Original Article

A novel in vitro cell culture model of human foreskin to study the immunogenicity of live attenuated influenza vaccine

Waleed Mahallawi[1,2] and Hamdi H. Almaramhy[3]

1.Department of Clinical Laboratory Sciences, Taibah University, Madinah 42362, Saudi Arabia

2. Department of Clinical Infection, Microbiology and Immunology, University of Liverpool, United Kingdom

3 .Pediatric surgery division, Department of surgery, college of medicine, Taibah University, Madinah 42362, Saudi Arabia

Received on 14.3.2019, accepted for publication on 15.11.2019, (Doi : dx.doi.org/10.5455/mjhs.2020.02.003)

Corresponding author :Waleed Mahallawi , Department of Clinical Laboratory Sciences, Taibah University, Madinah 42362, Saudi Arabia

Abstract

Background:
The foreskin of the penis is a part of the mucosal immune system, an important first line of defense against antigens and infection. We tested cell culture derived from human foreskin tissues to observe the immunogenicity of live attenuated influenza vaccine (LAIV). The aims of this study is to use a novel in vitro cell culture model of human foreskin to assess LAIV in priming humoral as well as cellular immune responses. Also, to provide a new model that could be used to investigate the immunogenicity of the intranasal vaccines in foreskin tissue.

Methods:
Foreskin samples (n=21) were obtained from infant boys who had undergone circumcision at Pediatric urology department in Madinah Children and Maternity Hospital, Saudi Arabia. The samples were collected within for weeks of time. No clinical conditions were found during the time of surgery for those who gave foreskin samples i.e., all were healthy. We isolated a single cell suspension from fresh foreskin tissues and measured the frequency of total T cell subsets (CD4 and CD8) using multiparameter flow cytometry. ELISA was also used to measure the antibody titrations in cell culture supernatants following stimulation.

Results:
Significant higher antibody levels were detected ($p < 0.05$) by ELISA following stimulation of foreskin mononuclear cells (MNCs) with LAIV when compared with unstimulated negative controls. Furthermore, there was a significant increase in total CD4+ and CD8+ T-cell responses ($p < 0.05$) after stimulation with LAIV. Additionally, Interferon gamma (IFNγ) was also detected in a significant manner after LAIV stimulation.

Conclusions:
Understanding mucosal immunity to respiratory tract infections such as influenza viruses may provide important support to the improvement of effective intranasal vaccines against other respi**ratory** infections. LAIV is found to be immunogenic to trigger cellular and humoral immune response in MNCs derived from human foreskin.

Keyword:
Foreskin, Humoral Immunity, Cellular Immunity, Cell culture model LAIV.

الملخص

مقدمة:
يعتبر الجلد الذي يزال خلال عملية ختان الذكور(القلف) أحد أهم مكونات جهاز المناعة. قمنا في هذه الدراسة باستخدام الخلايا المناعية الموجودة في هذا الجلد كنموذج لدراسة مدي مقدرة تطعيم الانفلونزا على استحثاث الخلايا المناعية.

طريقة البحث:
تم عزل الخلايا الليمفاوية من القلف لعدد عشرين طفلا بعد خضوعهم لعملية الختان في مستشفى الأطفال في المدين وذلك بعد أخذ موافقة الوالدين. تم اختيار الأطفال الأصحاء الذين ليس لديهم أي أعراض ظاهرية أثناء الختان وتم فصل خلاياهم واستحثاثها بعد إضافة تراكيز محددة من تطعيم الانفلونزا وتم استخدام تقنية التدفق الخلوي لقياس استجابة الخلايا التائية بنوعيها ٤ و٨. كما تمت دراسة مقدرة التطعيم على افراز الخلايا البائية للأجسام المضادة وقياس ذلك بتقنية الأليزا.

النتائج:
أظهرت النتائج نجاح استخدام هذا النموذج كنظام مناعي متكامل لديه المقدرة على التعامل مع فيروس الانفلونزا الموجود في التطعيم. فكان هناك استجابة مناعية قوية للخلايا التائية والبائية بعد الاستحثاث مقارنة بالخلايا التي لم يتم استحثاثها.

الخلاصة:
إن فهم خصائص الجهاز المناعي الموجود في خلايا الجلد بعد الختان يلعب دورا هاما في الدراسات المستقبلية لمثل هذا النوع من الأبحاث حيث يمكن أن يستخدم مستقبلا لدراسة ميكروبات أكثر لاسيما الفيروسات التي تصيب الجهاز التنفسي. أيضا نجاح هذا النموذج يفتح مجالا واسعا لتطوير النموذج واستخدامه على نطاق أوسع.

Introduction

Circumcision is a cultural, medical, and religious process in which the human foreskin (HFS) is either partially or entirely removed by surgery. Cells segregated from the circumcised tissues are called foreskin cells. These cells can serve as feeder cell lines for embryonic stem cells used in research.[1] Circumcision is beneficial in decreasing the risk of infection [2-4]. For example, a recent study confirmed that circumcision lowers HIV infection rate in heterosexual men by 50-60%. [5] Therefore, WHO and UNAIDS have recommended male circumcision as a vital measure of a broad HIV prevention bundle for males. [6] In addition, it has been proven that among infants <1 year of age with urinary tract infections (UTIs), about 86% were uncircumcised boys. [7] Based on this evidence, the American Academy of Pediatrics (AAP) and the CDC strongly recommend male circumcision.[8] In the USA, the CDC estimates that 81% of males aged 14 to 59 years are circumcised. [9] In Saudi Arabia, circumcision for boys is obligatory according to Sharia Law [10], which ensures a supply of foreskin for scientific use. Both humoral and cellular immune responses in the genital mucosa have a vital role in the prevention of sexually transmitted infections (STIs), including infection with HIV-1.[11] Mucosal immunity is an important compartment of the immune system and the foreskin is an important component of the mucosal immune system. However, its role in mucosal immunity has not been fully characterized. [12] Cell cultures initiated from HFS tissues have been used for isolation of viruses from diagnostic specimens because the tissues are readily available, and preparation is not difficult. Investigators have recommended greater use in diagnosis and research.[13] In this study, we investigated the potential use of foreskin-derived cell culture as a novel human model to study immune response to live attenuated influenza vaccine (LAIV) as an alternative to currently used animal models. [2, 14, 15] Foreskin-derived cell culture has been shown to be useful in characterizing the levels of CD4+ and CD8+ T cells, memory T cells, and the production of pro-inflammatory cytokines in HIV infection. [16] In this study, we developed an HFS cell culture model to observe the immunogenicity of LAIV. We isolated a single cell suspension from fresh foreskin tissues and measured the frequency of foreskin T cell subsets using multiparameter flow cytometry and ELISA for antibody detection. By studying immune cells from HFS, we aimed to determine if HFS could serve as an in vitro human model to assess immune response to different influenza viruses.

Methods

Foreskin samples

A total of twenty-one foreskin tissues were obtained from male infants (6 -30 months of age) undergoing circumcision at local hospitals in the Madinah region after signed consent obtained from parents. Foreskins were immediately placed in HANKS transport medium,

containing glutamine (2mM), penicillin (50 U/mL), and streptomycin (50µg/mL) (Sigma-Aldrich) and transferred to the laboratory for processing and isolation of MNCs.

Ethical statement: the study has been approved by Taibah University Ethical Committee (Ref No. MLT 2018050).

Isolation of foreskin mononuclear cells

Cell suspensions were prepared using a modified cell suspensions protocol from a previously described method that was used to isolate MNCs from the nose-associated lymphoid tissues (NALT). [17, 18] Foreskin samples were processed within one hour of surgery. The tissues were cut into small pieces, then transferred to an 8 cm-diameter sterile Petri dish and checked grossly. Each sample was mechanically disrupted and minced using a single sterile scalpel. The samples were then enzymatically digested with dispase II (Sigma-Aldrich) to release cells into the medium. The tissue–dispase II mixture was then kept in a water bath shaker for 2 hours at 37°C at 100 rpm. The remaining tissues were then cut, minced and incubated in cell release medium (RPMI 1640 medium with HEPES supplemented with 10% fetal bovine serum [FBS], glutamine [2mM], penicillin [50U/mL], and streptomycin [50µg/mL], Sigma-Aldrich). The cell suspension was then passed through a 70-µm sterile nylon mesh to eliminate debris and then centrifuged at 400 g for 10 min. The cells were washed twice in sterile phosphate-buffered saline (PBS) and divided into two portions. One was cultured in 3 mL of RPMI complete medium for antibody production and the second was processed for T cell proliferation assay Carboxyfluorescein succinimidyl ester (CFSE) staining.

Intranasal vaccine FluMist (LAIV)

FluMist vaccine LAIV Formula (2010-11) includes the following influenza strains; A/H1N1/2009; A/H3N2 and B influenza strains (BEI Resources, American Type Culture Collection (ATCC)).

Cell culture and foreskin MNCs stimulation for antibody production

Following foreskin MNCs isolation, each cell suspension was adjusted to contain 4 x105 cells/mL. Foreskin MNCs were co-cultured in RPMI complete medium in the presence and absence of LAIV. Unstimulated cells were used as a negative control. After preparation, a volume of 250 µL of cell suspension were cultured in a sterile 96 well cell culture plate (Costar) and placed in the incubator in a humidified, 5% CO2 atmosphere at 37°C. Cell culture supernatants were then collected at different time points and assayed by ELISA for measuring antibodies induced in response to LAIV stimulation.

Carboxyfluorescein succinimidyl ester (CFSE) staining

Foreskin MNCs were prepared and resuspended in sterile PBS in 45 ml sterile tube. CFSE was prepared at 5mM just before use by adding 5 µL of neat to 10 ml sterile PBS. After that, 3 ml of the preparation were added to the

cells then kept into the incubator in 5% CO2 at 37°C for 8 min. Adding of 10 ml ice cooled RPMI 1640 complete medium to block the reaction and then centrifuged at 400 x g for 10 min and then the supernatant discarded. The pellet was resuspended into 2 ml RPMI complete and then cells number was calculated via a haemocytometer. After adjusting cells concentration to 4 x105 cells/ml, cells were stimulated with LAIV and then incubated with 5% CO2 at 37°C for four days. After incubation, cell culture supernatants were collected for cytokines analysis and cells collected for T cell proliferation assay by flow cytometer.

Measurement of LAIV induced antibody levels by ELISA

Antibodies induced by LAIV stimulation were analyzed following the ELISA procedure as previously described [18]. In brief, ELISA plates were coated with 100 µL of LAIV (2 µg/mL) and incubated overnight at 4°C. After 5 washings, plates were blocked with 200-µL of 10% FBS followed by incubation of cell culture supernatants at different dilutions for 1.5 hour and then discarded and blotted on dry tissues. Then 50 µL of alkaline phosphatase conjugate of anti-human IgG, IgM and IgA (Sigma) were added and incubated for 1.5 hour. Afterward 5 washings, 50 µL of p-nitrophenyl phosphate (PNPP) substrate was added. Optical density (OD) was measured
at 405 nm.

T cells proliferation assay

Following foreskin MNCs stimulation with LAIV and incubation, percentage of proliferative T cell subpopulations including CD4+ and CD8+ T cells subsets was analyzed by CFSE (FITC) and T cell marker staining followed by flow cytometry (BD FACScalibur (BD Biosciences)). CellQuest software was used for flow cytometer data acquisition. Data analysis was performed using WinMDI 2.9 software. Subsequent stimulation, cells were harvested in 0.02% BSA-PBS buffer (Sigma-Aldrich). Cells were washed and centrifuged at 400xg for 8 min. The supernatants were thrown away then the cell pellet was resuspended in 50 µl 0.02% BSA for surface staining. A volume of 5 µl of mouse anti-human CD4-PE-Cy5 and 5 µl of mouse anti-human CD8-PE (BD) were added to stain CD4+ as well as CD8+ T cells. The cells with the antibodies were incubated at 4°C for 30 min. Then the cells were washed tow times with 500 µl 0.02% BSA and then centrifuged for 8 min at 400g at 4°C. The cells were then resuspended in 300 µl 0.02% BSA and transported to FACS tube (Falcon) for analysing flow cytometer.

Statistical analysis

All the calculations and statistical analysis were performed using GraphPad Prism statistical software (version 5, USA). Data were expressed as mean ± standard deviation (SD). Two-group comparisons were performed using paired two test. A $P < 0.05$ was considered significant for all tests.

Results

LAIV elicits for stronger humoral immune t response

LAIV primes for stronger humoral immune response in the foreskin cell culture model. ELISA was used to measure the antibody levels in cell culture supernatants at different time points. Significant antibody levels were detected at day 10 following stimulation of MNCs with LAIV when compared with unstimulated negative control. Interestingly, all antibody classes were detected. Antibody class IgA was dominant over the other two classes whereas IgM was higher than IgG class antibody; (Fig. 1, $p < 0.05$, n=21 (a) IgG, (b) IgM and (c) IgA).

LAIV induces cellular immune response

The percentage of total T cell proliferation index using CFSE staining shows significant increase in total T cell responses following LAIV stimulation in CD4+ (Fig. (2 a), $p < 0.05$, n=21) and CD8+(Fig. (2 b), $p < 0.05$, n=21) when compared with unstimulated negative control. CD4+ T cell proliferation index was used to quantify the CD4+ T cell response following LAIV stimulation. Gating strategy illustrated in figure 3. Lymphocyte and CD4+ and CD8+ T cell gates were used in this analysis during flow cytometry. Lymphocytes were gated on the basis of their forward scatter and side scatter properties. As shown in figure (3 a) the lymphocytes were gated on region 1 (R1). CD4+ T cell gate was then set as region 2 (R2) as in (figure 3 b). Finally, R2-gated CD4+ T cells were shown in a new window as in (figure 3 c) with CD4-PE on y axis against CFSE (FITC) on x axis. The CD4+ T cell proliferative index was defined as the percentage of proliferating CD4+ T cell of total CD4 T cells = A/A+B, A= upper left quadrant, B=upper right quadrant). Representative proliferative index of CD4+ T cell following stimulation with LAIV (figure 3 d) was shown as compared to unstimulated negative control (3 c). Moreover, stimulation with LAIV induced also significant increases in IFNγ in the foreskin-derived MNCs ($p<0.01$, n=21) (data not shown).

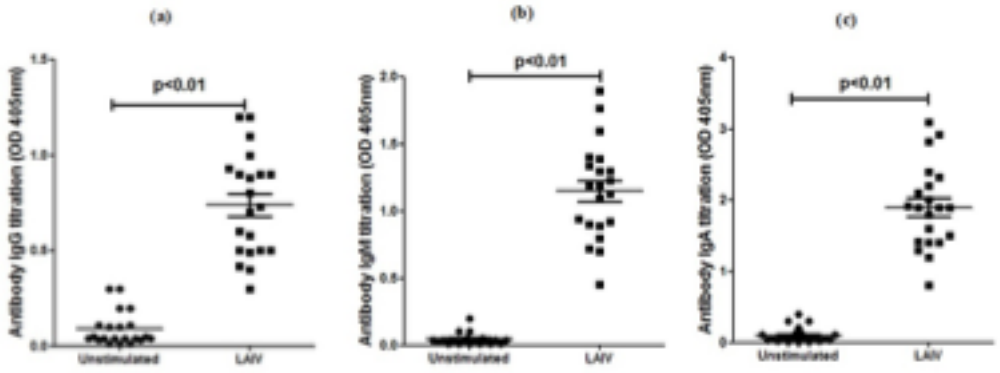


Figure 1: Significant antibody levels were detected ($p < 0.05$, n=21) following stimulation of MNCs with LAIV when compared with unstimulated negative controls. Interestingly, all antibody classes were detected (IgG (a), IgM (b) and IgA(c)).

Discussion

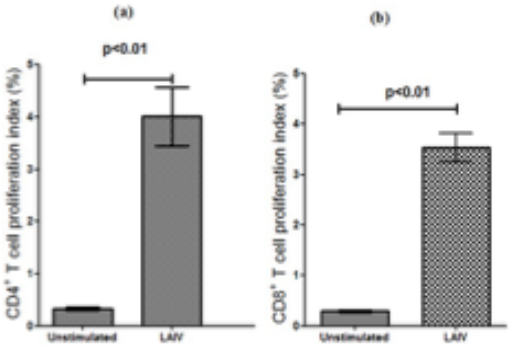


Figure 2: Significant increase in total T-cell responses after stimulation ($p < 0.05$, n=21). Stimulation with LAIV induced significantly increases in total CD4+ (a) and CD8+ (b) T cells when compared with unstimulated negative control.

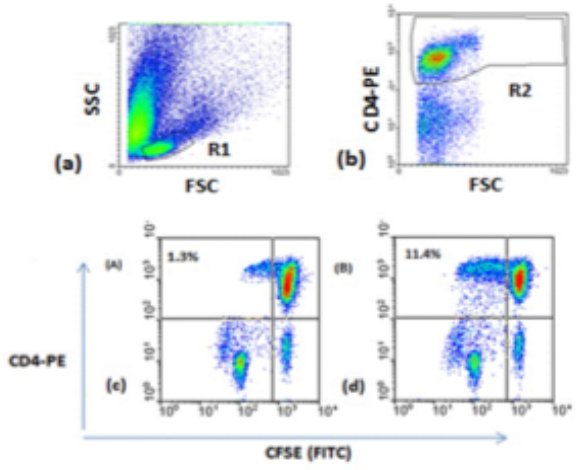


Figure 3:Representative CD4+ T of proliferative index following stimulation with LAIV 3 (c) was shown as compared to unstimulated negative control 3(d)). Representative CD4+ T cells and gating strategy used in quantifying CD4+ and CD8+ proliferation index using CFSE staining.

To the best of our knowledge, the present study is the first to report immune cell densities in the HFS using LAIV as immunogen for stimulation. We also provided the first evidence that foreskin-derived MNCs can be used as a model for an in vitro human cell culture model for studying mucosal immune response to respiratory pathogens such as influenza viruses. Influenza is a very transmissible acute respiratory illness initiated by influenza virus. The virus infects the mucosa of the host respiratory tract. Intranasal immunization with activated vaccine is more potent than intravenous inactivated vaccine and is a more biologically appropriate means of vaccination against respiratory infections such as influenza since it imitates natural infection. [19] Our results show a significant antibody production following stimulation with LAIV. As presented, IgA class antibody shows to be dominant over the other two classes, which is consistence with other study, which showed higher antibody levels in mucosal system. [19] IgG class antibody level appears to be the lowest among all classes which is expected as all subjects are infants less than three years. Therefore, they were expectedly lees exposed to influenza viruses during their lives compared with adult. LAIV provokes secretory IgA, the major antibody secreted at mucosal surfaces, which plays an essential role in protecting against influenza and is a critical part of the immune system, being the second line of defense against pathogens. [20-22] . LAIV seems to be superior in eliciting nasal IgA and CD4 T cells when compared with natural infection and inactivated influenza vaccine. However, LAIV is about as strong as natural infection in providing cross-protective immunity. [19]

The local mucosal immune tissue critically influence intranasal vaccination. [23] Human adenoids and tonsils are the main mucosal immune organs, specifically the NALT in hu-

mans and are recognized as essential points of induction of mucosal (and systemic) immunity against upper respiratory tract pathogens. [24, 25] Natural influenza infection elicits a wide-range of immune responses, which include humoral and cellular components. [26, 27] Mucosal antibodies are potent defenders of the upper respiratory airways and points of viral access. Serum antibodies mostly defend the lower respiratory tract Existing inactivated influenza vaccines induce strain-specific antibodies and not CD8+ T cells. [28] Our results also show significant increase in cellular immune responses after LAIV stimulation namely, CD4+ and CD8+ T cells. This result is in accordance with another study showed that LAIV mimics natural infection through viral replication in the upper respiratory tract and provokes many immune responses, comprising antibodies and CD4+ and CD8+ T cells. [29]

Several models have been used to study and evaluate infections caused by several influenza viruses. In a previous study, we showed that NALT from human adenoids and tonsils is one of the most successful models for that purpose. [18] Now we show that an HFS model is an alternative and novel in vitro cell human model. The present study demonstrates the ability of MNC-derived from human foreskin to be used as a model for studying humoral and cellular immune response. An advantage of HFS is that all immune cells are present in these tissues, which is well documented. [1, 30] Additionally, a previous study investigated mucosal response of tissues from the penises of transformed males following penal removal and found that the penis is a site of efficient mucosal response [31] . Studies in animal models have found that the presence of CD4 T cells in the foreskin of infected monkeys played a crucial role in fighting the HIV virus. [2] The role of IFNγ is very essential in fighting viral infection as influenza virus. IFNγ is released in large quantities in several immune compartments such as, macrophages, activated CD8 T cells, NK T cells, and Th1 CD4 T cells. [32, 33] Our data shows the ability of HFS model to produce IFNγ, which mimics the natural viral infection, and this is consistence with several studies that demonstrated the ability of LAIV to induce the secretion of IFNγ. [34-36]

Our data can be added to the accumulation of evidences to verify the appropriateness of foreskin to study the interactions between influenza virus and the immune system. This result is consistent with a study in children when T cells were produced abundantly, which might offer clinical protection. [26] In addition, another study reported similar results after intranasal LAIV. [28] The finding of significant antibody levels following stimulation of MNCs with LAIV means the model is valid for other studies to assess the immune responses to different pathogens that cause infection in humans such as MERS-CoV, pneumonia and many others. This result is also consistent with several studies of the NALT model. [37-40]

Conclusion

This study using an in vitro cell culture model of HFS to measure humoral as well as cellular immune responses to LAIV provides a new model to attest and study of intranasal vaccines. Use of human-derived cells is a more realistic model for investigation of the immune response against pathogens that cause human infection such as influenza viruses. Understanding mucosal immunity to respiratory tract infections such as influenza viruses may lead to meaningful improvements in effective intranasal vaccines against other respiratory infections.

Acknowledgement

We thank the BEI Resources company for providing us by FluMist vaccine for free.

Author Contributions

All authors having contributed equally to the work.

Disclosure of potential conflicts of interest

The authors claim no conflict of interest.

References

[1] Somuncu OS, Tasli PN, Sisli HB, Somuncu S, Sahin F. Characterization and Differentiation of Stem Cells Isolated from Human Newborn Foreskin Tissue. Appl Biochem Biotechnol. 2015;177(5):1040-54.

[2] Lei JH, Liu LR, Wei Q, Yan SB, Yang L, Song TR, et al. Circumcision Status and Risk of HIV Acquisition during Heterosexual Intercourse for Both Males and Females: A Meta-Analysis. PLoS One. 2015;10(5):e0125436.

[3] McDaid LM, Weiss HA, Hart GJ. Circumcision among men who have sex with men in Scotland: limited potential for HIV prevention. Sex Transm Infect. 2010;86(5):404-6.

[4] Siegfried N, Muller M, Deeks JJ, Volmink J. Male circumcision for prevention of heterosexual acquisition of HIV in men. Cochrane Database Syst Rev. 2009(2):CD003362.

[5] Anderson D, Politch JA, Pudney J. HIV infection and immune defense of the penis. Am J Reprod Immunol. 2011;65(3):220-9.

6] Tynan A, Vallely A, Kelly A, Kupul M, Naketrumb R, Aeno H, et al. Building social currency with foreskin cuts: a coping mechanism of Papua New Guinea health workers and the implications for new programmes. Health Policy Plan. 2014;29(7):902-11.

[7] Schoen EJ, Colby CJ, Ray GT. Newborn circumcision decreases incidence and costs of urinary tract infections during the first year of life. Pediatrics. 2000;105(4 Pt 1):789-93.

[8] Morris BJ, Kennedy SE, Wodak AD, Mindel A, Golovsky D, Schrieber L, et al. Early infant male circumcision: Systematic review, risk-benefit analysis, and progress in policy. World J Clin Pediatr. 2017;6(1):89-102.

[9] Introcaso CE, Xu F, Kilmarx PH, Zaidi A, Markowitz LE. Prevalence of circumcision among men and boys aged 14 to 59 years in the United States, National Health and Nutrition Examination Surveys 2005-2010. Sex Transm Dis. 2013;40(7):521-5.

[10] Alahmad G, Dekkers W. Bodily integrity

and male circumcision: an islamic perspective. J IMA. 2012;44(1).

[11] Garulli B, Kawaoka Y, Castrucci MR. Mucosal and systemic immune responses to a human immunodeficiency virus type 1 epitope induced upon vaginal infection with a recombinant influenza A virus. J Virol. 2004;78(2):1020-5.

[12] Rothaeusler K, Ma ZM, Qureshi H, Carroll TD, Rourke T, McChesney MB, et al. Antiviral antibodies and T cells are present in the foreskin of simian immunodeficiency virus-infected rhesus macaques. J Virol. 2012;86(13):7098-106.

[12] Rothaeusler K, Ma ZM, Qureshi H, Carroll TD, Rourke T, McChesney MB, et al. Antiviral antibodies and T cells are present in the foreskin of simian immunodeficiency virus-infected rhesus macaques. J Virol. 2012;86(13):7098-106.

[13] Sharon N. The use of human foreskin cell cultures for isolation of herpesvirus group in the diagnostic laboratory. Am J Clin Pathol. 1976;66(1):65-72.

[14] Fahrbach KM, Barry SM, Anderson MR, Hope TJ. Enhanced cellular responses and environmental sampling within inner foreskin explants: implications for the foreskin's role in HIV transmission. Mucosal Immunol. 2010;3(4):410-8.

[15] Johnson KE, Redd AD, Quinn TC, Collinson-Streng AN, Cornish T, Kong X, et al. Effects of HIV-1 and herpes simplex virus type 2 infection on lymphocyte and dendritic cell density in adult foreskins from Rakai, Uganda. J Infect Dis. 2011;203(5):602-9.

[16] Prodger JL, Gray R, Kigozi G, Nalugoda F, Galiwango R, Hirbod T, et al. Foreskin T-cell subsets differ substantially from blood with respect to HIV co-receptor expression, inflammatory profile, and memory status. Mucosal Immunol. 2012;5(2):121-8.

[17] Lu FX, Abel K, Ma Z, Rourke T, Lu D, Torten J, et al. The strength of B cell immunity in female rhesus macaques is controlled by CD8+ T cells under the influence of ovarian steroid hormones. Clin Exp Immunol. 2002;128(1):10-20.

[18] Mahallawi WH, Kasbekar AV, McCormick MS, Hoschler K, Temperton N, Leong SC, et al. Infection with 2009 H1N1 influenza virus primes for immunological memory in human nose-associated lymphoid tissue, offering cross-reactive immunity to H1N1 and avian H5N1 viruses. J Virol. 2013;87(10):5331-9.

[19] Sridhar S, Brokstad KA, Cox RJ. Influenza Vaccination Strategies: Comparing Inactivated and Live Attenuated Influenza Vaccines. Vaccines (Basel). 2015;3(2):373-89.

[20] Belshe RB, Coelingh K, Ambrose CS, Woo JC, Wu X. Efficacy of live attenuated influenza vaccine in children against influenza B viruses by lineage and antigenic similarity. Vaccine. 2010;28(9):2149-56.

[21] Caspard H, Gaglani M, Clipper L, Belongia EA, McLean HQ, Griffin MR, et al. Effectiveness of live attenuated influenza vaccine and inactivated influenza vaccine in children 2-17 years of age in 2013-2014 in the United States. Vaccine. 2016;34(1):77-82.

[22] McLean HQ, Caspard H, Griffin MR, Poehling KA, Gaglani M, Belongia EA, et al. Effectiveness of live attenuated influenza vaccine and inactivated influenza vaccine in children during the 2014-2015 season. Vaccine. 2017;35(20):2685-93.

[23] Barbey-Martin C, Gigant B, Bizebard

T, Calder LJ, Wharton SA, Skehel JJ, et al. An antibody that prevents the hemagglutinin low pH fusogenic transition. Virology. 2002;294(1):70-4.

[24] Kiyono H, Fukuyama S. NALT- versus Peyer's-patch-mediated mucosal immunity. Nat Rev Immunol. 2004;4(9):699-710.

[25] Shikina T, Hiroi T, Iwatani K, Jang MH, Fukuyama S, Tamura M, et al. IgA class switch occurs in the organized nasopharynx- and gut-associated lymphoid tissue, but not in the diffuse lamina propria of airways and gut. J Immunol. 2004;172(10):6259-64.

[26] Mohn KG, Brokstad KA, Pathirana RD, Bredholt G, Jul-Larsen A, Trieu MC, et al. Live Attenuated Influenza Vaccine in Children Induces B-Cell Responses in Tonsils. J Infect Dis. 2016;214(5):722-31.

[27] Mohn KG, Bredholt G, Brokstad KA, Pathirana RD, Aarstad HJ, Tondel C, et al. Longevity of B-cell and T-cell responses after live attenuated influenza vaccination in children. J Infect Dis. 2015;211(10):1541-9.

[28] Hoft DF, Babusis E, Worku S, Spencer CT, Lottenbach K, Truscott SM, et al. Live and inactivated influenza vaccines induce similar humoral responses, but only live vaccines induce diverse T-cell responses in young children. J Infect Dis. 2011;204(6):845-53.

[29] He XS, Holmes TH, Mahmood K, Kemble GW, Dekker CL, Arvin AM, et al. Phenotypic changes in influenza-specific CD8+ T cells after immunization of children and adults with influenza vaccines. J Infect Dis. 2008;197(6):803-11.

[30] Slivka SR, Landeen LK, Zeigler F, Zimber MP, Bartel RL. Characterization, barrier function, and drug metabolism of an in vitro skin model. J Invest Dermatol. 1993;100(1):40-6.

[31] Sennepin A, Real F, Duvivier M, Ganor Y, Henry S, Damotte D, et al. The Human Penis Is a Genuine Immunological Effector Site. Front Immunol. 2017;8:1732.

[32] Villegas-Mendez A, de Souza JB, Murungi L, Hafalla JC, Shaw TN, Greig R, et al. Heterogeneous and tissue-specific regulation of effector T cell responses by IFN-gamma during Plasmodium berghei ANKA infection. J Immunol. 2011;187(6):2885-97.

[33] Arany I, Tyring SK, Brysk H, Brysk MM. Induction by interferon-gamma of its receptor varies with epithelial differentiation and cell type. Arch Dermatol Res. 1998;290(6):331-4.

[34] Bhat P, Leggatt G, Waterhouse N, Frazer IH. Interferon-gamma derived from cytotoxic lymphocytes directly enhances their motility and cytotoxicity. Cell Death Dis. 2017;8(6):e2836.

[35] Horvath KM, Brighton LE, Herbst M, Noah TL, Jaspers I. Live attenuated influenza virus (LAIV) induces different mucosal T cell function in nonsmokers and smokers. Clin Immunol. 2012;142(3):232-6.

[36] Chirkova TV, Naykhin AN, Petukhova GD, Korenkov DA, Donina SA, Mironov AN, et al. Memory T-cell immune response in healthy young adults vaccinated with live attenuated influenza A (H5N2) vaccine. Clin Vaccine Immunol. 2011;18(10):1710-8.

[37] Ahmed MS, Derbyshire S, Flanagan B, Loh C, McCormick M, Barocchi M, et al. Immune responses to pneumococcal pilus RrgA and RrgB antigens and their relationship with pneumococcal carriage in humans. J Infect. 2014;68(6):562-71.

[38] Mubarak A, Ahmed MS, Upile N,

Vaughan C, Xie C, Sharma R, et al. A dynamic relationship between mucosal T helper type 17 and regulatory T-cell populations in nasopharynx evolves with age and associates with the clearance of pneumococcal carriage in humans. Clin Microbiol Infect. 2016;22(8):736 e1-7.

[39] Mullin J, Ahmed MS, Sharma R, Upile N, Beer H, Achar P, et al. Activation of cross-reactive mucosal T and B cell responses in human nasopharynx-associated lymphoid tissue in vitro by Modified Vaccinia Ankara-vectored influenza vaccines. Vaccine. 2016;34(14):1688-95.

[40] Aljurayyan A, Puksuriwong S, Ahmed M, Sharma R, Krishnan M, Sood S, et al. Activation and Induction of Antigen-Specific T Follicular Helper Cells Play a Critical Role in Live-Attenuated Influenza Vaccine-Induced Human Mucosal Anti-influenza Antibody Response. J Virol. 2018;92(11).