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B Lymphocyte Activation by Human Papillomavirus-Like Particles Directly Induces Ig Class Switch Recombination via TLR4-MyD88

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Vaccination with human papillomavirus type 16 (HPV16) L1 virus-like particles (VLP) induces both high titer neutralizing IgG and protective immunity. Because protection from experimental infection by papillomavirus is mediated by neutralizing IgG, we sought the mechanisms that trigger humoral immunity to HPV16 L1 VLP. We find that HPV16 L1 VLP bind to murine B lymphocytes thereby inducing activation-induced cytidine deaminase expression and Ig class switch recombination to cause the generation of IgG. HPV16 L1 VLP also activate production of proinflammatory factors IFN-α, IL-6, MIP-1α, RANTES, and KC, up-regulate the expression of costimulatory molecules by naive B cells, and increase the B1 B cell subpopulation. These B cell responses to HPV16 L1 VLP are dependent upon MyD88. Although MyD88 up-regulate the expression of costimulatory molecules by naive B cells, and increase the B1 B cell subpopulation. These B cell responses are MyD88 dependent. Notably, TLR4 mutant C3H/HeJ mice exhibited significantly reduced HPV16 VLP-specific IgG1, IgG2a, IgG2b, and IgG3 titers after vaccination as compared with the control C3H/HeOuJ mice. HPV16 L1 VLP directly activated class switch recombination and costimulatory molecule expression by B cells of C3H/HeOuJ mice but not C3H/HeJ mice. Thus HPV16 L1 VLP directly activate B cells to induce CD4⁺ T cell independent humoral immune responses via TLR4- and MyD88-dependent signaling. The Journal of Immunology, 2005, 174: 7912–7919.

Neutralizing Abs are critical mediators of immunity to microbial challenge. These Abs can be rapidly generated by CD4⁺ Th cell-independent mechanisms or more slowly as CD4⁺ Th cell-dependent adaptive immune responses (1). Innate recognition of pathogen-associated molecule patterns signals APCs, typically dendritic cells (DCs), to express costimulatory molecules and secrete cytokines. These DC-derived signals can drive the polarization of naive CD4⁺ Th type 1 and type 2 cells toward the Th1 or Th2 phenotype (2). Th1 cells produce IFN-γ and TNF-α that direct B cells to produce Ag-specific IgG2a, whereas Th2 cells express IL-4, IL-5, IL-9, and IL-13 to promote IgG1 and IgE class switching. The TNF superfamily members B lymphocyte-stimulating factor and a proliferation-inducing ligand (APRIL) expressed by monocytes and DCs also directly regulate Ig class switch recombination (CSR) (3). IFN-α directly induces their expression on the surface of DCs (4). However, in the absence of T cell help, certain Ags induce Ag-specific B cell responses, termed Th cell-independent humoral immunity (5, 6). Th cell-independent responses can provide the host with the specificity of adaptive immune system and the speed of the innate immunity, enabling rapid production of neutralizing Abs to control invading microorganisms (7, 8). Several viruses, such as vesicular stomatitis virus (9), polyomavirus (10), and rotavirus (11) have been shown to be largely Th cell-independent in generating neutralizing Ab.

Splenic B1 B cells located in marginal zone are considered to play a critical role in Th cell-independent humoral responses (1). These cells express CD5 and IgM (12). Recently it has been suggested that TLRs play a role in signaling events in B1 B cells and T cell-independent immune responses (1). TLRs mediate the recognition of pathogen-associated molecule pattern and subsequent activation of innate immunity (7, 13, 14) via an adaptor molecule MyD88 that plays a critical role in resistance to pathogens (15–18). Members of the TLR family recognize particular pathogen-associated molecular patterns, e.g., TLR4 recognizes bacterial LPS (15, 19), although it also mediates recognition of viruses including mouse mammary tumor virus (MMTV) and respiratory syncytial virus (20–22).

The highly ordered, close-packed xenogeneic structure of HPV16 L1 VLP induces both high titer protective Ab (23–25) and potent cell-mediated immune responses (26). Indeed, i.m. vaccination of patients who have genital warts with HPV6 L1 VLP may enhance regression (27). Similarly, vaccination of women with HPV16 L1 VLP was 91.2% (confidence interval 80–97%) protective against transient HPV16 infection, but 100% (90–100%) protective against persistent HPV16 infection, suggesting possible L1-mediated immune clearance of breakthrough infection (28).

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3 Abbreviations used in this paper: DC, dendritic cell; CSR, class switch recombination; VLP, virus-like particle; HPV, human papillomavirus; AID, activation-induced cytidine deaminase; MMTV, mouse mammary tumor virus; GAG, glycosaminoglycan.
Furthermore, the immunogenicity of weak foreign or self-Ags is dramatically enhanced by coupling to VLP (29–32). However, the mechanisms by which HPV16 L1 VLP trigger such potent humoral and cellular immunity without significant side effects in patients is poorly understood.

Several groups have demonstrated that HPV16 VLP can activate innate responses in human monocyte-derived DCs (33), mouse bone marrow-derived DCs (34), and DC subpopulations from mouse splenic cells (35) to produce the proinflammatory factors that regulate adaptive immunity. For example, HPV16 L1 VLP trigger production of type I IFN by bone marrow-derived DCs, and type I IFN receptor-deficient mice exhibit reduced humoral immune responses to HPV16 L1 VLP (35, 36). We demonstrate that HPV16 L1 VLP can also directly activate B cells to induce activation-induced cytidine deaminase (AID) and Ig CSR to cause the generation of IgGs via TLR4- and MyD88-dependent signaling pathway. This ability of HPV16 VLP to rapidly induce Th cell-independent immune responses independently of CD4+ T cells may play an important role in early control of virus spread and mediate immunity in patients lacking CD4+ T cells that are more prone to HPV-related disease.

Materials and Methods

Mice

129/Sv-C57BL/6-MyD88+/− and MyD88−/− mice were maintained by Akira and colleagues (our study). The 6- to 8-wk-old male 129S6-BAC (B6.129/Epk susceptible to LPS/CD40Ag, TLR7/8, and MyD88 family) Abs were used to stain the stimulated B cells. Cells were then washed twice before being resuspended in PBS containing 1% paraformaldehyde and 1% FCS and kept at 4°C before flow cytometric analysis (FACScan; Becton Dickinson). The methodology is fully described (35).

Preparation of HPV16 L1 VLP

HPV16 L1 VLP were generated with recombinant baculoviruses and purified as previously reported (39).

Stimulation on naive and VLP-activated B cells

Enriched population of splenic B cells were obtained from naive or HPV16 VLP-immunized mice using CD45R-specific (B220) (RA3-6B2, MACS) or CD19-specific (ID3, MACS) beads according to the manufacturer’s instructions (Miltenyi Biotec) (40). The cells were cultured in 24-well flat-bottom plates at 106 cells/ml in RPMI 1640 medium supplemented with 10% FCS. To induce isotype switching, B cells were stimulated either with 25 μg/ml VLP alone or with 1000 U/ml recombinant mouse IL-4 or 10 μg/ml anti-CD40 Ab (HM40-3; BD Pharmingen). Cells were harvested on day 4 for flow cytometric or RT-PCR analyses. Supernatants were harvested on day 6 for Ig isotype analysis by capture ELISA.

Flow cytometry of mouse splenic B cells

Freshly isolated splenic B cells were collected in ice-cold PBS and surface marker phenotypes were analyzed using PE-conjugated anti-IgM (331.1), anti-IgG1 (A85.10), anti-IgG2a (R19-15), anti-IgG2b (R12-13), anti-IgG3 (R80-42), or anti-IgA (C10-3) (BD Pharminogen). The supernatants were collected at the different time points and analyzed by the chemiluminescence secreted alkaline phosphatase assay according to manufacturer’s protocol (BD Clontech).

Expression profiling using DNA microarrays

The methodology is fully described (35).

Results

Generation of CD4+ Th cell-independent responses upon vaccination with HPV16 VLP via MyD88

To test for the role of CD4+ T help in the generation of humoral responses to HPV16 VLP, we immunized CD4−/− and CD4+/+ mice with HPV16 VLP or, as a control, the T cell-dependent Ag OVA. Specific reactivity for each Ab isotype in pooled sera from groups of mice (n = 6) was measured by ELISA. CD4−/−, but not CD4+ mice produced a robust specific IgG response to OVA as expected (Fig. 1A). However, HPV16 VLP vaccination induced specific IgG in CD4−/− mice, although the titers were higher in CD4+/+ mice (Fig. 1B). Thus HPV16 L1 VLP vaccination induces both CD4+ independent and dependent humoral responses. Furthermore, consistent with other Th cell-independent Ags (43), no IgE response to HPV16 VLP was observed (data not shown). Analogous results were obtained in mice upon CD4+ T cell depletion with anti-CD4 Ab (Fig. 1C). To investigate the role of TLR signaling in inducing the Th cell-independent response to HPV16 VLP, we also vaccinated mice lacking a key component of the TLR signal cascade, MyD88 (20). MyD88−/− mice failed to produce HPV16 VLP-specific IgG after vaccination, although the IgM response was retained (Fig. 1D and data not shown). To address a possible role for a defect in B cell development in MyD88−/− mice, we compared the ratio of different splenic cell populations, including IgM−, IgD+ B220+, CD5−, and CD19− B cells in MyD88−/− and MyD88+/+ mice. Consistent with previous reports, the proportions of each of these B cell subsets were similar in MyD88−/− and MyD88+/+ mice (data not shown).

CD5 IgM+ B cells correspond to the B1 subset that is associated with Th cell-independent humoral immunity. Upon comparison of splenocytes pooled from each group of mice (n = 6), we noted that the splenic population of CD5− IgM+ B cells doubled (1.14 vs 2.76%) after vaccination with HPV16 L1 VLP but not with PBS (Fig. 2B). Interestingly, this increase in the splenic B1 cell population did not occur in HPV16 VLP vaccinated MyD88−/− mice, suggesting that MyD88 signaling is critical for B1 cell expansion in the development of Th cell-independent humoral immunity to HPV16 VLP.

Costimulatory molecules play a critical role in the induction of immunity, including Th cell-independent responses (44). We detected an elevated proportion of B220+ B cells with high expression of CD40 and CD80 in MyD88+/+ mice after vaccination with
HPV16 L1 VLP (Fig. 2C). Notably, this did not occur in HPV16 VLP vaccinated MyD88<sup>−/−</sup> mice (Fig. 2C). Thus, our findings suggest that HPV16 L1 VLP directly activate B cells and induce CD4<sup>+</sup> Th cell-independent humoral immune responses via MyD88.

**MyD88<sup>−/−</sup> B cells are impaired for activation of Ig CSR by HPV16 L1 VLP**

To address whether HPV16 L1 VLP directly induce CD4<sup>+</sup> Th cell-independent immunity, we confirmed direct binding of HPV16 L1 VLP to naive CD19<sup>+</sup> B cells by immunofluorescent staining and analysis by fluorescence microscopy or flow cytometry (Fig. 3, A1–A6). Heparinase pretreatment of naive B cells reduced binding by HPV16 VLP (Fig. 3, A5 and A6). We then tested for the ability of HPV16 L1 VLP trigger naive CD19<sup>+</sup> B cells to undergo CSR, as assessed by up-regulated expression of IgG1, IgG2a, IgG2b, and IgG3 H chain transcripts and down-regulation of μ chain transcripts (Fig. 3B). Exposure to HPV16 L1 VLP caused naive B cells to undergo CSR and express IgG1, IgG2a, IgG2b, and IgG3 H chain transcripts. Expression of IgG was also confirmed by FIGURE 2. Up-regulation of the B1 subset and B cell costimulatory markers after HPV16 VLP vaccination is dependent upon MyD88. MyD88<sup>−/−</sup> and MyD88<sup>+/+</sup> mice (n = 6) were immunized i.v. with 10 µg of OVA or PBS on days 0, 7, and 14, then sera were harvested on day 24. The pooled sera were tested for Ag-specific IgG1, IgG2a, IgG2b, and IgG3 responses. B, Same as in A, but the mice were vaccinated with HPV16 VLP. C, Same as in B, except that CD4<sup>+/−</sup> mice, either with or without CD4<sup>+</sup> T cell depletion, were vaccinated with HPV16 VLP. The CD4<sup>+</sup> T cell depletion protocol was validated by flow cytometric analysis. D, MyD88<sup>−/−</sup> and MyD88<sup>+/+</sup> mice were immunized i.v. with 10 µg of HPV16 VLP or PBS on days 0, 7, and 14, then sera were harvested on day 24. The pooled sera (n = 6) were tested for HPV16 VLP-specific IgG1, IgG2a, IgG2b, and IgG3 responses. Representative data of three or more experiments are shown.
ELISA and isotype-specific flow cytometric staining (Fig. 3C and data not shown). To examine whether MyD88 signaling is also critical for direct induction of CSR by HPV16 VLP, splenic B cells were purified by CD19-specific MACS from MyD88<sup>+/+</sup> and MyD88<sup>-/-</sup> mice and incubated with HPV16 L1 VLP or PBS alone. The HPV16 L1 VLP bound equally well to MyD88<sup>-/-</sup> and MyD88<sup>+/+</sup> CD19<sup>+</sup> B cells (Fig. 3A). However, whereas MyD88<sup>+/+</sup> B cells expressed α, γ, and µ Ig H chain transcripts in response to HPV16 VLP, only the µ transcript was present in the MyD88<sup>-/-</sup> B cells (Fig. 3B). Further, HPV16 VLP induced transcription and production of IgA, IgG1, IgG2a, IgG2b, and IgG3, and IgM in MyD88<sup>+/+</sup> purified B cells, but only IgM in MyD88<sup>-/-</sup> B cells (Fig. 3C). Notably, MyD88<sup>-/-</sup> B cells secreted IgG in response to CD40 ligation and IL-4, demonstrating their viability and ability to undergo MyD88-independent CSR (Fig. 3D).

Because B cells undergo MyD88-dependent activation upon binding to HPV16 L1 VLP, we investigated their ability to generate other innate immune responses. Therefore we compared the transcriptional response of B cells using Affymetrix U74A, U74B, and U74C microarrays 12 h after exposure to HPV16 VLP or LPS (Fig. 4). The microarray analysis indicated numerous common transcriptional changes in B cells activated by HPV16 VLP and LPS, including induction of IFN-α mRNA by 9.9-fold. Production of IFN-α was quantified by ELISA and found to be elevated in supernatants of B cell cultures stimulated by HPV16 VLP (Fig. 3E).

To test for production of other proinflammatory mediators, we harvested supernatants 24 h after exposure to either HPV16 VLP or PBS for comparison to untreated B cells (45, 46). We concurrently analyzed these supernatants for the presence of IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12 (p40), IL-12 (p70), IL-17, TNF-α, IFN-γ, GM-CSF, and G-CSF regulated upon activation of normal T expressed and secreted (RANTES), cytokine-induced neutrophil chemotactic (KC/CXCL1), and MIP-1α using the Bio-Plex 18 cytokine assay (Bio-Rad). HPV16 VLP elevated production of MIP-1α, RANTES, KC, G-CSF and especially IL-6 by B cells (Fig. 3E). Notably, MyD88<sup>-/-</sup> B cells were impaired in their production of proinflammatory mediators after exposure to HPV16 L1 VLP (Fig. 3E). We also examined whether HPV16 L1 VLP act directly upon B cells to up-regulate the co-stimulatory molecules CD40, CD80, and CD86. Incubation of naive splenic B cells in vitro with HPV16 VLP significantly up-regulated surface expression of CD40, CD80, and CD86 (Fig. 3F). Again, MyD88<sup>-/-</sup> B cells exhibited reduced expression of these co-stimulatory molecules, especially CD86. Surprisingly, up-regulation of CD86 was not observed in the B cells of HPV16 VLP.

**FIGURE 3.** HPV16 VLP directly activates CSR in B cells via MyD88-dependent pathway. A, Flow cytometric analysis showing binding of HPV16 VLP to MyD88<sup>+/+</sup> (A1, A2, A5, and A6) and MyD88<sup>-/-</sup> B cells (A3 and A4) isolated from naive mice. B cells (A6) were pretreated with heparinase for 1 h at 37°C (48). Splenic B cells were incubated with PBS (A1) or HPV16 VLP (A2–A6) for 1 h at 4°C in Dulbecco’s PBS, and then stained for L1 VLP (H16.V5) PE- and FITC-labeled anti-CD19. The cells were analyzed by flow cytometry (A1–A3) or confocal scanning fluorescence microscopy (A4–A6). B, HPV16 VLP directly induces Ig CSR in vitro. B cells were purified from mouse splenic cells by CD19-MACS and incubated for 4 days in the presence of IL-4 (25 μg/ml). Transcription of AID and Ig was analyzed by RT-PCR. C, ELISA for specific isotypic Abs in supernatant of MyD88<sup>+/+</sup> and MyD88<sup>-/-</sup> B cell cultures, 6 days after stimulation with HPV16 VLP or PBS. D, MyD88 splenic B cells were purified by CD19 MACS from naive mice and then incubated for 4 days in the presence of 500 U/ml IL-4 or 10 μg/ml CD40 Ab. Individual Ab isotypes were analyzed by flow cytometry using isotype control Ab (D1), anti-IgG1 (D2), or anti-IgG2a (D3). E, B cell-based innate immune-like responses induced by HPV16 VLP in MyD88<sup>-/-</sup> B cells in vitro. B cells were stimulated with 25 μg/ml HPV16 VLP, control crude Sf9 cell lysate from same purification scheme or PBS. The supernatants were harvested after 24 h and assayed for IFN-α (PBL Biomedical Laboratories), IL-6, G-CSF, MIP-1α, RANTES, and KC (Bio-Rad). F, Expression of CD40, CD80, and CD86 on B cells after culture in vitro for 36 h. Isotypic control (black histogram); unstimulated (red histogram, crude Sf9 cell lysate); stimulated with 25 μg/ml HPV16 VLP (green histogram) are shown.
vaccinated mice (data not shown), implying more complex regulation in vivo than in vitro.

Endotoxin contamination, which could potentially induce CSR, was below the level of detection in the HPV16 VLP 1.05 mg/ml stock preparation (<0.058 EU/ml by Limulus assay E-Toxate; Sigma-Aldrich). Further, crude S9 cell lysate that was subjected to the same purification scheme with HPV16 L1 VLP failed to promote CSR. This suggests that potential contamination of the HPV16 L1 VLP preparation with endotoxin or S9 cell proteins does not account for the observed induction of CSR. To examine the potential for indirect activation of B cells in the purified population via VLP activation of contaminating cell types such as monocytes, macrophages, or DCs, the B cell preparation was stained with CD14, CD11c, and CD11b. Only 0.004% cells were positive after staining with FITC Ab to CD14, CD11c, and CD11b in splenic B cells purified by CD19-MACS after repeated selection, suggesting that the observed responses were the result of direct activation of the B cells by HPV16 VLPs. Studies described elsewhere (36) suggest that the HPV16 L1 VLP preparations lack contamination unmethylated DNA that could potentially activate B cells via TLR9.

**TLR4 is involved in HPV16 VLP-directed Ig CSR**

Our previous studies indicated that HPV16 L1 VLP activated NF-κB-dependent transcription in RAW264.7 cells via MyD88 (36). To screen for TLRs that function in innate recognition of HPV16 L1 VLP, we developed selectable small interfering RNA (siRNA) constructs to individually knockdown the mRNA levels for each TLR. Because CD4+ T cell-derived signals enhance HPV16 VLP-induced CSR (Fig. 5, A1 and A2). Interestingly, knockdown of TLR4 mRNA partially inhibited the activation of NF-κB-dependent transcription by HPV16 L1 VLP, as well as the TLR4 ligand LPS (Fig. 5A3) implicating TLR4 in innate recognition of HPV16 L1 VLP.

C3H/HeJ mice contain a dominant-negative point mutation within the cytoplasmic portion of TLR4 rendering them insensitive to bacterial LPS (19). To further investigate whether TLR4 is involved in the generation of humoral immunity induced by HPV16 L1 VLP, we immunized C3H/HeJ and control mice C3H/HeOuJ mice with HPV16 VLP or the control protein OVA (Fig. 5, B and C). The TLR4 mutant C3H/HeJ mice exhibited significantly reduced HPV16 VLP-specific IgG2a, IgG2b, and IgG3 titers as compared with the control C3H/HeOuJ mice (Fig. 5, B and C). Notably, there were no significant differences among B cell populations, including IgM+, IgD+, B220+, CD5+, and CD19+ B cells, between C3H/HeJ and C3H/HeOuJ mice suggesting that gross differences in B cell development do not account for their differential response to HPV16 VLP (data not shown). Furthermore, the humoral response to OVA was equivalent in C3H/HeJ and C3H/HeOuJ mice (Fig. 5B2). To address the role of TLR4 signaling in Th cell-independent immune responses to HPV16 L1 VLP, we examined their ability to produce CSR and IgG as well as up-regulate co-stimulatory molecules on purified CD19+ B cells derived from C3H/HeJ and C3H/HeOuJ mice. In vitro, HPV16 VLP induced production of IgA, IgG1, IgG2a, IgG2b, and IgG3 and IgM in CD19+ B cells of C3H/HeOuJ mice, but only IgM in B cells of C3H/HeJ mice with defective TLR4 signaling (Fig. 5D, E). Similarly, HPV16 VLP also up-regulated expression of CD40 and CD80 in CD19+ B cells of C3H/HeOuJ mice, but not in B cells of C3H/HeJ mice (Fig. 5E), further implicating TLR4 signaling in Th cell-independent responses to HPV16 VLP.

**T cell-derived signals enhance HPV16 VLP-induced CSR**

Because CD4+ T cell-derived signals provide a significant contribution to the generation of HPV16 VLP-specific IgG (Fig. 1, B and C), we also examined the influence of key T cell-derived signals, IL-4 and CD40 ligation, upon HPV16 VLP-induced CSR in vitro (Fig. 6A). IL-4 and CD40 ligation enhanced HPV16 VLP-induced CSR as well as production of IgG1 and, to a lesser extent, other IgG isotypes by purified B cells (Fig. 6). Expression of IgG1 was also confirmed by ELISA and isotype-specific flow cytometric staining (Fig. 6, B and C). Coadministration of anti-CD40 Ab with HPV16 VLP enhanced both the transcription of AID and γ Ig, whereas μ transcripts were down-regulated (Fig. 6).

**Discussion**

Innate responses triggered by the TLR-MyD88 signaling pathway promote adaptive immune responses and mediate adjuvant effects. We demonstrate that vaccination with HPV16 L1 VLP can rapidly induce specific immune responses via TLR4- and MyD88-mediated signaling. Because the highly ordered structure of HPV16 L1 VLP is potently immunogenic in humans even without adjuvant and provides protective immunity, HPV16 L1 VLP represent an important model for the development of subunit vaccines. Indeed, the repetitiveness of many viral Ags is a key factor responsible for the efficiency of early and rapid B cell amplification for potent IgM responses and also for efficient switching to IgG (47). For example,
B cells were unresponsive to the poorly organized vesicular stomatitis virus-G (IND) present as self-Ag but responded promptly to the same Ag presented in a highly organized form (23). The spacing and density of the epitopes also is critical in the response to papillomavirus VLP (32). The close-packed HPV16 L1 VLP structure directly activates B cells to induce Ig CSR and the

**FIGURE 5.** TLR4 is involved in the Ig CSR induced by HPV16 VLP. A, Effect of knockdown of TLR4 by small interfering RNA on TLR4 mRNA levels estimated by semiquantitative RT-PCR (A1), for TLR4 protein levels as measured by MTS510 (eBioscience) Ab staining and flow cytometry (A2) and by its influence upon the NF-κB-dependent transcriptional response to 25 μg/ml HPV16 VLP, 0.1 μg/ml LPS (026:B6), 10 μg/ml peptidoglycan Staphylococcus aureus (InvivoGen), 25 μg/ml poly(I:C) (InvivoGen), 5 μM CpG (ODN 1826; InvivoGen), or 25 μg/ml zymosan A (Sigma-Aldrich) (A3). B, ELISA reactivity by isotype of serum Abs from C3H/HeJ (TLR4 mutant) and C3H/HeOuJ mice after immunization by HPV16 VLP (B1) or OVA (B2). C3H/HeJ (TLR4 mutant) and C3H/HeOuJ mice were immunized with HPV16 VLP or OVA (10 μg), or PBS, respectively, on days 0, 7, and 14. Day 24 pooled sera (n = 6) were analyzed for reactivity to HPV16 VLP by ELISA. C, Titration by isotype of serum Abs from C3H/HeJ (TLR4 mutant) and C3H/HeOuJ mice after immunization by HPV16 VLP. D, ELISA for specific Ab isotypes in supernatant of C3H/HeJ (TLR4 mutant) and C3H/HeOuJ B cell cultures, 6 days after stimulation with HPV16 VLP or control, crude sf9 cell lysates. E, Splenocytes from HeOuJ and HeJ mice, vaccinated with either PBS or HPV16 VLP, were subjected to double staining with PE-labeled B220 and FITC-labeled anti-CD40 or CD80 for flow cytometric analysis. Representative data of three or more experiments are shown.

B cells were unresponsive to the poorly organized vesicular stomatitis virus-G (IND) present as self-Ag but responded promptly to the same Ag presented in a highly organized form (23). The

**FIGURE 6.** Direct expression of IgG induced by HPV16 VLP is enhanced by T cell-derived signals. A, Splenic B cells were purified by CD19 MACS from mice immunized by HPV16 VLP, and then incubated for 4 days in the presence of VLP (25 μg/ml), with or without 500 U/ml IL-4 or 10 μg/ml CD40 Ab. Total RNAs were analyzed by RT-PCR using Ab isotype-specific and AID primers. B, ELISA measurement of isotypic-specific Ab in supernatants from B cells after 6 days of stimulation with HPV16 VLP. C, B cells were treated with VLP and after 4 days surface expression of CD19 and individual Ab isotypes were analyzed by flow cytometry. Representative data of three or more experiments are shown.
production of IgG. Such Th cell-independent responses provide the host with the specificity of adaptive immune system and the speed of the innate immune, enabling rapid production of neutralizing Abs to control invading microorganisms (7, 8).

Immune recognition of HPV16 VLP via MyD88 suggests signaling via TLRs. Indeed, studies in TLR4-defective mice suggest that TLR4 contributes to the recognition of HPV16 VLP. It is also notable that three viruses, namely respiratory syncytial virus, MMTV, and HPV16 that have been shown to bind heparan sulfate on the cell surface (21, 22, 48) are also recognized by TLR4-dependent pathways (49, 50). Although viruses use many different surface molecules to initiate infection, heparan sulfate glycosaminoglycan (GAG) represents an important surface binding receptor for a number of medically important viral pathogens (51). Given that cell surface GAGs are the primary receptors for HPV16 VLP, and TLR4 is important in immune recognition of HPV16 VLP, it is intriguing that GAG oligosaccharides activate DCs via TLR4 (50). Heparin pretreatment prevents binding of HPV16 VLP to B cells, also suggesting that activation of TLR4 is secondary to B cell binding via surface GAGs, as described for interaction of TLR4 and MMTV after binding to its primary surface receptor. Indeed, heparin blocks activation of human monocyte-derived DCs by HPV16 VLPs (52).

Murine B cells express both TLR4 and TLR9, whereas human B cells express only the latter (53). Therefore, Th cell-independent responses to HPV16 VLPs in mice and humans may differ. However, DCs also trigger Th cell-independent class switching (4) and HPV16 VLPs are potent activators of human myeloid and plasmacytoid DCs (54–56) as well as murine DC subsets (35, 36). HPV16 L1 VLPs induce class switching to IgG1, IgG2a, IgG2b, and IgG3, but not IgE. Notably, this pattern of isoforms is distinct from that observed upon TLR9-dependent CpG activation of B cells, which specifically fails to induce IgG1 (57, 58). This may reflect additional MyD88-independent signals generated via TLR4, but not TLR9 (59).

AID is the only B cell-specific factor that is required for somatic hypermutation, gene conversion, and CSR. It is only expressed in activated B cells such as germinal center B cells (42) and B cells undergoing CSR or somatic hypermutation in vitro (60, 61). IL-4 and CD40L promote AID expression (62). Our studies indicate that MyD88 is also involved in the up-regulation of AID expression. This pathway is likely significant for early Th cell-independent humoral responses against Ags such as HPV16 L1 VLP that are recognized by TLRs on B cells.

HPV16 VLP induce expression of type I IFN, proinflammatory cytokines, chemokines, and costimulatory molecules, which can potentially amplify the B cell response by feedback or paracrine pathways or interaction with Th cells. For example, HPV16 VLP up-regulate CD40 on the surface of B cells (Fig. 3F), which can be stimulated by CD40L, classically on the surface of Th cells. Significantly, CD40L on the surface of adjacent B cells may provide functional activation of CD40 in Th cell-independent responses (44). Paracrine or autocrine signaling by MIP-1α, IFN-α, and IL-6 may regulate the Ab isotypes induced by HPV16 VLP and are consistent with a defense response (35, 63, 64). IFN-α can promote isotopic switching by stimulating DCs and acts as a potent adjuvant of humoral immunity to monomeric Ag (4, 64, 65). Indeed, type I IFN receptor-deficient mice exhibit blunted CSR and specific Ab production after vaccination with HPV16 VLP whereas coadministration of IFN-α with either OVA or HPV16 VLP enhances the humoral immune response to these Ags (35).

Although HPV16 L1 VLP vaccination i.v. and live polyomavi-rus inoculation of mice induce Th cell-dependent humoral responses, i.p. vaccination with polyomavirus V1P VLP does not (66). This likely reflects the ability of HPV16 L1 VLP and the failure of human polyomavirus V1P VLP to directly activate DC maturation (34) and B cells, suggesting differences in their recognition by the innate immune system.

CD4+ CD8- αβ T cells or γδ T cells can substitute for CD4+ T cell help to induce CSR after vaccination with formalin-inactivated influenza virus or inoculation with vesicular stomatitis virus, respectively (67, 68). It is also possible that these T cell subpopulations are also involved in T cell-dependent humoral immunity induced by HPV16 VLP. However, the ability to generate HPV16 VLP-specific IgGs in the absence of CD4+ T help suggests that it may be possible to induce immunity even in patients with impaired T cell function such as those undergoing organ transplantation or patient with HIV infection. These patients are particularly susceptible to HPV-induced cancers and thus even transient immune could be beneficial.

Disclosures

R. B. S. Roden is a paid consultant of Knobbe, Martens, Olson and Bear, LLC and an arrangement regarding a patent dispute to HPV16 VLP is being managed by the Office of Policy Coordination, Johns Hopkins University. The remaining authors have no financial conflict of interest.

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