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Intramuscular vaccination of guinea pigs with the live-attenuated human herpes simplex vaccine VC2 stimulates a transcriptional profile of vaginal Th17 and regulatory Tr1 responses

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ABSTRACT

Herpes simplex virus is a common causative agent of oral and genital diseases. Novel vaccines and therapeutics are needed to combat herpes infections especially after the failure of subunit vaccines in human clinical trials. We have shown that the live-attenuated HSV-1 VC2 vaccine strain is unable to establish latency in vaccinated animals and produces a robust immune response capable of completely protecting mice against lethal vaginal HSV-1 or HSV-2 infections. The guinea pig represents the best small animal model of genital HSV-2 disease. Reported here, twenty-one female Hartley guinea pigs received intramuscular injection with either the VC2 vaccine, or equal volume of conditioned tissue culture media. Animals received 2 booster vaccinations at 21 day intervals following the initial vaccination. After vaccination, animals were challenged with the highly virulent HSV-2 (G) strain. Histologically, VC2 vaccinated animals had little to no apparent inflammation/disease following challenge. Unvaccinated animals developed moderate to severe erosive and ulcerative vaginitis. Quantitative reverse-transcriptase PCR analysis in VC2 vaccinated and challenged animals identified transcriptional signatures of Th17 and regulatory Tr1 cells associated with the inflammatory response primed by VC2 vaccination. Treatment of cultured human vaginal epithelial cells (VK2 cells) with a combination of IL-17A and IL-22 resulted in the significant induction of beta-defensin 3 expression. Further, treatment of VK2 cells with IL-17A, IL-22, IL-36 or beta-defensin 3 resulted in diminished HSV-2 replication. Overall, these results suggest that intramuscular vaccination with the live-attenuated vaccine VC2 primes a mucosal immune response predisposing the adaptive expression of transcripts associated with a Th17 response to challenge and these responses contribute to antiviral immunity.

1. Introduction

Human herpes simplex virus type 1 and type 2 (HSV-1 and HSV-2 respectively) are common human pathogens that are typically associated with infection of the oral and genital mucosa. Infection with either of these viruses results in a life-long latent infection with sporadic reactivation and shedding of infectious virus. Herpes associated disease can manifest pathologically as blistering and ulceration at the initial site of infection, but more severe and debilitating pathologies can manifest as permanent stromal keratitis of the cornea and meningoencephalitis [1]. HSV-2 infection has a high global prevalence. Recent estimates from the world health organization show that approximately 536 million individuals were living with HSV-2 infection in 2003 with 23.6 million new cases in the same year [2]. In the United States, increased prevalence of HSV-2 infection is significantly associated with lower income [3]. Data from a study conducted between 2003 and 2013 indicated that the incidence of HIV/HSV-2 co-infection was significantly increased, while both HIV infections and hepatitis C/HIV co-infections declined. There were no significant changes observed in the seroprevalence of HSV-2 through the same time period indicating that sexual practices were not changed. Overall, these data suggest that HSV-2 infection is a significant cofactor for HIV infection [4].

Previously, our laboratory has shown that the live-attenuated herpes simplex VC2 vaccine strain is safe and effective at preventing genital HSV-1 and HSV-2 disease in mice. The VC2 vaccine contains 2 truncating mutations in the genes coding for glycoprotein K (gK) and the membrane protein UL20 [5]. The 37 amino acid deletion in the amino terminus of gK results in a virus incapable of entering into the axonal termini of cultured neurons [6]. While

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the 18 amino acid deletion in UL20 is attenuating the exact mechanism has yet to be established (Stanfield and Kousoulas, unpublished observations). Despite the majority of preclinical herpes vaccine research being conducted utilizing mice [reviewed in: [7]], very few vaccine candidates progress from the preclinical to the clinical environment and currently no vaccine has proven effective at preventing disease in humans. Unfortunately, mice are not optimal models for this disease, largely because they do not accurately depict immunological and inflammatory characteristics of disease observed in humans [8–10]. In contrast, guinea pigs offer a model of genital HSV-2 disease closely mimicking human disease [8,11–13]. Recently, our laboratory has demonstrated that the VC2 vaccine is safe and immunogenic in rhesus macaques [14]. In macaques VC2 is capable of stimulating the expansion of vaginal $CD4^+$ and $CD8^+$ T cells and germinal center responses resulting in the sustained production of highly cross neutralizing antibodies against HSV-1 and HSV-2 after intramuscular vaccination [14].

A variety of evidence in experimental animals and humans indicate that both humoral and cellular immune responses are responsible for providing protective immunity against genital herpes [7]. In the murine model of genital HSV-2 infection, it has been reported that $CD4^+$ T cells played a critical role in preventing disease [15], most likely by clearing infectious virus at neural sites via a non-lytic mechanism [16]. Recently, the importance of IL-17A in the adaptive response to genital HSV-2 infection has been demonstrated in mice [17]. T helper 17 cells (Th17) are a subset of pro-inflammatory $CD4^+$ T cells defined by their production of interleukin 17 (IL-17). Th17 cells also secrete cytokines such as IL-17A, IL-17F, IL-21, and IL-22. These cytokines act through the IL-17 receptor (IL-17R) found on the surface of a variety of cell types including epithelial cells. Interaction of IL-17A with IL-17R initiates a signaling cascade through ACT1 and TRAF6 to stimulate the production of proinflammatory IL-36 family cytokines [reviewed in: [18]]. These IL-36 family cytokines and specifically IL-36γ then signal via the IL-36 receptor to stimulate the secretion of antimicrobial peptides [19]. Genital Th17 responses are known to be important for the clearance of bacterial pathogens [10,20–22]. A variety of evidence suggests that Th17 cells play important roles in vaccine-induced protection against a variety of pathogens entering the host via mucosal surfaces [23,24]. Recently estradiol has been shown to prime vaginal dendritic cells to induce Th17 responses demonstrating increased vaccine efficacy with estradiol treatment against genital HSV-2 challenge and IL-17 knock-out mice were more susceptible to HSV-2 challenge [25].

To understand the immune response generated by intramuscular injection with the VC2 vaccine, we utilized the guinea pig to model genital HSV-2 infection. The guinea pig is widely regarded as the best small animal model of genital HSV-2 disease. Herein, we show that the live-attenuated HSV-1 VC2 vaccine strain induces mucosal IL-17A responses that contribute to protection against lethal challenge with virulent HSV-2 through the induction of the antiviral peptide beta-defensin 3.

2. Materials and methods

2.1. Ethics statement

This work was approved by the Louisiana State University School of Veterinary Medicine IACUC protocol number 14-040. The Office of Laboratory Animal Welfare of the National Institutes of Health (NIH) has approved LSU regarding the use of animals in research with an approval Assurance Statement (#A3612-01).

2.2. Cells and viruses

Green African Monkey Kidney cells (Vero) (ATCC, CCL-81) were maintained in DMEM (ThermoFisher) containing 50 mg Primocin (ThermoFisher) and 10% heat inactivated fetal bovine serum (FBS) (ThermoFisher). Stock HSV-2 (G) was grown to high titer and titrated in confluent monolayers of Vero cells. The live-attenuated VC2 vaccine strain was described previously, as described previously [5]. Human vaginal mucosa epithelial cells (VK2/E6E7) (ATCC, CRL-2616) were cultured in Keratinocyte-SFM (ThermoFisher) containing 1% heat inactivated FBS (ThermoFisher).

2.3. Preparation of vaccine

Serum-free stock VC2 virus for multiple injections was prepared by infection of Vero cells at an MOI of 0.001 followed by replacement of culture media with serum free DMEM (ThermoFisher). Infection was allowed to progress for 48 h and then virus was collected and aliquoted for administration to the animals.

2.4. Vaccination and challenge scheme

Twenty-one, 250–300 g, female Hartley guinea pigs (Charles River) were housed in groups of 3 per cage. Groups were then divided randomly into the three treatment groups. Guinea pigs were mildly anesthetized by inhalation of 2–3% isoflurane prior to vaccination and were euthanized by CO_2 asphyxiation.

A total of 21 animals were used in this study. They were divided in three groups as follows: Group 1 had three animals that were left unvaccinated and unchallenged; group 2 had nine animals that received intramuscular injection with conditioned media; group 3 had nine animals that were vaccinated with the VC2 virus. On day 0 of the study animals received intra-muscular injection of either 100 μL of conditioned media (unvaccinated), or 100 μL of serum free media containing 2×10^6 PFU of VC2 vaccine. Animals received booster vaccines on days 21 and 42 post initial inoculation. On day 63 of the study, animals to be challenged were lightly anesthetized by inhalation of 2–3% isoflurane and then inoculated intravaginally with HSV-2 (G strain) by first clearing the mucus plug from the vagina with a cotton swab, followed by a second cotton swab inside the vaginal vault to further dry the walls of the vagina, and finally, using a micropipette, instilling the vaginal vault with 40 μL complete DMEM containing 1×10^6 PFU of HSV-2 (G).

2.5. Tissue collection and euthanasia

At 1 h, 3 days, and 8 days post challenge, animals were anesthetized by inhalation of 2–3% isoflurane and bled via cardiac puncture. Maximum volume of blood was collected and animals were euthanized by 2 asphyxiation. Vaginal tissue samples were collected immediately following euthanasia, placed in RNAlater (Ambion) and stored at −20 °C until use.

2.6. Histology

All animals were examined for the identification of macroscopic lesions. All tissues and organs were collected and fixed in 10% buffered formalin. Sections of tissues from the genital tracts and lymphoid organs were dehydrated in progressive alcoholic solutions and xylene and paraffin embedded. Five micron tissue sections were obtained, lined on glass slides, stained with hematoxylin and eosin and examined with light microscopy.

2.7. Vaginal RNA/DNA isolation and analysis

DNA was extracted from vaginal biopsies using the Qiagen DNeasy Blood & Tissue Kit as per the manufacturer's instructions. The eluted DNA was quantified using a Nanodrop 1000 spectrophotometer. Equal amounts of DNA from each sample were used to perform quantitative real-time PCR analysis on an Applied Biosystems 7900HT Fast Real-Time PCR System. Viral DNA from purified HSV-2(G) was used as positive control. The following primer/probe combinations were used to specifically detect HSV-2 (G). HSV2gDFP (CCGCGGGTACGTACCTGCGGCTAG); HSV2 Probe (HEX-GGCCC GCGC/ZEN/CTCCTGCAAGTACGCTCT–IABkFQ); HSV2gDRP (GCCCTGTTGGTAGGCCTT CGAGGTG). To determine the sensitivity of the qPCR assay, a known amount of HSV-2 gD expression plasmid (CMV14gD2) was subjected to ten-fold serial dilutions ranging from 10^5 to 1 molecules, and subsequently were used in Taqman PCR reactions. Quantitative PCR was performed on the Applied biosystems 7900HT Fast Real-Time PCR System. The sensitivity of this qPCR assay has been assessed and determined to a limit of detection of 3 copies per reaction [5].

Total RNA was extracted from 30 mg of vaginal biopsy tissue using the Macherer-Nagel NucleoSpin RNA isolation Kit as per the manufacturer's instructions. RNA samples were used to synthesize cDNA using the High-Capacity cDNA Reverse Transcription Kit (ThermoFisher). cDNA was then used for single transcript qPCR using the Power SYBR Green Master Mix (ThermoFisher). Reactions were assayed on the 7900HT Fast Real-time PCR system (Applied Biosystems). CT values for the various transcripts assayed were normalized to their respective CT value for S18 rRNA. A list of primers used and their sequences specific for the indicated guinea pig transcript is included in Table 1. Statistical significance was calculated using Student's T test.

2.8. Human vaginal epithelial cell stimulation and analysis

VK2 cells were either left unstimulated (NA) or stimulated for 24 h with 10 ng/mL recombinant human IL-17A, IL-22, or both IL-17A and IL-22 (7955-IL-025/CF, 782-IL-010/CF R&D Systems). RNA was isolated using the NucleoSpin RNA isolation Kit (Macherer-Nagel) and used to synthesize cDNA using the High-Capacity cDNA Reverse Transcription Kit (ThermoFisher). cDNA was then used in single transcript qPCR using Taqman primer sets purchased from ThermoFisher (18S rRNA housekeeping gene Hs99999901_s1, IL-36α Hs00205367_ml, IL-36β Hs_00205359_ml, IL-36γ Hs00219742_ml, and DEFB103 Hs00218678_ml). Transcripts were quantified using the 7900HT Fast Real-time PCR system (Applied Biosystems). Kinetics of DEFB103 (DEFB3) expression were monitored by stimulating cells with either fresh tissue culture media (NA) or 10 ng/mL IL-17A and IL-22. Total RNA was extracted from cultured cells at 0, 3, 6, 24, 30, and 48 h post stimulation and subjected to cDNA synthesis and qPCR analysis using the 18S rRNA housekeeping gene Hs99999901_s1 and DEFB103 Hs00218678_ml predesigned primer probe sets. Antiviral cytokine assay was conducted by pre-stimulation of confluent monolayers of VK2 cells with 100 ng/mL either IL-17A, IL-22, IL-36 gamma (200 ng/mL) (6835-IL-010/CF R&D Systems), Human beta-defensin 3 (4435-BD-050, R&D Systems) or the indicated combination. Cells were treated for three hours and then infected with ~100 PFU of the HSV-2 (G) strain. Virus was allowed to attach to the cell surface for 1-h rocking at room temperature to allow for attachment to the cell surface and synchronize infection after which the media was replaced with fresh media (NC) or cytokine containing media. The infection was allowed to progress for 24 h at 37 °C and then frozen at −80 °C until being titrated by standard plaque assay on Vero cells. Statistical significance was calculated using Student's T test.

Table 1
List of transcripts and primers.

Gene	Direction	Sequences
S18	Forward	AAT AGC CTT TGC CAT CAC TGC C
	Reverse	CTT GGT GAG GTC GAT GTC TGC
IL-17A	Forward	CCA CAT CAC CTT GGA CTC TCC ACC G
	Reverse	GCA GGA CCA GGA TCT CTT GCT GG
IL-23A	Forward	CAG ACC CGG GAC GTC TCT GG
	Reverse	GAG TCC TTG TGG ATC ACA GC
IL-22	Forward	GGC TCC ACA ACT TCC AGG AG
	Reverse	CAA TGA GAC GGA CAT CTG TG
IL-36γ	Forward	GGA TCT GTA CAA CCA GCC TG
	Reverse	GTC TGC AGT GAG GAA GAT AGG
IL-36β	Forward	AAC ATC ATG GAC CTG TAC TGG
	Reverse	GGA TCT AAA TAG AAG TTG GTG C
DEFB3	Forward	AAG GCA TCT CCC ACT GTC AC
	Reverse	CTG CCC TTT CCT GCC TTC CAC
FoxP3	Forward	CGC ACA AGG AGC GTA GGC GG
	Reverse	CCT TCA GTG CCT ACC TCC CTG CC
IL-10	Forward	CAC CGC TGC TAT GTT GCC TG
	Reverse	CGT TGT CCA GCT GAT CCT
TGFβ	Forward	CTT CAA CAC GTC AGA GCT CC
	Reverse	GCT GGT TGC TGA GGT AGC GC
STAT3	Forward	CAG AAC GAC CTG CAG CAA TAC C
	Reverse	CAC AAG CTG GGA GAT GCT CTG G
T-Bet	Forward	CTG GAC CCA ACT GTC AAC TCC
	Reverse	CTG TTC CAT TGA GCT GAG TCC
IRF4	Forward	GAG CAT CTT CCG CAT CCC GTG G
	Reverse	CTC TTG TTC AAA GCG CAT CGC

3. Results

3.1. Vaccination and intravaginal challenge of guinea pigs

Animals were divided into groups and vaccinated as described in Fig. 1. Briefly, 3 animals were left unvaccinated to serve as untreated controls for transcriptional analysis, 9 animals were administered IM 100 μL of conditioned media (unvaccinated), and 9 animals received 100 μL of serum free VC2 vaccine. Animals received booster vaccinations on days 21 and 42 of the study. No clinical disease was observed over this time period. All animals except the untreated controls were challenged intravaginally with 1×10^6 PFU HSV-2 (G strain) on day 63 of the study. For tissue collection and virological analysis animals were sacrificed at 1 h, 3 days, and 8 days post challenge. Significant gross pathological disease was observed at day 8 post challenge in unvaccinated animals (Fig. 2A). While no apparent disease was observed in VC2 vaccinated animals (Fig. 2B). Viral loads in infected tissues were analyzed by total DNA extraction from vaginal tissue samples. Quantitative PCR (QPCR) analysis of HSV-2 genome copies assayed demonstrated viral load peaks at day 3 post challenge in all animals analyzed. Viral genome copy numbers were significantly reduced in VC2-vaccinated groups at day 3 post challenge, and 2/3 animals in the VC2 vaccinated group had undetectable genome copies at day 8 post challenge (Table 2).

3.2. Histological evaluation of disease

Grossly, vaginal lesions were only observed in the unvaccinated guinea pigs challenged intra-vaginally with HSV-2 (Fig. 2A and B). Microscopically, vaginal epithelium visualized at 1 h post infection are representative of healthy genital tissue. The most severe vaginal disease, characterized by complete destruction of the vaginal epithelium, was observed in the unvaccinated group at day 3 post challenge. Evidence of viral cytopathic effects (CPE, syncytia) was apparent only in the unvaccinated group at day 3 post challenge (Fig. 3: top panels). Significant infiltration of neutrophils, and

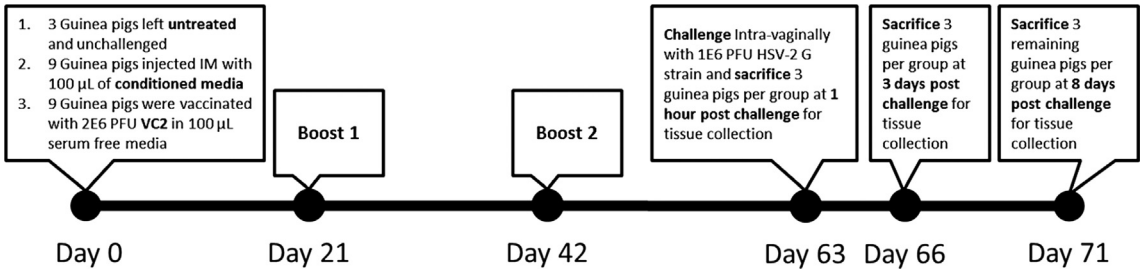

Fig. 1. Study design.

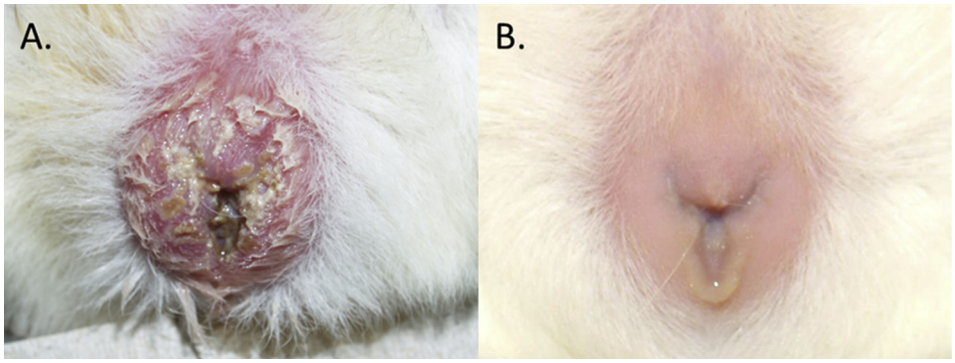

Fig. 2. Gross vaginal disease. Photographs of animals exhibiting gross disease 8 days after intra vaginal HSV-2 challenge with 1×10^6 PFU of either (A) Unvaccinated or (B) VC2-Vaccinated guinea pigs.

Table 2
Viral load in vaginal tissue.

Time post challenge	Unvaccinated			VC2		
1 Hour	1.89[a]	0.00[a]	0.00[a]	0.00[a]	1.02[a]	0.00[a]
3 Days	243.28	663.17	785.59	14.61	155.88	1.184[a]
8 Days	56.52	39.33	37.17	0.00[a]	1.33[a]	0.00[a]

[a] Below the limits of detection.

macrophages were apparent at days 3 and 8 post challenge in the unvaccinated group at these time points (Fig. 3). Vaginal tissue sections were subjected to immuno-histological analysis with antibodies specific to Iba1, a marker for the macrophage lineage (Fig. 3 bottom panels) to assess the level of tissue inflammation. Unvaccinated animals demonstrated a massive infiltration of $Iba1^+$ cells at day 3 post challenge, which persisted through day 8 of the study. In comparison, the $Iba1^+$ staining cells remained relative constant in the tissues of VC2-vaccinated animals.

3.3. qRTPCR analysis of vaginal tissue

Total RNA was extracted from vaginal tissue as described in Materials and Methods and analysis was conducted by single transcript quantitative reverse transcriptase PCR (qRTPCR). As compared to unvaccinated, challenged animals we noted induction of transcripts associated with Th17 and regulatory T cell responses after challenge of VC2-vaccinated guinea pigs (Fig. 4). Specifically, SYBR green qRTPCR assays were developed to quantify the levels of expression for guinea pig IL-17A, IL-22, IL-23A, IL-36β, IL-36γ, and DEFB3 for Th17 related cytokines (Table 1). Also, SYBR green assays were developed to analyze the regulatory T cell response targeting transcripts for guinea pig FoxP3, IL-10, TGFβ, IRF4, STAT3, and T-bet. CT values were normalized to guinea pig 18S rRNA. These assays were validated for specific amplification of single amplicons by PCR and gel electrophoresis. PCR products from amplified cDNA using the indicated primer sets (Table 1) were also confirmed for their target specificity by Sanger sequencing (data not shown). Significant increases in RNA from VC2 vaccinated animals were observed as early as 1 h post challenge for IL-17A, IL-23A, FoxP3, IL-10, STAT3, and T-bet transcripts. Downstream effects of Th17 signaling were observed at 3 days post challenge with significant increases in IL-36β, IL-36γ, as well as the antimicrobial peptide DEFB3 (Fig. 4).

3.4. In-vitro vaginal epithelial culture

To investigate whether similar responses would be induced in human cells, cultured human vaginal epithelial cells were stimulated with 10 ng/mL IL-17A, 10 ng/mL IL-22, or a combination of 10 ng/mL IL-17A and 10 ng/mL IL-22, or left unstimulated. Total RNA was extracted at 24 h post stimulation and used in predesigned human qRTPCR assays for IL-36α, IL-36β, IL-36γ, and DEFB3. A significant increase in DEFB3 transcripts was only observed after combined stimulation with IL-17A and IL-22 (Fig. 5A). Specifically, DEFB3 transcripts were rapidly upregulated after stimulation with a combination of IL-17A and IL-22 with peak expression being observed within the first 3 h of stimulation (Fig. 5B). A 3 h pre-stimulation with IL-17, IL-22, IL-36γ, and purified human beta defensin 3 (DEFB3) was able to significantly reduce HSV-2 infectious virus produced 24 h post infection by approximately two thirds. Previous experiments demonstrated a

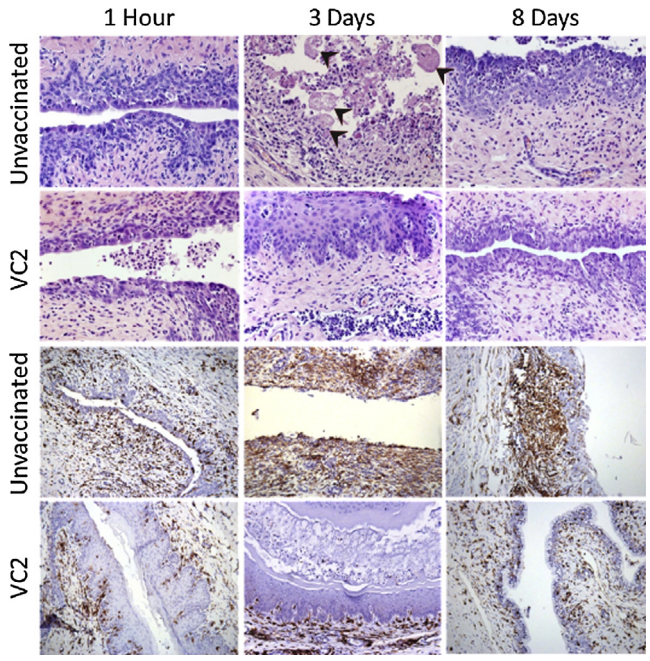

Fig. 3. Histology of vaginal tissue from unvaccinated and VC2-vaccinated animals after challenge with HSV-2. Unvaccinated animals developed moderate to severe erosive and ulcerative vaginitis at 3 days post challenge (H and E staining in top panels) and a significant infiltration of activated macrophage by Iba1 IHC (Bottom panels).

significantly reduced plaque size in pretreated and infected VK2 cells indicating a potential mechanism of antiviral response limiting the spread of infection (data not shown). The combination of IL-17 and IL-22 had the most pronounced effect on infectious virus production reducing viral titers to 1/3rd the untreated cell titer (Fig. 5C).

4. Discussion

Previously we have shown that the live-attenuated herpes simplex type 1 VC2 vaccine protects against virulent HSV-1 and HSV-2 challenge in the mouse model of lethal genital infection [5]. This work demonstrated that the VC2 vaccine stimulated a robust cellular and humoral immune response after single intramuscular vaccination sufficient to completely protect vaccinated animals from lethal HSV-1 and HSV-2 infection [5]. Importantly, neurons of mice were protected from infection by both HSV-1 and HSV-2 as evidenced by the inability to detect viral genomes in the sacral dorsal root ganglia of vaccinated and challenged animals [5]. Also, we have demonstrated that intramuscular vaccination with the VC2 vaccine stimulated the proliferation of $CD4^+$ and $CD8^+$ T cells in the vaginal mucosa and produced robust and long lasting cross reactive neutralizing antibodies in rhesus macaques [14]. Here we show that immunization of guinea pigs with VC2 produces an efficacious immune response capable of protecting guinea pigs from acute genital HSV-2 infection characterized by the rapid induction of IL-17A responses as early as 1 h post challenge in VC2 vaccinated animals resulting in the production of the antiviral peptide beta-defensin-3.

VC2 vaccinated animals showed significant protection against HSV-2 challenge characterized by reduction in viral replication in mucosal surfaces and a lack of pathogenic symptoms. Two-out-of-the-three VC2 vaccinated animals in the day 8 group had cleared the challenge virus from detectable levels in the vaginal tissue by the time of analysis. Histologically, the VC2-vaccinated animals demonstrated unremarkable pathogenesis over the study period (data not shown). The asymptomatic infection and lack of any histological pathogenesis or inflammation indicate that VC2 vaccination induced the immune system to respond rapidly and effectively to clear virus without the immunopathogenesis typically associated with wild-type HSV infection. qRTPCR analysis identified transcripts that were upregulated immediately upon challenge in the VC2 vaccinated animals in comparison to naïve animals suggesting that the encoded genes may be involved in the observed VC2-induced protection against HSV-2 genital infection. Interestingly, testing of the VC2 vaccine for its ability to protect against recurrent HSV-2 infection in the guinea pig model has indicated a significant reduction in recurrent vaginal lesions and virus shedding (Cardin, Bernstein, Stanfield and Kousoulas, in preparation).

IL-17A is a highly proinflammatory cytokine and when unregulated can lead to chronic inflammatory states and autoimmune diseases such as multiple sclerosis, collagen induced arthritis, experimental colitis, and psoriasis. The chronic inflammatory response in psoriasis requires the interaction of IL-17A and IL-22 with the IL-17 receptor (IL-17R) on the surface of epithelial cells and keratinocytes which then secrete pro-inflammatory IL-36 family molecules [26]. These molecules then signal to stimulate the secretion of antimicrobial peptides and promote the inflammatory response in the skin [27,28]. Regulation of Th17 responses is important to subvert the chronic inflammatory state observed in psoriatic lesions. This can be mediated by IL-10 secreting regulatory T cells, which are dependent on the phosphorylation of STAT3 [29]. Also, STAT3 is essential for the differentiation of immature T cells into effector $CD4^+$ Th17 cells [30–32].

In rhesus macaques, simian immunodeficiency virus depletion of Th17 cells results in the erosion of mucosal barriers and increased dissemination to mesenteric lymph nodes by *S. typhimurium* infection [33]. IL-17 confers protection to enteric pathogens by two distinct mechanisms. (1) By stimulating the secretion of CXC family chemokines that function in the recruitment of neutrophils, and (2) in the context of IL-22 together interacting with cells at the site of infection to produce antimicrobial peptides and defensins [34]. Memory Th17 responses have been shown to respond to both single antigen vaccines and in the complex context of microbial infection [35]. Polarizing dendritic cells to stimulate Th17 responses has demonstrated increased vaccine efficacy against genital HSV-2 infection and IL-17 KO mice are significantly more susceptible to HSV-2 infection demonstrating the importance of IL17 in mediating genital HSV-2 disease [25].

Human anti-microbial peptides have been shown to have antiviral activity against HSV-2. Specifically, all six alpha defensins and beta-defensin-3 exhibit anti-herpes activity [36]. However, the current understanding of tissue specific secretion of beta-defensin-3 and the mechanism by which beta-defensin-3 is produced to potentially counteract genital HSV-2 infection has not been explored. Anti-microbial peptides are secreted as an innate response to infection; however, under conditions primed by an adaptive Th17 response, epithelial cells at mucosal sites of infection can be stimulated to substantially increase production of anti-microbial peptides (Fig. 6). It is known that cells of the innate immune system can secrete IL-17A. Specifically, γδ T cells [37–39], NK cells expressing RORγτ+NKp46+ [40,41], and lymphoid-tissue inducer like cells [42] produce IL-17A and IL-22. However, innate immune responses of immunologically naïve animals should respond the same as the VC-2 vaccinated animals. These data suggest that intramuscular vaccination must stimulate herpes specific Th17 lymphocyte differentiation and migration to the genital mucosa where they mount an effective antiviral immune response limiting virus replication, spread and resultant immunopathogenesis.

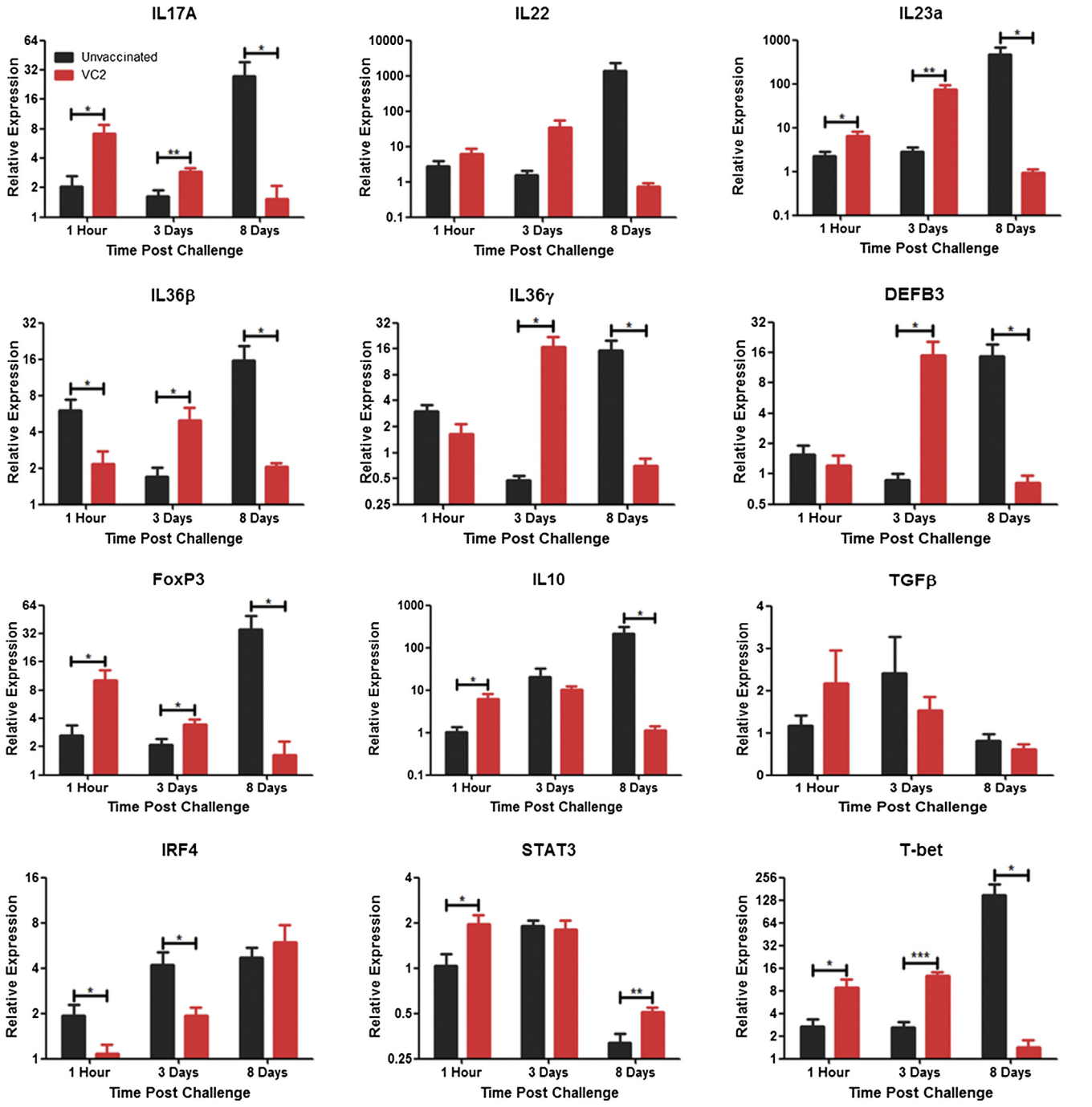

Fig. 4. Vaginal gene expression profiles of unvaccinated and VC2-vaccinated Guinea pigs. RNA isolated from vaginal tissue at the indicated time point post challenge was used in single transcript qRTPCR assays to analyze the Th17 (IL-17A, IL-22, and IL-23a) and regulatory T cell (FoxP3, IL-10, TGFβ, IRF4, STAT3, and T-bet) response. Downstream effects of Th17 signaling on epithelial cells was examined by analyzing the expression of IL-36β, IL-36γ, and beta defensin 3 (DEFB3).* $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$. Statistical analysis was conducted by the students T-test.

Here we demonstrate for the first time that a rapid IL-17 dependent response in the genital mucosa results in the production of beta-defensin-3 and contributes to inhibition of HSV-2 viral replication and resultant immunopathogenesis in vaginal mucosal surfaces of guinea pigs. This IL-17 induced innate response must work synergistically with humoral and cellular immune responses generated by the VC2 vaccine resulting in enhanced protection against HSV-2 disease. In addition, beta-defensin-3 inhibition of viral replication and spread may prevent efficient infection of neuronal termini in mucosal surfaces and establishment of HSV-2 latency in the guinea pig dorsal root ganglionic neurons.

Author summary

Human herpes simplex virus type 2 (HSV-2) is a ubiquitous human pathogen infecting ~50% of the global population. Infection with HSV-2 is typically associated with genital ulcerations and blisters which occur sporadically throughout the lifetime of the infected individual. HSV-2 is spread by direct skin to skin contact and can be transmitted in the absence of disease. Currently, there is no vaccine to prevent HSV-2 infection and mechanisms of protective immunity remain controversial. Here, we describe that intramuscular vaccination with a live-attenuated HSV vaccine

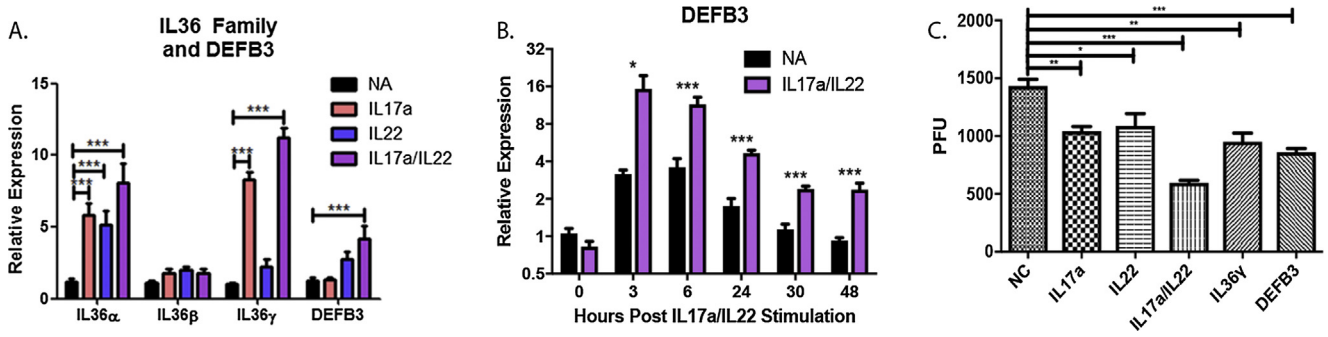

Fig. 5. In-vitro human vaginal epithelial culture. (A) Cultured human vaginal epithelial cells (VK2 cells) were left untreated (NA), or stimulated with either IL-17A, IL22, or both for 24 h and then subjected to qRTPCR analysis for IL36α, IL36β, IL36γ, and beta-defensin 3 (DEFB3). (B) The kinetics of DEFB3 expression in VK2 cells after IL-17A/IL22 stimulation was analyzed by qRTPCR analysis. (C) Antiviral effect of IL-17A/IL22, IL36 and DEFB3 stimulation of VK2 cells on HSV-2 virus titer 24 h post infection. Cells were pretreated for 3 h with indicated cytokine(s) and subsequently infected with ~100 pfu HSV-2 G. 24 h post infection lysates were collected and viral titers were determined via plaque assay. ** $p < 0.01$ *** $p < 0.001$. Statistical analysis was conducted by the Students T-test.

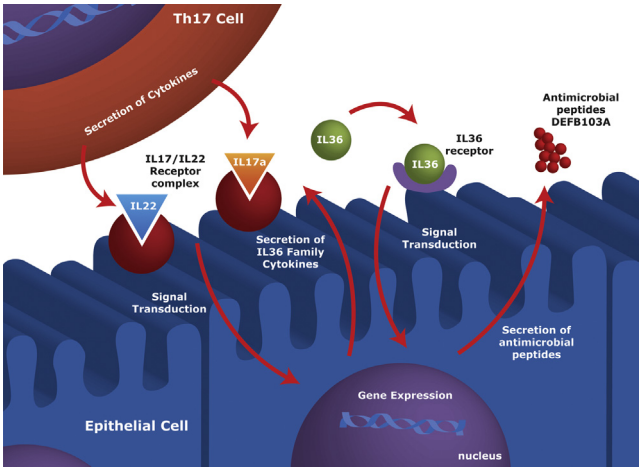

Fig. 6. Schematic of Th17-dependent antimicrobial peptide secretion. Th17 cells secrete IL-17A and IL22, which interact with their cognate receptors on epithelial cells resulting in the production of the IL36 family of cytokines that cause antimicrobial peptide secretion through an autocrine mechanism.

(VC2) stimulated vaginal IL-17A dependent antimicrobial peptide immune responses in the guinea pig model of genital HSV-2 infection. These responses were robust and protected animals from acquiring any appreciable disease. Human vaginal epithelial cells responded similarly after stimulation with IL-17A and IL-22. This stimulation was able to significantly reduce the amount of progeny HSV-2 produced after infection of human vaginal epithelial cells. Taken together, these data suggest a novel anti-herpes immune response which will aid the development of future genital herpes vaccines.

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References

[1] Gupta R, Warren T, Wald A. Genital herpes. Lancet 2007;370(9605):2127–37.
[2] Looker KJ, Garnett GP, Schmid GP. An estimate of the global prevalence and incidence of herpes simplex virus type 2 infection. Bull World Health Organ 2008;86(10):805–812, A.
[3] Fleming DT, McQuillan GM, Johnson RE, Nahmias AJ, Aral SO, Lee FK, et al. Herpes simplex virus type 2 in the United States, 1976 to 1994. N Engl J Med 1997;337(16):1105–11.
[4] Patel EU, Laeyendecker O, Hsieh YH, Rothman RE, Kelen GD, Quinn TC. Parallel declines in HIV and hepatitis C virus prevalence, but not in herpes simplex virus type 2 infection: a 10-year, serial cross-sectional study in an inner-city emergency department. J Clin Virol: Off Publ Pan Am Soc Clin Virol 2016;80:93–7.
[5] Stanfield BA, Stahl J, Chouljenko VN, Subramanian R, Charles AS, Saied AA, et al. A single intramuscular vaccination of mice with the HSV-1 VC2 virus with mutations in the glycoprotein K and the membrane protein UL20 confers full protection against lethal intravaginal challenge with virulent HSV-1 and HSV-2 strains. PLoS One 2014;9(10):e109890.
[6] Jambunathan N, Charles AS, Subramanian R, Saied AA, Naderi M, Rider P, et al. Deletion of a predicted beta-sheet domain within the amino terminus of herpes simplex virus glycoprotein K conserved among alphaherpesviruses prevents virus entry into neuronal axons. J Virol 2015;90(5):2230–9.
[7] Stanfield B, Kousoulas KG. Herpes simplex vaccines: prospects of live-attenuated HSV vaccines to combat genital and ocular infections. Curr Clin Microbiol Rep. 2015;2(3):125–36.
[8] Dasgupta G, BenMohamed L. Of mice and not humans: how reliable are animal models for evaluation of herpes CD8(+)-T cell-epitopes-based immunotherapeutic vaccine candidates? Vaccine 2011;29(35):5824–36.
[9] Mestas J, Hughes CC. Of mice and not men: differences between mouse and human immunology. J Immunol 2004;172(5):2731–8.
[10] Seok J, Warren HS, Cuenca AG, Mindrinos MN, Baker HV, Xu W, et al. Genomic responses in mouse models poorly mimic human inflammatory diseases. Proc Natl Acad Sci U S A 2013;110(9):3507–12.
[11] Prichard MN, Kaiwar R, Jackman WT, Quenelle DC, Collins DJ, Kern ER, et al. Evaluation of AD472, a live attenuated recombinant herpes simplex virus type 2 vaccine in guinea pigs. Vaccine 2005;23(46–47):5424–31.
[12] Stanberry LR. Evaluation of herpes simplex virus vaccines in animals: the guinea pig vaginal model. Rev Infect Dis 1991;13(Suppl 11):S920–3.
[13] Visalli RJ, Natuk RJ, Kowalski J, Guo M, Blakeney S, Gangolli S, et al. Vaccination with a HSV-2 UL24 mutant induces a protective immune response in murine and guinea pig vaginal infection models. Vaccine 2014;32(12):1398–406.
[14] Stanfield BA, Pahar B, Chouljenko VN, Veazey R, Kousoulas KG. Vaccination of rhesus macaques with the live-attenuated HSV-1 vaccine VC2 stimulates the proliferation of mucosal T cells and germinal center responses resulting in sustained production of highly neutralizing antibodies. Vaccine 2017;35(4):536–43.
[15] Kuklin NA, Daheshia M, Chun S, Rouse BT. Role of mucosal immunity in herpes simplex virus infection. J Immunol 1998;160(12):5998–6003.
[16] Johnson AJ, Chu CF, Milligan GN. Effector CD4+ T-cell involvement in clearance of infectious herpes simplex virus type 1 from sensory ganglia and spinal cords. J Virol 2008;82(19):9678–88.
[17] Bagri P, Anipindi VC, Nguyen PV, Vitali D, Stampfli MR, Kaushic C. Novel role for interleukin-17 in enhancing type 1 helper T cell immunity in the female genital tract following mucosal herpes simplex virus 2 vaccination. J Virol 2017;91(23).
[18] Guglani L, Khader SA. Th17 cytokines in mucosal immunity and inflammation. Curr Opin HIV AIDS 2010;5(2):120–7.
[19] Winkle SM, Throop AL, Herbst-Kralovetz MM. IL-36gamma augments host defense and immune responses in human female reproductive tract epithelial cells. Front Microbiol 2016;7:955.

[20] Feinen B, Jerse AE, Gaffen SL, Russell MW. Critical role of Th17 responses in a murine model of Neisseria gonorrhoeae genital infection. Mucosal Immunol 2010;3(3):312–21.
[21] Moore-Connors JM, Fraser R, Halperin SA, Wang J. CD4(+)CD25(+)Foxp3(+) regulatory T cells promote Th17 responses and genital tract inflammation upon intracellular Chlamydia muridarum infection. J Immunol 2013;191(6):3430–9.
[22] Vicetti Miguel RD, Quispe Calla NE, Pavelko SD, Cherpes TL. Intravaginal *Chlamydia trachomatis* challenge infection elicits TH1 and TH17 immune responses in mice that promote pathogen clearance and genital tract damage. PLoS One 2016;11(9):e0162445.
[23] Aujla SJ, Dubin PJ, Kolls JK. Th17 cells and mucosal host defense. Semin Immunol 2007;19(6):377–82.
[24] Conti HR, Shen F, Nayyar N, Stocum E, Sun JN, Lindemann MJ, et al. Th17 cells and IL-17 receptor signaling are essential for mucosal host defense against oral candidiasis. J Exp Med 2009;206(2):299–311.
[25] Anipindi VC, Bagri P, Roth K, Dizzell SE, Nguyen PV, Shaler CR, et al. Estradiol enhances CD4+ T-cell anti-viral immunity by priming vaginal DCs to induce Th17 responses via an IL-1-dependent pathway. PLoS Pathog 2016;12(5):e1005589.
[26] Carrier Y, Ma HL, Ramon HE, Napierata L, Small C, O'Toole M, et al. Inter-regulation of Th17 cytokines and the IL-36 cytokines in vitro and in vivo: implications in psoriasis pathogenesis. J Invest Dermatol 2011;131(12):2428–37.
[27] Blumberg H, Dinh H, Trueblood ES, Pretorius J, Kugler D, Weng N, et al. Opposing activities of two novel members of the IL-1 ligand family regulate skin inflammation. J Exp Med 2007;204(11):2603–14.
[28] Johnston A, Xing X, Guzman AM, Riblett M, Loyd CM, Ward NL, et al. IL-1F5, -F6, -F8, and -F9: a novel IL-1 family signaling system that is active in psoriasis and promotes keratinocyte antimicrobial peptide expression. J Immunol 2011;186(4):2613–22.
[29] Chaudhry A, Samstein RM, Treuting P, Liang Y, Pils MC, Heinrich JM, et al. Interleukin-10 signaling in regulatory T cells is required for suppression of Th17 cell-mediated inflammation. Immunity 2011;34(4):566–78.
[30] Adamson AS, Collins K, Laurence A, O'Shea JJ. The current STATus of lymphocyte signaling: new roles for old players. Curr Opin Immunol 2009;21(2):161–6.
[31] Littman DR, Rudensky AY. Th17 and regulatory T cells in mediating and restraining inflammation. Cell 2010;140(6):845–58.
[32] Xu S, Cao X. Interleukin-17 and its expanding biological functions. Cell Mol Immunol 2010;7(3):164–74.
[33] Raffatellu M, Santos RL, Verhoeven DE, George MD, Wilson RP, Winter SE, et al. Simian immunodeficiency virus-induced mucosal interleukin-17 deficiency promotes Salmonella dissemination from the gut. Nat Med 2008;14(4):421–8.
[34] Blaschitz C, Raffatellu M. Th17 cytokines and the gut mucosal barrier. J Clin Immunol 2010;30(2):196–203.
[35] Becattini S, Latorre D, Mele F, Foglierini M, De Gregorio C, Cassotta A, et al. T cell immunity. Functional heterogeneity of human memory CD4(+) T cell clones primed by pathogens or vaccines. Science 2015;347(6220):400–6.
[36] Hazrati E, Galen B, Lu W, Wang W, Ouyang Y, Keller MJ, et al. Human alpha- and beta-defensins block multiple steps in herpes simplex virus infection. J Immunol 2006;177(12):8658–66.
[37] Lockhart E, Green AM, Flynn JL. IL-17 production is dominated by gammadelta T cells rather than CD4 T cells during Mycobacterium tuberculosis infection. J Immunol 2006;177(7):4662–9.
[38] Martin B, Hirota K, Cua DJ, Stockinger B, Veldhoen M. Interleukin-17-producing gammadelta T cells selectively expand in response to pathogen products and environmental signals. Immunity 2009;31(2):321–30.
[39] Umemura M, Yahagi A, Hamada S, Begum MD, Watanabe H, Kawakami K, et al. IL-17-mediated regulation of innate and acquired immune response against pulmonary Mycobacterium bovis bacille Calmette-Guerin infection. J Immunol 2007;178(6):3786–96.
[40] Cella M, Fuchs A, Vermi W, Facchetti F, Otero K, Lennerz JK, et al. A human natural killer cell subset provides an innate source of IL-22 for mucosal immunity. Nature 2009;457(7230):722–5.
[41] Satoh-Takayama N, Vosshenrich CA, Lesjean-Pottier S, Sawa S, Lochner M, Rattis F, et al. Microbial flora drives interleukin 22 production in intestinal NKp46+ cells that provide innate mucosal immune defense. Immunity 2008;29(6):958–70.
[42] Takatori H, Kanno Y, Watford WT, Tato CM, Weiss G, Ivanov II, et al. Lymphoid tissue inducer-like cells are an innate source of IL-17 and IL-22. J Exp Med 2009;206(1):35–41.