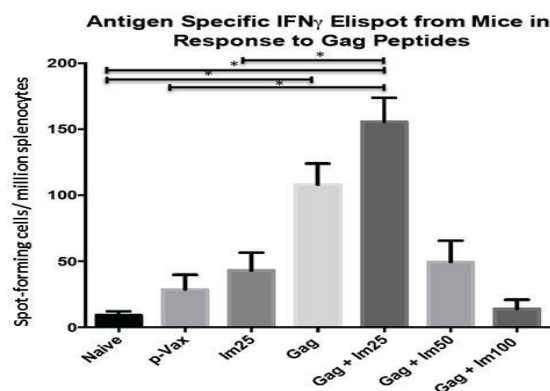


Figure 4: Frequencies of Gag-specific IFN- γ spot forming cells per million splenocytes after DNA vaccination using 25nM, 50nM, and 100nM Imiquimod combined with 100 μ g Gag. * $p < 0.001$.

Cytokine production

We performed an ELISA to measure IFN- γ levels in each of the groups. The mean cytokine IFN- γ response was 14.20 ± 2.66 pg/mL in naive mice and, 13.67 ± 2.13 , 12.54 ± 2.45 , 32.19 ± 4.06 , 53.74 ± 3.55 , 19.11 ± 3.3 , and 17.06 ± 1.18 pg/mL in mice vaccinated with pVax, Gag, Gag plus 25 nM Imiquimod, Gag plus 50 nM Imiquimod, and Gag plus 100 nM Imiquimod, respectively. Significantly higher IFN- γ production was observed in the group immunized with Gag plus 25 nM Imiquimod (Figure 5).

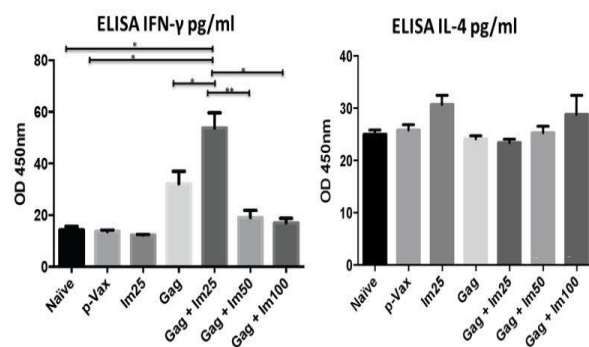


Figure 5: (A) The cytokine IFN- γ response induced in mice after immunization. The IFN- γ responses were assayed by screening for supernatant of splenocytes using the ELISA assay. Significant differences are labeled as * $p < 0.001$ or ** $p < 0.01$. (B) IL-4 response in mice after immunization. No significant differences were observed.

We also performed an ELISA to quantify IL-4 levels in each group ($n=4$). Our data shows that the mean IL-4 cytokine response was 23.71 ± 1.48 for the naive mice group (Figure 5B). Mean IL-4 levels for mice vaccinated with pVax, Gag, Gag plus 25 nM Imiquimod, Gag plus 50 nM Imiquimod, and Gag plus 100 nM Imiquimod were 25.79 ± 1.68 , 31.18 ± 3.55 , 24.15 ± 3.55 , 23.41 ± 1.52 , 25.32 ± 3.11 , and 28.78 ± 5.76 pg/ml, respectively. We observed a mild increase in IL-4 production in mice treated with Gag plus 100 nM Imiquimod, only

marginally significant. A similar mild increase in IL-4 levels was observed when only administering 25 nM Imiquimod.

Discussion

We tested the efficiency of 25 nM, 50 nM, and 100 nM of Imiquimod, an immunomodulator, when used as an adjuvant for a HIV-1 p55 Gag DNA vaccine. Imiquimod is a known specific activator (agonist) of TLR7 toll-like receptors. TLR7 receptors are mainly located on DCs in humans and mice. Its ligands activate DCs by upregulating Retinoic Acid-Inducible Gene I (RIG-I) like receptors (RLRs), which are key sensors for viral infections [25-28]. Using this strategy, we pre-activated the antigen-presenting cells that modulate T-cell immune response, DCs. It is known that DCs are very involved in immune tolerance mechanisms because they participate in the negative selection of autoreactive T cells in the thymus [29-32]. However, there is a gap in knowledge regarding whether overactivation of DCs results in tolerance.

In our experiments, Gag precursor protein production after immunization with Gag vaccine alone was practically the same as with 50 nM of Imiquimod, while 25 nM of Imiquimod increased Gag expression by approximately 30%. In contrast, the addition of 100 nM of Imiquimod, reduced Gag production by about 30%.

This effect may be explained if there is the positive link between the activation of TLR7 in DC by Imiquimod and the Gag expression from the plasmid but with negative feedback which depends on the concentration of Imiquimod as the agonist of TLRs. It is known that DNA vaccine transcription occurs mainly in DC [33-35]. Actually, it is the usual behavior of receptors to be activated at low concentrations of the agonist, while being suppressed (desensitized) by higher concentrations, especially for G protein coupled receptors (GPCRs) like TLR7 and TLR8 [36-38].

Immune response to the Gag antigen in our experiments followed the same pattern. The frequencies of Gag-specific IFN- γ spot forming cells (most probably T-cells) were slightly increased for the Gag plus 50 nM Imiquimod combination when compared to Gag without an adjuvant, while the lowest dose of Imiquimod adjuvant (25 nM) significantly increased the response by about 25%. The highest dose of Imiquimod (100 nM) significantly reduced the frequencies by about 55% when compared to the Gag vaccination. These results suggest that higher dose of Imiquimod adjuvant elicits tolerance.

Different mechanisms were proposed why TLR agonists sometimes produce tolerance: (1) it has been hypothesized TLR agonists may hamper vaccine particles internalization by abrogating micropinocytosis. (2) It is also possible that the inhibitory effect of these TLR ligands on protein expression is mediated by type I IFN-dependent antiviral defense because of the induction of I IFN receptors on DCs. In addition, the (3) specific response of DCs to inflammatory stimuli promoting DC maturation may also contribute to the negative outcome of TLR activation [39].

Hyperimmunisation with antigen can cause inversion of the immune response; however, our data suggest it may also be caused by adjuvant. Consequently, the concentrations of IFN- γ splenocytes supernatants depended on the Imiquimod concentration in a very similar way. Remarkably, the IL-4 concentrations (a marker of T-cell activation) do not follow the same pattern and were statistically inconsistent [40]. For example, an enhanced IL-4 response was observed by administering 25 nM Imiquimod alone. Our data

correlated with previously known properties of Imiquimod alone, which has been shown to indirectly influence the production of several Th1 cytokines (IFN- α , IL-12, and IFN- γ) and subsequently affect the immune response. The addition of topical Imiquimod augments the effectiveness of vaccination against HPV-16 oncoproteins E6 and 7, using a vaccine that has Freund's adjuvant, by reducing neoplasia lesions in patients with high HPV-16 specific T-cell count, and it is difficult to say, if it is the activation of the vaccine or the tolerance to some inflammation [41]. Also, Imiquimod in a high (5%) concentration has been reported to decrease vulvar intraepithelial neoplasia in 81% of patients, and reduce pain and itching, suggesting that it suppresses inflammation which may be explained by tolerance induction. Adjuvant effect of topical imiquimod for HIV DNA vaccine was also shown, as well as the imiquimod/montanide mix was used to boost DNA vaccine generating synthetic long peptides representing HIV conserved regions [42]. The use of Imiquimod before HIV infection was reported to be the most beneficial in preventing overall viral entry into the primary human macrophages, which could also suggest that DCs are suppressed [43].

Conclusion

Planning our experiments, we have not expected any tolerance, but only enhancement at all concentrations of adjuvant. The adjuvant concentration we opted to test was based on the fact that 50 nM is a standard concentration of imiquimod used in previous studies a slight increase in the immune response was reported.

Now, analyzing our data, we may suggest that activation/hyperactivation of Gag production and immune response depends on the concentration of TLR7 agonist imiquimod added as an auxiliary component (adjuvant) to a vaccine, and that it is a biphasic dependence balancing around 50 nM of the adjuvant. In addition, high concentrations of imiquimod reduce the vaccination effectiveness and suppress the immune response. It was known before, that hyperimmunisation with antigen can cause inversion of the immune response, but our novel data suggest it may also be caused by adjuvant.

Although our results were unexpected, as we first anticipated a less complicated response and planned experiments accordingly, they suggest that the adjuvant effects of Imiquimod are dependent on the concentration used. Our next study definitely will include the assessment of DC-specific interleukins (for example, IL-12). We strongly believe that our results support future studies focused on examining the DC-mediated induction of T-cell responses, including possible induction of immunotolerance by hyper-activation of DCs.

Supplementary Materials

The following supporting information can be downloaded: Figure S1: title; Originals Western-Blots.

Author Contributions

Conceptualization, J.A. and M.I.; methodology, J.A.; validation, J.A., V.T. and M.I.; formal analysis, J.A.; investigation, J.R.-H, E. M.-V., K.R.-F., J.A.; data curation, G.M.-M.; writing-original draft preparation, J.A.; writing-review and editing, M.I.; visualization, J.A.; supervision, J.A., R. H.-M.; project administration, J.A and R.H.-M.; funding acquisition, J.A. and M.I. All authors have read and agreed to the published version of the manuscript.

Funding

Research reported in this publication was supported Hispanic Alliance for Clinical and Translational Research of the National Institutes of Health under award number U54GM133807, which provided funding to J.A. and M.I., and NIH NIGMS RCMI award number G12MD007583 granted to the UCC. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Institutional Review Board Statement

The animal study protocol was approved by the Institutional Care and Use Committee (IACUC) of UCC (Approval #041-2014-1100PHA).

Data Availability Statement

All data in this article are presented within the article.

Acknowledgments

Authors want to thank Dr. Miguel Otero for inspiration of this work and a gift of vaccines, and Dr. Eddy Ríos-Olivares for his constant support.

Conflicts of Interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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