Toll-like Receptors: Important Immune Checkpoints in the Regression of Cervical Intra-epithelial Neoplasia 2

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Abstract

Toll-like receptors (TLRs) are innate immune defenders thought to be critical for the clearance of Human Papillomavirus (HPV) infections hence preventing the development of HPV-associated high-grade cervical intra-epithelial neoplasia (CIN2 or 3), a potential cervical cancer precursor. However, the role of TLRs in the regression of established cervical lesions, such as CIN2, is hindered by a lack of prospective design studies. Using SYBR green real-time PCR assays, we have examined the gene expression of TLR2, TLR3, TLR7, TLR8, and TLR9, in cytobrush collected endocervical cells of 63 women diagnosed with CIN2 at study entry (baseline) and followed over a 3-year period. Wilcoxon rank-sum test was used to examine the association between TLR expression levels, measured at baseline, and CIN2 outcome (regression versus persistence/progression) over time. HPV genotyping was performed using Roche Linear Array Assay detecting 37 HPV types. Women with CIN2 regression showed significantly higher baseline levels of TLR2 (p=0.006) and TLR7 (p=0.007), as well as a non-significant trend for a higher TLR8 expression (p=0.053) compared to women with CIN2 persistence/progression. Six women with CIN2 regression, who presented with an HR-HPV DNA-negative CIN2 lesion at study entry, had significantly higher baseline levels of TLR2 (p=0.005), TLR7 (p=0.013), and TLR8 (p=0.012), compared to women with CIN2 persistence/progression, suggesting their role in clearance of HPV prior to clearance of the lesion. Our results confirm a key role of TLRs in regression of CIN2 and support the potential use of TLR-agonists for treatment of these lesions.

Keywords

Toll-like receptors; Human Papillomavirus; Cervical Intra-epithelial neoplasia 2; CIN2 regression

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INTRODUCTION

Persistent infection with one of the 12 carcinogenic or high-risk human papillomavirus (HR-HPV) types, is a prerequisite for the development of cervical cancer [1]. Cervical cancer develops through a series of molecular events which are reflected by histologic changes. True pre-cancers are considered to be represented by histologic alterations referred to as cervical intra-epithelial neoplasia (CIN) grades 2 and 3 whereas CIN grade 1 is considered a benign reversible lesion, with regression rate of 90% among adolescents and young women [2, 3]. CIN2 lesions can also spontaneously regress though at the lower rate [4–6]. Ours and other studies have shown that 70% of CIN2 cases in young women regress within three years without any intervention [3, 6, 7]. Underlying immune mechanisms critical for regression of such established lesions have not been well defined. Therefore, identification of targets for treatment of CIN2 and/or novel biomarkers to predict lesion progression, are continuously being sought out in order to prevent overtreatment and provide more specific/personalized medical care.

The host immune responses to cervical HPV are considered instrumental in shaping the natural history of HPV infection. Key components of that response include the initial pathogen recognition by the innate immune system, cytokines that bridge the innate and adaptive responses and mediate the latter, cytotoxic effector mechanisms, and regulatory cells and mechanisms that modulate the strength and duration of the response [8–13]. Among these, innate immune cells and receptors, such are toll-like receptors (TLRs) have been garnering increasing attention as therapeutics for treatment of HPV-associated diseases [14]. However, the roles of different TLRs in the natural history of CIN have not been studied in detail.

TLRs are central molecules of the innate immune system engaged in the continuous detection of highly conserved, non-self, structural motifs known as pathogen-associated microbial patterns (PAMPs), exclusively expressed by microbial pathogens. Besides PAMPs, TLRs can also sense endogenous molecules released from necrotic or dying cells known as danger-associated molecular patterns (DAMPs). Of the ten TLRs characterized to date in humans, several are particularly important in viral infections and are also expressed in keratinocytes within the female genital tract (the cells HPV infects) [15]. Intracellularly localized TLRs recognize nucleic acid motifs and include TLR3 (dsRNA), TLR7 (ssRNA), TLR8 (ssRNA), and TLR9 (CpG DNA); while cell surface-localized TLRs, such are heterodimers of TLR2, are important bacterial lipoprotein and viral protein sensors [16–18]. It has been described that viral dsDNA, or viral dsRNA (potential intermediate during viral replication), as well as viral proteins such as HPV L1 and L2 capsid proteins, can act as potential PAMPs which trigger TLR signaling [13, 19, 20].

We have previously demonstrated that high cervical mucosal expression levels of TLR2, TLR3, TLR7, TLR8 and TLR9 upon incident HPV16 infection were critical for HPV16 clearance by the following (4-month) visit [13, 21], suggesting both a role for TLRs in the successful host response to HPV and a mechanism by which HPV16 can evade immune recognition, persist, and drive lesion progression. In the present study, we explored the role of these TLRs in the regression of an established cervical neoplastic lesion, CIN2. We have

measured, in a cross-sectional design, cervical mRNA expression of TLR2, TLR3, TLR7, TLR8, and TLR9 in adolescent and young women with biopsy-confirmed CIN2 at the study entry (baseline), and followed over a 3-year period. We hypothesized that TLR baseline levels would be the highest in women whose CIN2 lesions regressed and who at the same time cleared their baseline HR-HPV infection.

MATERIALS AND METHODS

Study design.

The recruitment of women for the parental study on the Natural History of CIN2 took place from 2002 – 2007 in Northern California, USA [6, 22]. The study was approved by the institutional review boards of the University of California, San Francisco (UCSF) and Kaiser Permanente, Northern California (KPNC), respectively. Women aged 13 to 25 years referred for abnormal cytology while visiting one of the 12 KPNC participating clinics, with CIN2 diagnosed on H&E histology, were recruited and followed at 4 – 6 month intervals for on an average of 3 years. Exclusion criteria included previous treatment for CIN, immunosuppression, pregnancy, or planning to leave the area within 3 years. Analysis included histologically confirmed CIN2 and at least one follow-up visit resulting in the participation of 95 women. Details of the study design have been previously published [6]. Biological samples obtained at each of the visits included a cytobrush collected endocervical cells stored in RNAlater for RNA analysis. Cervical biopsies were collected at the study entry (for all 95 women) and the study exit (for 68% of 95 participating women), and at the intermediate visits if the colposcopist was concerned about progression, or if the cytology on any visit suggested progression [6]. Two pathologists reviewed each cervical biopsy collected at the baseline, while CIN3 diagnosis, if established, was confirmed by a 3rd pathologist and women were exited for treatment. All study cytology and histology samples during follow-up were sent to the centralized laboratory to be reviewed by a single pathologist (TMD). During the interval of our study p16INK4a immunostaining was not a recommended/ standard practice for confirmation of CIN2 diagnosis. The study entry biopsies could not be retrieved from the original centers for p16INK4a immunostaining after The Lower Anogenital Squamous Terminology (LAST) Standardization Project for HPV-Associated Lesions was introduced in 2012 [23].

HPV testing.

Samples for cytology and HPV were immediately placed into liquid based media (PreservCyt; Hologic Corp, Marlborough, MA) and were sent to the UCSF laboratory where 9 mL was removed for amplification and genotyping as described [6, 22]. HPV testing was performed using the Roche Linear Array Assay (Roche Molecular Diagnostics, In, Alameda, CA) which detects 37 different HPV types: 12 HR-HPV (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59), nine probable/possible (p)HR-HPV (26, 53, 66, 67, 68, 69, 70, 73, and 82), and 16 low-risk (LR-)HPV types (6, 11, 40, 42, 54, 55, 61, 62, 64, 71, 72, 81, 83, 84, IS39, and CP6108) [1].

Definition of CIN2 lesion regression, persistence, or progression.

Definitions of CIN2 regression, persistence or progression for this cohort have been previously described [6]. In brief, the definition of regression was based on having three consecutive visits with negative cytology, and negative biopsy on any of these visits (CIN0), if available. If there was an insufficient follow-up (i.e., only a single visit with negative cytology), the analysis was censored at the last visit with abnormal cytology or histology. Women who continued to have a low grade (L) or high grade (H) squamous intraepithelial lesion (SIL) on cytology, or CIN1 or 2 on histology at the end of follow-up, were considered non-regressors, i.e. having persistent CIN2. The definition of progression required a biopsy-proven CIN3 or greater at any visit after the baseline diagnosis of CIN2. Time to CIN2 regression or progression was defined as the time between the time point of CIN2 detection and the first of the three consecutive visits with normal cytology/histology (CIN0/LSIL), or the first CIN3 diagnosis [6].

RNA Extraction.

Of 95 women who fulfilled requirements for participation in the study on Natural History of CIN2 [6, 22], 63 women had available endocervical cell specimens from the baseline visit for TLRs mRNA analysis. Specimen collection by cytology brush and subsequent RNA extraction using TRI Reagent (Molecular Research Center, Cincinnati, Ohio) have been previously described [24]. One μg total mRNA was DNase-treated using TURBO DNA-free Kit (Ambion, Austin, TX, USA), following the manufacturer's instructions. Reverse transcription was performed using 0.5 μg DNase-treated RNA in a 20 μl reaction volume with random hexamer (37 ng/μl final concentration, Promega, Billerica, Massachusetts) and random nonamer (25 μM, Gene Link, Hawthorne, New York) priming, RNasin RNase inhibitor (10 U/reaction, Promega), and OmniScript RT (4 U/reaction, Qiagen, Valencia, California) at 37°C for 60 min, following the manufacturer's instructions.

TLR Testing by Quantitative Reverse-Transcription PCR.

Quantitative PCR was performed in triplicate in a 384-well plate format on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, California) using Power SYBR Green Master Mix (Applied Biosystems), forward and reverse primers (Integrated DNA Technologies, Coralville, Iowa) at 150 nM final concentration, and either cervical unknown cDNA template (12.5 ng/reaction), standard, or water for the no-template control. Primer sequences for TLRs selection and assay validation have been previously described [24]. Standard curves for each plate were prepared from Stratagene qPCR Human Reference Total RNA (Stratagene, La Jolla, California), reverse transcribed identically to the cervical RNA samples, and diluted in 4-fold steps from 80 ng/reaction (final concentration) to 0.078 ng/reaction. The amplification program was run as described [24]. Quantitative (threshold cycle) PCR values were obtained during exponential amplification. TLR expression, in relative units calculated by the efficiency-corrected method using amplification efficiencies determined from standard-curve slopes [25], was normalized to the geometric mean [26] of two reference genes previously validated for cervical samples in HPV studies; Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH), and Ribosomal Protein Lateral stalk subunit P0 (RPLP0) [24].

Data Analysis.

Because normalized TLR expression levels were not normally distributed, Wilcoxon rank-sum test was used to examine associations between follow-up (independent variable) and TLR expression (dependent variable). GraphPad Prism 7.0 software was used for statistical calculations and preparation of figures. Fisher exact probability test was used to examine the significance of the contingency between different groups (CIN2 regression vs. CIN2 persistence/progression).

RESULTS

Study population

This study included 63 young women (average age 20.9, SD 2.3 years; range 16.3 – 25.0 years), with histologically confirmed CIN2 diagnosis at study entry (baseline). Women were followed on average for 19.2 months (range 4.9 – 34.4 months). At the end of the follow-up, 67% (42/63) of women showed CIN2 regression (CIN2 → CIN0), 20% (13/63) showed lesion persistence (CIN2 → CIN1 or 2) and 13% (8/63) presented with CIN2 progression (CIN2 → CIN3), respectively.

There were no differences in any of characteristics examined between women with CIN2 lesion persistence/progression vs. CIN2 regression (Table 1).

HPV Type(s) at the Baseline and Relation with CIN2 Regression vs. CIN2 Persistence/ Progression

At baseline, 90% (57/63) of women were positive for HPV DNA of one or more HR-HPV types. Six (10%) women with CIN2 had no detectable HR-HPV type at the baseline or at any other CIN2 visit. Among CIN2 lesions positive for a single HR-HPV type (n=31), the most common type was HPV16 (n=12), followed by HPV31 (n=4), HPV18 (n=3), and HPV52 (n=3). Infections with pHR- or LR-HPV types occurred in 43% (26/61) of all HPV DNA+ cases.

HR-HPV persistence was more common in women with CIN2 persistence/progression compared to those with CIN2 regression (43% vs. 17%; p=0.027). HPV16 persistence was also more common among women with persistence/progression vs. those with regression (64% vs. 27%; p=0.099). All six women who were negative for any of the 12 HR-HPV types at the baseline had CIN2 regression. These results are summarized in Table 2.

TLR Expression in Women with CIN2 Regression vs. CIN2 Persistence/Progression

Women with CIN2 regression had significantly higher levels of TLR2 (p=0.006) and TLR7 (p=0.007) and showed a non-significant trend for a higher expression of TLR8 (p=0.053) compared to women with CIN2 persistence/progression (Figure 1A). We separated out the six women who were negative for HR-HPV DNA at their baseline visit. Each of these six women showed CIN2 regression and presented with significantly higher levels of TLR2 (p=0.005), TLR7 (p=0.013), and TLR8 (p=0.012), and a trend for a higher expression of TLR9 (p=0.110) compared to women with CIN2 persistence/progression (Figure 1B). TLR2 (p=0.018) and TLR7 (p=0.017) levels remained significantly higher among CIN2 regressors

who were positive for HR-HPV at baseline compared to women with CIN2 persistence/ progression (Figure 1B).

DISCUSSION

Established cervical cancer precursors such as CIN2 have a regression rate of up to 70%, especially in young women [4, 27, 28]. Since the precise immune mechanisms by which such HPV-associated pre-cancers resolve are not well understood, we evaluated the role of TLRs (TLR2, 3, 7, 8 and 9) in the regression of CIN2. Our data suggest that high levels of TLR2, TLR7, and potentially TLR8 in cervical mucosa are important for CIN2 regression. As this study examined a single, baseline time point, it was not possible to define if it was the TLRs upregulation that lead to lesion regression or, TLRs downregulation that lead to lesion progression/persistence. The levels of TLRs 2, 7, 8, as well as TLR9, were even higher in the small subset of six women who were regressors, had CIN2 at the H&E histology, but were negative for any of the 12 HR-HPV types [1] at the time point of CIN2 diagnosis. This finding underscores the importance of TLRs in viral clearance as suggested before [21], and necessity of viral recognition for the upregulation of these TLRs.

We previously showed that mRNA expression of protein-sensing TLR2, along with viral nucleic acid-sensing TLR3, TLR8, and TLR9 increases upon incident HPV infection (over a pre-infection baseline measurement) in women who show HPV16 clearance on 4-months follow-up compared with those who do not [21]. This finding significantly correlated with increases in interferon-alpha2 in cervical lavage specimens, suggesting a key role for TLRs and downstream cytokines in viral clearance [21]. As an extension of that incident study, we also examined the association of TLR expression in the clearance of HPV16 persistent infections and we found similar associations with elevated TLRs 3, 7, 8 and 9 but only in presence of measurable cell-mediated immune response to HPV16 E6 in the peripheral blood [13]. This finding suggested an important link between innate and adaptive immunity in the control of HPV infections following periods of persistence [13]. The important role of TLRs in HPV persistence and lesion progression is supported by *in vitro* studies demonstrating direct downregulation of TLRs by HPV types, specifically downregulation of TLR9 by HPV16 [29] as well as studies suggesting that specific TLR polymorphisms may be genetic factors contributing to cervical cancer development [30–33].

Mechanisms involving TLR expression and regression of CIN2 are likely complex. TLR activation results in expression of co-stimulatory molecules on dendritic cells and production of inflammatory cytokines that polarize T-helper (Th)1 immune responses to viral infections [34]. Our laboratory has previously demonstrated that a Th1 pattern (expression of interferon-gamma (IFN-gamma) mRNA and absence of IL-4 mRNA) precedes clearance of HPV infection in young women without an HPV-associated lesion supporting our *in vivo* findings [35]. The finding associated with TLR2 was of particular interest since its thought to be primarily involved in recognition of bacterial cell wall components. TLR2 has a critical role in expansion of regulatory T cells (Tregs) [36–39] and previous studies from our group showed that CIN2/3 lesions had higher levels of mucosal Forkhead box P3 (Foxp3), a transcription regulator of Tregs, than samples with normal histology or CIN1 [40]. Therefore, *in vivo*

studies incorporating other immune markers, including known inhibitory molecules such are Foxp3, PD-1 and PD-L1, would be informative.

Due to their anti-tumor potential, many TLR agonists have been studied in *in vitro* and animal studies, and developed either as monotherapy, as adjuvants to vaccination, or other therapeutic modalities [41, 42]. In the context of HPV, synthetic TLR7-agonist imiquimod is of particular interest for the treatment of HPV-related disease [14]. The successful use of imiquimod in the treatment of genital warts was first demonstrated two decades ago [43]. Since then, application of imiquimod was also shown to be effective in the treatment of cervical, vaginal and vulvar intra-epithelial neoplasia as well [14].

However, TLRs seem to have complex roles in development of different cancer types including both tumor-suppressive and tumor-promoting effects [44, 45]. As mentioned, continuous upregulation of TLRs might result in the activation of several inflammatory and tumor-promoting pathways via NF-κB pathway [34], including upregulation of Tregs, or PD-L1; immunosuppressants which are known to be upregulated in cervical pre-cancer and cancer [46, 47]. These concepts are supported by multiple studies demonstrating significantly higher expression of TLRs in cervical cancer cell lines and invasive cervical cancer biopsies compared to cervical dysplasia and/or normal cervix, respectively [48]. Interestingly, DeCarlo and colleagues have demonstrated that the upregulation of TLR2, 7, 8, and 9 in cervical cancer seem to be associated with stromal and not epithelial compartment, while in cervical precancerous lesions both stromal and epithelial compartments exhibited upregulation of most TLRs [49].

Our study has few limitations. One of them is sample size. Because most women 25 years old are expected to clear their CIN2 lesion within 12 – 36 months observation period [4, 27, 28, 50] the number of women in the persistence/progression group was relatively small. Also, at the time of this study, p16INK4a immunostaining was not a standard practice for confirmation of CIN2 diagnosis on H&E, which is a less reproducible stand-alone diagnosis than CIN3 [51]. The Lower Anogenital Squamous Terminology (LAST) Standardization Project for HPV-Associated Lesions, which recommends when p16INK4a immunostaining should be used in combination with histology to describe HPV-associated precancerous lesions, was only introduced in 2012 [23]. In addition, the source of TLRs (i.e. macrophages, dendritic, epithelial or other cell types) remains unknown in our study and it is possible that the proportion of dysplastic to normal cells might influence TLR expression. Unfortunately, identification of specific cell types was not possible. Therefore, an important step for future studies would be to define the source of TLR mRNA and whether the epithelial make-up of the samples influences TLR expression. Another limitation was the lack of suitable specimens in the parent study design for studying downstream cytokine protein levels (e.g., interferon alpha2) to document activation of these TLRs [21]. Finally, this study examined a single baseline time point with many women requiring several years before lesion regression. Therefore, studying the expression of TLRs closer to the time point of lesion regression may be more informative.

To our knowledge, ours is the first study reporting on the association between cervical TLRs expression and CIN2 regression. Combined with our previous findings demonstrating the

importance of TLRs in the clearance of incident HPV infections (specifically HPV16) in women without an established cervical disease, this study highlights the important protective role of the innate immune system at different stages of HPV infection and sequelae. Detailed understanding of host immune responses, as well as viral factors that influence whether a lesion progresses or regresses, is important to make the right choice of today's available immunotherapeutics for HPV-associated disease [14, 52]. In addition, cytology and HPV testing are more powerful biomarkers of cervical lesion progression or regression when combined than as stand-alone biomarkers [53, 54] and a recent study suggested that measuring multiple clinical biomarkers might be helpful in accurately predicting regression of cervical lesions [55]. Our data suggest that measurement of TLR levels in cervical mucosa of women with HR-HPV DNA+ CIN2 lesions might be suitable in helping differentiate women who require only careful monitoring over time vs. those for whom excisional procedures would be the most beneficial early option, and support the potential use of TLR-agonists for treatment of these lesions.

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Abbreviations:

HPV	Human Papillomavirus
TLR	Toll-like receptor
CIN	Cervical Intra-epithelial Neoplasia

REFERENCES

1. IARC. Human papillomaviruses. IARC Monogr Eval Carcinog Risks Hum. Rev Hum Carcinog Part B Biol Agents 100B:1–475 2011.

2. Cox JT, Schiffman M, Solomon D, Group A-LTS. Prospective follow-up suggests similar risk of subsequent cervical intraepithelial neoplasia grade 2 or 3 among women with cervical intraepithelial neoplasia grade 1 or negative colposcopy and directed biopsy. Am J Obstet Gynecol 2003;188: 1406–12. [PubMed: 12824970]

3. Moscicki AB, Shiboski S, Hills NK, Powell KJ, Jay N, Hanson EN, Miller S, Canjura-Clayton KL, Farhat S, Broering JM, Darragh TM. Regression of low-grade squamous intra-epithelial lesions in young women. Lancet 2004;364: 1678–83. [PubMed: 15530628]

4. Trimble CL, Piantadosi S, Gravitt P, Ronnett B, Pizer E, Elko A, Wilgus B, Yutzy W, Daniel R, Shah K, Peng S, Hung C, et al. Spontaneous regression of high-grade cervical dysplasia: effects of human papillomavirus type and HLA phenotype. Clin Cancer Res 2005;11: 4717–23. [PubMed: 16000566]

5. Castle PE, Schiffman M, Wheeler CM, Solomon D. Evidence for frequent regression of cervical intraepithelial neoplasia-grade 2. Obstet Gynecol 2009;113: 18–25. [PubMed: 19104355]

6. Moscicki AB, Ma Y, Wibbelsman C, Darragh TM, Powers A, Farhat S, Shiboski S. Rate of and risks for regression of cervical intraepithelial neoplasia 2 in adolescents and young women. Obstet Gynecol 2010;116: 1373–80. [PubMed: 21099605]

7. Moore K, Cofer A, Elliot L, Lanneau G, Walker J, Gold MA. Adolescent cervical dysplasia: histologic evaluation, treatment, and outcomes. Am J Obstet Gynecol 2007;197: 141 e1–6. [PubMed: 17689626]

8. Kanodia S, Fahey LM, Kast WM. Mechanisms used by human papillomaviruses to escape the host immune response. Curr Cancer Drug Targets 2007;7: 79–89. [PubMed: 17305480]

9. Fahey LM, Raff AB, Da Silva DM, Kast WM. Reversal of human papillomavirus-specific T cell immune suppression through TLR agonist treatment of Langerhans cells exposed to human papillomavirus type 16. J Immunol 2009;182: 2919–28. [PubMed: 19234187]

10. Stanley M HPV - immune response to infection and vaccination. Infect Agent Cancer 2010;5: 19. [PubMed: 20961432]

11. Stanley MA. Epithelial cell responses to infection with human papillomavirus. Clin Microbiol Rev 2012;25: 215–22. [PubMed: 22491770]

12. Hibma MH. The immune response to papillomavirus during infection persistence and regression. Open Virol J 2012;6: 241–8. [PubMed: 23341859]

13. Scott ME, Ma Y, Farhat S, Moscicki AB. Expression of nucleic acid-sensing Toll-like receptors predicts HPV16 clearance associated with an E6-directed cell-mediated response. Int J Cancer 2015;136: 2402–8. [PubMed: 25346143]

14. de Witte CJ, van de Sande AJ, van Beekhuizen HJ, Koeneman MM, Kruse AJ, Gerestein CG. Imiquimod in cervical, vaginal and vulvar intraepithelial neoplasia: a review. Gynecol Oncol 2015;139: 377–84. [PubMed: 26335596]

15. Nasu K, Narahara H. Pattern recognition via the toll-like receptor system in the human female genital tract. Mediators Inflamm 2010;2010: 976024. [PubMed: 20396665]

16. Takeuchi O, Sato S, Horiuchi T, Hoshino K, Takeda K, Dong Z, Modlin RL, Akira S. Cutting edge: role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins. J Immunol 2002;169: 10–4. [PubMed: 12077222]

17. Xagorari A, Chlichlia K. Toll-like receptors and viruses: induction of innate antiviral immune responses. Open Microbiol J 2008;2: 49–59. [PubMed: 19088911]

18. Henrick BM, Yao XD, Rosenthal KL, team Is. HIV-1 Structural Proteins Serve as PAMPs for TLR2 Heterodimers Significantly Increasing Infection and Innate Immune Activation. Front Immunol 2015;6: 426. [PubMed: 26347747]

19. Diebold SS, Kaisho T, Hemmi H, Akira S, Reis e Sousa C. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. Science 2004;303: 1529–31. [PubMed: 14976261]

20. Weber F, Wagner V, Rasmussen SB, Hartmann R, Paludan SR. Double-stranded RNA is produced by positive-strand RNA viruses and DNA viruses but not in detectable amounts by negative-strand RNA viruses. J Virol 2006;80: 5059–64. [PubMed: 16641297]

21. Daud II, Scott ME, Ma Y, Shiboski S, Farhat S, Moscicki AB. Association between toll-like receptor expression and human papillomavirus type 16 persistence. Int J Cancer 2011;128: 879–86. [PubMed: 20473890]

22. Moscicki AB, Ma Y, Wibbelsman C, Powers A, Darragh TM, Farhat S, Shaber R, Shiboski S. Risks for cervical intraepithelial neoplasia 3 among adolescents and young women with abnormal cytology. Obstet Gynecol 2008;112: 1335–42. [PubMed: 19037044]

23. Darragh TM, Colgan TJ, Cox JT, Heller DS, Henry MR, Luff RD, McCalmont T, Nayar R, Palefsky JM, Stoler MH, Wilkinson EJ, Zaino RJ, et al. The Lower Anogenital Squamous Terminology Standardization Project for HPV-Associated Lesions: background and consensus recommendations from the College of American Pathologists and the American Society for Colposcopy and Cervical Pathology. Arch Pathol Lab Med 2012;136: 1266–97. [PubMed: 22742517]

24. Daud II, Scott ME. Validation of reference genes in cervical cell samples from human papillomavirus-infected and -uninfected women for quantitative reverse transcription-PCR assays. Clin Vaccine Immunol 2008;15: 1369–73. [PubMed: 18632922]

25. Bookout AL, Cummins CL, Mangelsdorf DJ, Pesola JM, Kramer MF. High-throughput real-time quantitative reverse transcription PCR. Curr Protoc Mol Biol 2006;**Chapter 15**: Unit 15 8.**Chapter 15**

26. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 2002;3: RESEARCH0034.

27. Discacciati MG, de Souza CA, d'Otavianno MG, Angelo-Andrade LA, Westin MC, Rabelo-Santos SH, Zeferino LC. Outcome of expectant management of cervical intraepithelial neoplasia grade 2 in women followed for 12 months. Eur J Obstet Gynecol Reprod Biol 2011;155: 204–8. [PubMed: 21193261]

28. Moscicki AB, Cox JT. Practice improvement in cervical screening and management (PICSM): symposium on management of cervical abnormalities in adolescents and young women. J Low Genit Tract Dis 2010;14: 73–80. [PubMed: 20043357]

29. Hasan U Human papillomavirus (HPV) deregulation of Toll-like receptor 9. Oncoimmunology 2014;3: e27257. [PubMed: 24605267]

30. Pandey S, Mittal B, Srivastava M, Singh S, Srivastava K, Lal P, Mittal RD. Evaluation of Toll-like receptors 3 (c.1377C/T) and 9 (G2848A) gene polymorphisms in cervical cancer susceptibility. Mol Biol Rep 2011;38: 4715–21. [PubMed: 21132533]

31. Roszak A, Lianeri M, Sowinska A, Jagodzinski PP. Involvement of Toll-like Receptor 9 polymorphism in cervical cancer development. Mol Biol Rep 2012;39: 8425–30. [PubMed: 22714906]

32. Zidi S, Verdi H, Yilmaz-Yalcin Y, Yazici AC, Gazouani E, Mezlini A, Atac FB, Yacoubi-Loueslati B. Involvement of Toll-like receptors in cervical cancer susceptibility among Tunisian women. Bull Cancer 2014;101: E31–5. [PubMed: 25373689]

33. Jin Y, Qiu S, Shao N, Zheng J. Association of toll-like receptor gene polymorphisms and its interaction with HPV infection in determining the susceptibility of cervical cancer in Chinese Han population. Mamm Genome 2017;28: 213–9. [PubMed: 28497211]

34. Lu H TLR Agonists for Cancer Immunotherapy: Tipping the Balance between the Immune Stimulatory and Inhibitory Effects. Front Immunol 2014;5: 83. [PubMed: 24624132]

35. Scott M, Stites DP, Moscicki AB. Th1 cytokine patterns in cervical human papillomavirus infection. Clin Diagn Lab Immunol 1999;6: 751–5. [PubMed: 10473530]

36. Sutmuller R, Garritsen A, Adema GJ. Regulatory T cells and toll-like receptors: regulating the regulators. Ann Rheum Dis 2007;66 Suppl 3: iii91–5. [PubMed: 17934105]

37. Dai J, Liu B, Li Z. Regulatory T cells and Toll-like receptors: what is the missing link? Int Immunopharmacol 2009;9: 528–33. [PubMed: 19539562]

38. Sutmuller RP, den Brok MH, Kramer M, Bennink EJ, Toonen LW, Kullberg BJ, Joosten LA, Akira S, Netea MG, Adema GJ. Toll-like receptor 2 controls expansion and function of regulatory T cells. J Clin Invest 2006;116: 485–94. [PubMed: 16424940]

39. Liu H, Komai-Koma M, Xu D, Liew FY. Toll-like receptor 2 signaling modulates the functions of CD4+ CD25+ regulatory T cells. Proc Natl Acad Sci U S A 2006;103: 7048–53. [PubMed: 16632602]

40. Scott ME, Ma Y, Kuzmich L, Moscicki AB. Diminished IFN-gamma and IL-10 and elevated Foxp3 mRNA expression in the cervix are associated with CIN 2 or 3. Int J Cancer 2009;124: 1379–83. [PubMed: 19089920]

41. Smits EL, Cools N, Lion E, Van Camp K, Ponsaerts P, Berneman ZN, Van Tendeloo VF. The Toll-like receptor 7/8 agonist resiquimod greatly increases the immunostimulatory capacity of human acute myeloid leukemia cells. Cancer Immunol Immunother 2010;59: 35–46. [PubMed: 19449004]

42. Vasilakos JP, Tomai MA. The use of Toll-like receptor 7/8 agonists as vaccine adjuvants. Expert Rev Vaccines 2013;12: 809–19. [PubMed: 23885825]

43. Edwards L, Ferenczy A, Eron L, Baker D, Owens ML, Fox TL, Hougham AJ, Schmitt KA. Self-administered topical 5% imiquimod cream for external anogenital warts. HPV Study Group. Human PapillomaVirus. Arch Dermatol 1998;134: 25–30. [PubMed: 9449906]

44. Wolska A, Lech-Maranda E, Robak T. Toll-like receptors and their role in carcinogenesis and anti-tumor treatment. Cell Mol Biol Lett 2009;14: 248–72. [PubMed: 19096763]

45. Mikulandra M, Pavelic J, Glavan TM. Recent findings on the application of Toll-like receptors agonists in cancer therapy. Curr Med Chem 2017.

46. Visser J, Nijman HW, Hoogenboom BN, Jager P, van Baarle D, Schuuring E, Abdulahad W, Miedema F, van der Zee AG, Daemen T. Frequencies and role of regulatory T cells in patients with (pre)malignant cervical neoplasia. Clin Exp Immunol 2007;150: 199–209. [PubMed: 17937675]

47. Mezache L, Paniccia B, Nyinawabera A, Nuovo GJ. Enhanced expression of PD L1 in cervical intraepithelial neoplasia and cervical cancers. Mod Pathol 2015;28: 1594–602. [PubMed: 26403783]

48. Yang X, Cheng Y, Li C. The role of TLRs in cervical cancer with HPV infection: a review. Signal Transduct Target Ther 2017;2: 17055. [PubMed: 29263932]

49. DeCarlo CA, Rosa B, Jackson R, Niccoli S, Escott NG, Zehbe I. Toll-like receptor transcriptome in the HPV-positive cervical cancer microenvironment. Clin Dev Immunol 2012;2012: 785825. [PubMed: 22013487]

50. McAllum B, Sykes PH, Sadler L, Macnab H, Simcock BJ, Mekhail AK. Is the treatment of CIN 2 always necessary in women under 25 years old? Am J Obstet Gynecol 2011;205: 478 e1–7. [PubMed: 21872201]

51. Carreon JD, Sherman ME, Guillen D, Solomon D, Herrero R, Jeronimo J, Wacholder S, Rodriguez AC, Morales J, Hutchinson M, Burk RD, Schiffman M. CIN2 is a much less reproducible and less valid diagnosis than CIN3: results from a histological review of population-based cervical samples. Int J Gynecol Pathol 2007;26: 441–6. [PubMed: 17885496]

52. Lee SJ, Yang A, Wu TC, Hung CF. Immunotherapy for human papillomavirus-associated disease and cervical cancer: review of clinical and translational research. J Gynecol Oncol 2016;27: e51. [PubMed: 27329199]

53. Boone JD, Erickson BK, Huh WK. New insights into cervical cancer screening. J Gynecol Oncol 2012;23: 282–7. [PubMed: 23094132]

54. Herbert A Re: HPV testing alone is not as safe as cytology and selective HPV testing. BJOG 2016;123: 1561–2.

55. Koeneman MM, van Lint FHM, van Kuijk SMJ, Smits LJM, Kooreman LFS, Kruitwagen R, Kruse AJ. A prediction model for spontaneous regression of cervical intraepithelial neoplasia grade 2, based on simple clinical parameters. Hum Pathol 2017;59: 62–9. [PubMed: 27697590]

Novelty: We found high levels of viral protein-sensing TLR2, and viral nucleic acid-sensing TLR7 and TLR8, measured at the time point of CIN2 diagnosis, to be associated with CIN2 regression over time. The highest levels of these TLRs were observed in cervical mucosa of women whose CIN2 lesions did not contain measurable HR-HPV infection. Our data support the use of TLR agonists for treatment of CIN2.

TLR2

TLR3

Relative gene expression level in cervical mucosa
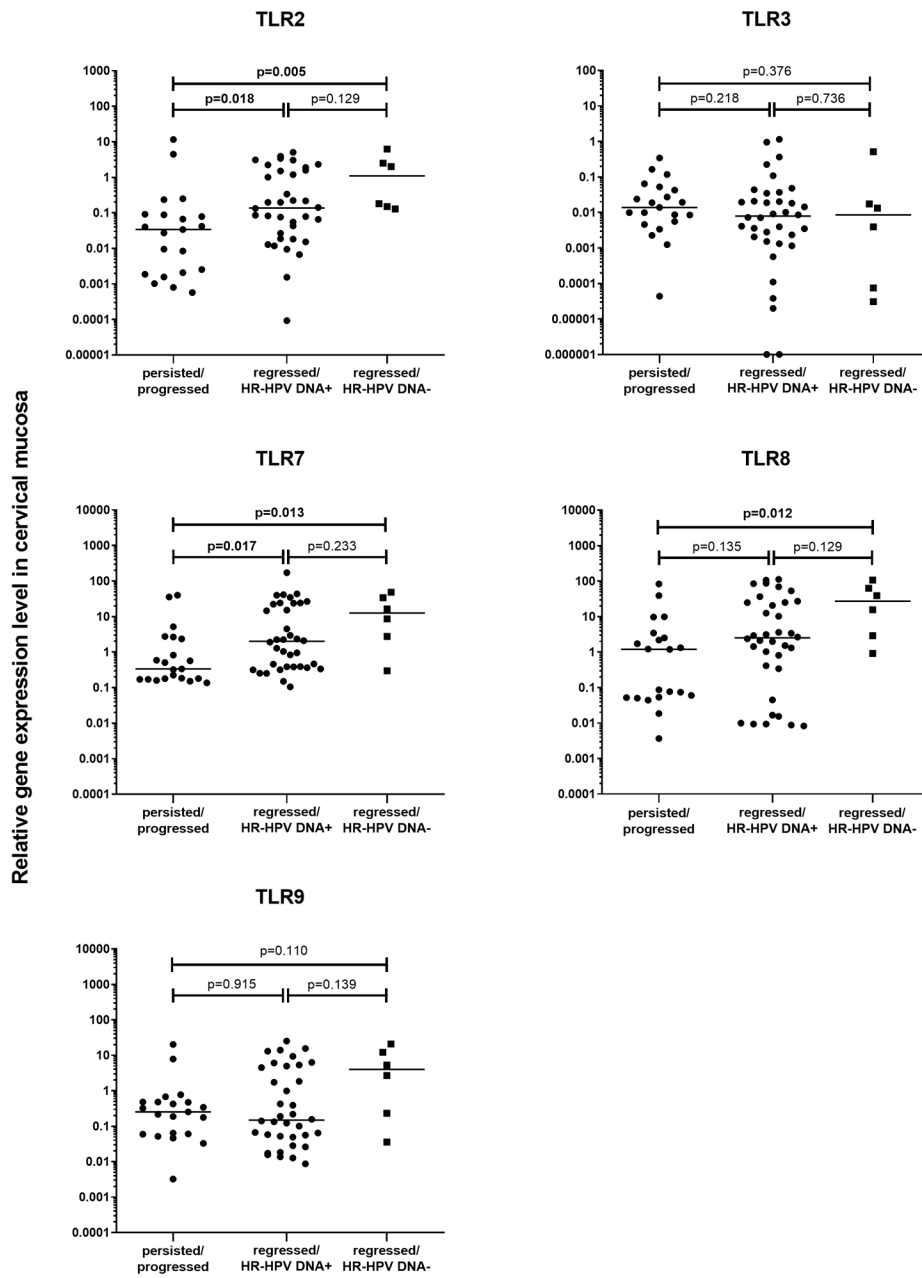
TLR7

TLR8

TLR9
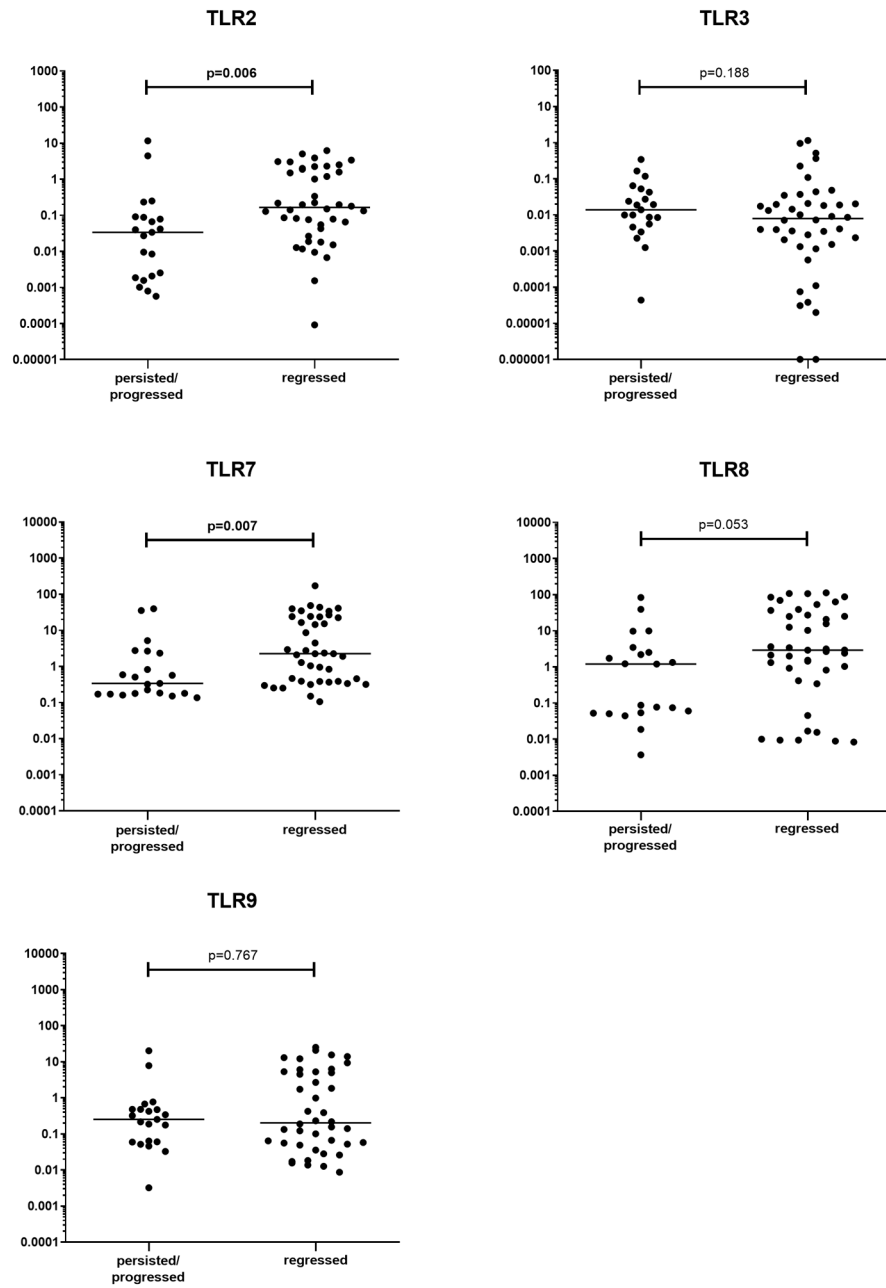
Figure 1.
A Expression of TLRs in CIN2 persistence/progression vs. regression group
B Expression of TLRs in CIN2 persistence/progression vs. regression group according to HR-HPV DNA genotyping results

Table 1.

Selected characteristics of the study population

	All women (n = 63)	CIN2 persistence/progression (n = 21)	CIN2 regression (n = 42)	p-value[a]
	N (%)	N (%)	N (%)	
Race				
White, non-Hispanic	27 (43)	5 (24)	22 (52)	
Black, non-Hispanic	16 (25)	6 (28)	10 (24)	p=0.057[b]
Latin/Hispanic	13 (21)	8 (38)	5 (12)	
Other race	7 (11)	2 (10)	5 (12)	
Age				
16 - 20	34 (54)	13 (62)	22 (52)	p=0.593
21 - 25	29 (46)	8 (38)	20 (48)	
Alcohol consumption				
ever	60 (95)	21 (100)	39 (93)	p=0.544
never	3 (5)	0 (0)	3 (7)	
Drug use[c]				
ever	53 (84)	16 (76)	37 (88)	p=0.279
never	10 (16)	5 (24)	5 (12)	
Pregnant				
ever	23 (37)	9 (43)	14 (33)	p=0.580
never	40 (63)	12 (57)	28 (67)	
Current smoker				
yes	17 (27)	5 (24)	12 (29)	p=0.770
no	46 (73)	16 (76)	30 (71)	
Current use of hormonal contraception				
yes	30 (48)	11 (52)	19 (45)	p=0.789
no	33 (52)	10 (48)	23 (55)	
Reported sexually transmitted diseases[d]				
yes	20 (32)	9 (45)	11 (55)	p=0.251
no	43 (68)	12 (28)	31 (72)	

[a] p-value <0.05 (Fisher exact probability two-tailed test) indicated no significant differences for any of the variables listed between CIN2 persistence/progression compared to CIN2 regression group.

[b] There was a non-significant trend for higher number of non-Caucasian women in CIN2 persistence/progression group (p=0.057).

[c] Drug use recorded for pot, hallucinogen or other drugs, with pot use being most common (96%), followed by pot/other drugs (49%), and pot/hallucinogen (32%), respectively.

[d] STI testing included detection of: Chlamydia trachomatis, Neisseria gonorrhoeae, Trichomonas vaginalis, Treponema pallidum, and Herpes simplex virus, respectively.

Table 2.

HPV types in CIN2 persistence/progression vs. CIN2 regression group

HR-HPV type at the baseline	#CIN2 persistence/progression			#CIN2 regression			p-value[a]
	All	HPV$_{persisted}$	HPV$_{cleared}$	All	HPV$_{persisted}$	HPV$_{cleared}$	
HPV16	11	7 *(64%)*	4 *(36%)*	11	3 *(27%)*	8 *(73%)*	0.099
other HR-HPV	10	2 *(20%)*	8 *(80%)*	25	4 *(16%)*	21 *(84%)*	0.563
no HR-HPV	0	0	0	6	0	6 *(100%)*	NA
Total #cases	21	9 *(43%)*	12 *(57%)*	42	7 *(17%)*	35 *(83%)*	0.027

[a]Fisher exact probability test