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Human Papillomavirus Type-16 Virus-Like Particles Activate Complementary Defense Responses in Key Dendritic Cell Subpopulations

Rongcun Yang,* Francisco Martinez Murillo,† Ken-Yu Lin,* William H. Yutzy IV,* Satoshi Uematsu,¶ Kiyoshi Takeda,¶ Shizuo Akira,¶ Raphael P. Viscidi,‡ and Richard B. S. Roden²*§

Human papillomavirus type-16 (HPV16) L1 virus-like particles (VLPs) activate dendritic cells (DCs) and induce protective immunity. In this study, we demonstrate, using global gene expression analysis, that HPV16 VLPs produce quite distinct innate responses in murine splenic DC subpopulations. While HPV16 VLPs increase transcription of IFN-γ and numerous Th1-related cytokines and chemokines in CD8α+CD11c+ DCs, CD4+CD11c+ DCs up-regulate only type I IFN and a different set of Th2-associated cytokines and chemokines. Type I IFN, but not IFN-γ, potentiates humoral immunity, notably production of VLP-specific IgG2a. However, HPV16 VLP-stimulated IL-12 production by CD8α+CD11c+ DCs is augmented by autocrine IFN-γ signaling. Thus, before adaptive immunity, HPV16 VLPs signal complementary defense responses in key DC subpopulations, indicating specialized DC lineages with predetermined polarization.


D endritic cells (DCs)² may be derived by in vitro culture of murine bone marrow (BM) DCs in GM-GSF (1) or directly isolated from lymphoid tissue. DCs comprise a heterogeneous cell population in naive mice that have been classified based on surface markers, functional potential and location, as Langerhans cells in the skin, mature tissue interstitial DCs present in secondary lymph nodes, and CD8α+CD4−, CD8α+CD4+, and CD8α−CD4− DC subsets in the spleen (2). The CD8α+ and CD8α− splenic DC subsets have been reported to play different roles in the polarization of adaptive immunity (3–8). For example, microbial stimulation of IL-12 production by CD8α+ DCs is associated with the induction of a Th1 response, whereas production of IL-10 by CD8α−DCs promotes Th2 polarization (6, 7).

Oncogenic human papillomaviruses (HPVs) represent the primary causative agent of cervical cancer, and are strongly associated with a subset of vulval, anal, and penile cancers (9). Although more than a dozen oncogenic HPV genotypes have been identified, HPV type-16 (HPV16) predominates and is present in over 50% of cervical cancers. The major papillomavirus capsid protein, L1, self-assembles to form empty capsids termed virus-like particles (VLPs) that are morphologically and immunologically very similar to native virions, but lack the oncogenic viral genome (10). VLPs exhibit a highly ordered, close-packed foreign structure (11) and engender both high titer protective Ab (12, 13) and cell-mediated immune responses (14). Vaccination with HPV16 L1 VLPs induces humoral, Th1-biased immune responses (15, 16) that protect women from natural acquisition of persistent HPV16 infection and cervical intraepithelial neoplasia (17). Furthermore, the immunogenicity of weak foreign or self Ags is dramatically enhanced by fusion with VLPs (18–22).

The identification of the immune mechanisms regulating innate and adaptive immune responses to clinically effective Ags is critical for rational development of vaccines. IL-12 and IFN-γ are known to promote Th1 polarization, whereas IL-4, IL-6, IL-9, IL-10, IL-13, and G-CSF are associated with Th2 responses. However, the source of the cytokines that regulate the polarization of immune responses has been a matter of debate (23). Recent studies demonstrate that HPV16 VLPs bind to DCs and stimulate their maturation, including up-regulation of MHC class I, class II, CD80, CD86, and CD40 and cytokine production (24–26).

In this study, we demonstrate by a global gene expression analysis that HPV16 VLPs effectively activate different subsets of splenic DCs to produce polarized responses. Notably, we found that CD4+CD11c− DC up-regulate transcription of IFN-α and Th2-related cytokines and chemokines, whereas IFN-γ and Th1-associated cytokines and chemokines are produced by CD8α+CD11c+ DCs in response to HPV16 VLPs. IFN-α, but not IFN-γ, -mediated signaling potently enhances VLP-specific Ab production and particularly IgG2a class switching. Thus, production of such opposing innate responses to a single Ag suggests specialized DC lineages with predetermined polarization. These emergency defense responses by specialized DCs may be critical for control of invading pathogens before the onset of adaptive immunity.
Materials and Methods

Mice
Six- to 8-wk male, IFN-γ knockout (B6.129S7-Ifngtm1ts6Cg) and control mice (The Jackson Laboratory, Bar Harbor, ME), 129-IfngFRT knockout mice and control mice (B&K Universal, Hull, U.K.) were maintained in a pathogen-free animal facility at least 1 wk before use. Experiments were performed in accordance with institutional guidelines.

Preparation of HPV16 VLPs
VLPs were generated by infection of Sf9 with recombinant baculoviruses expressing HPV16 L1 and purified as previously reported (10). VLPs were further purified by A-15 mGel (Bio-Rad, Hercules, CA) and a strong cation exchange packing column (PerSeptive Biosystems, Framingham, MA). Protein content was determined using a BCA kit (Pierce, Rockford, IL) and SDS-PAGE. Samples were absorbed onto carbon-coated grids and stained with 1% uranyl acetate. The grids were examined with a Philips CM120 transmission electron microscope (Eindhoven, The Netherlands) operating at 80 kV.

Preparation and flow cytometry of DC subsets of mouse splenic cells
Fresh mouse DC subsets were isolated based on a published protocol (27) with modification. Spleens were diced into small fragments, suspended in 10 ml PBS containing 0.02% FCS and 10 mM EDTA (pH 8.0), and digested at 37°C for 30 min with collagenase (1 mg/ml, type II; Worthington Biochemical, Freehold, NJ) and dispase (500 U/ml, type II; Boehringer Mannheim, Mannheim, Germany), and digested with intermittent agitation for 25 min at room temperature (22°C). EDTA at 0.01 M, pH 7.2, was added to the digest to disrupt DC-T cell complexes. Incubation with agitation was continued for 5 min. Undigested stromal fragments were then removed with a stainless steel sieve. All remaining procedures were performed on ice. The cells were removed from the digest by centrifugation. The spleen cell suspension was directly stained with FITC-labeled anti-CD11c, PE-labeled anti-CD4, or PE-labeled anti-CD8. After washing, cells were gated for DC characteristics, namely, high forward and side scatter and bright staining on different days, for 16 h at 45°C. For comparison between different chips, the product was purified by phenol-chloroform extraction, and biotinylated antisense cRNA was generated through in vitro transcription using the BioArray RNA High Yield Transcript Labeling kit (Enzo Biochem, New York, NY). Fifteen micrograms of the biotinylated cRNA were fragmented at 94°C for 35 min (100 mM Tris-acetate, pH 8.2, 500 mM KAc, 150 mM MgOAc), and 10 μg of total fragmented cRNA were hybridized to the Affymetrix murine genome GeneChip array (U74Av2; Santa Clara, CA), on different days, for 16 h at 45°C with constant rotation (60 rpm). Affymetrix Fluidics Station 400 was then used to wash and stain the chips, removing excess nonhybridized cRNA, and an anti-PE conjugate to stain the biotinylated cRNA. The staining was then amplified using goat IgG as blocking reagent and biotinylated anti-streptavidin Ab (goat), followed by a second staining step with a streptavidin-PE conjugate.

Fluorescence was detected using the Hewlett-Packard G2500 GeneArray Scanner (Palo Alto, CA) and image analysis of each GeneChip was done through Micro Array Suite 5.0 software (Affymetrix), using the standard default settings. For comparison between different chips, global scaling was used, scaling all probe sets to a user-defined target intensity of 150.

To ascertain the quality control of the total RNA from the samples, we used the Agilent Bioanalyzer (Palo Alto, CA), “lab on a chip” technology, and confirmed that all the samples had optimal rRNA ratios (1.2, for 18S and 2.0, for 28 S) and overall quality of the RNA in the form of cRNA and fragmented cRNA. To assess the quality control of the hybridization, GeneChip image, and comparison between chips, we studied the following parameters: scaling factor; background; percentage of present calls; housekeeping genes (3/5 ratios of GAPDH) and presence or absence of internal spike controls. To assess quality control interreplicates, we calculated the percentage of differential calls (up- or down-regulated) between pairwise comparisons.

The initial expression results were based on pairwise comparisons among the different experimental conditions represented by the samples. Any transcript that showed at least a 2-fold change in expression level between control and experimental samples was considered “significant”. For duplicated samples, the results were filtered independently for significance on each of the four pairwise comparisons. Transcripts that were consistently significant in at least two of the four iterative comparisons were selected for the final candidate list.

Analysis of IFN-α, -γ, and IL-12 by ELISA
The culture supernatants of different subsets of DCs were harvested at varying times after stimulation with VLPs (25 μg/ml), but also with LPS (1 μg/ml), Cpg (5 μM), or poly I:C (25 μg/ml). Commercial sandwich ELISA kits were used for quantification of IFN-γ (Pierce), IL-12p70 (R&D Systems, Minneapolis, MN), and IFN-α (PBL Biomedical Laboratories, New Brunswick, NJ). The OD of each of the sample was measured at 450 nm using a SpectraMax 190 ELISA plate reader (Molecular Devices, Sunnyvale, CA). Cytokine levels were quantified from two to three titrations using standard curves, and expressed in picograms per milliliter.

Expression profiling using DNA microarrays
Total RNA was isolated from DCs using TRIzol Reagent (Invitrogen Life Technologies) followed by RNA clean up with RNeasy Mini kit (Qiagen). The processing of the sample was done following Affymetrix specifications. Briefly, 5 μg of total RNA were used to synthesize first strand cDNA using 200 U of oligo (dT)20 primer and 1x M-MLV reaction buffer (Promega, Madison, WI). Biotin-11-UTP-labeled cRNA was generated using Sp6 transcriptase (Roche, Nutley, NJ) and 250 mM Mg2+ (Pierce), 50 mM MgOAc, and 1 μg of poly I:C. The labeled cRNA was purified by A-15 mGel (Bio-Rad, Hercules, CA) and a strong cation exchange packing column (PerSeptive Biosystems, Framingham, MA). Protein content was determined using a BCA kit (Pierce, Rockford, IL) and SDS-PAGE. Samples were absorbed onto carbon-coated grids and stained with 1% uranyl acetate. The grids were examined with a Philips CM120 transmission electron microscope (Eindhoven, The Netherlands) operating at 80 kV.

Preparation and flow cytometry of DC subsets of mouse splenic cells
DCs were incubated with VLPs for 1 h at 4 °C in Dulbecco’s PBS then washed with medium at 37 °C. At the time points indicated, the cells were washed with PBS, fixed with 3.7% formaldehyde solution for 10 min, permeabilized with 0.1% (v/v) Triton X-100 in PBS for 5 min, and blocked with PBS containing 1% BSA for 30 min. mAb H16.V5 was used at a 1/100 dilution for detection of HPV16 L1, and FITC-conjugated goat anti-mouse IgG (Sigma-Aldrich) was added sequentially at 5 μg/ml for 20 min at 4 °C. Actin was stained with rhodamine phalloidin (Molecular Probes, Eugene, OR). Samples were examined by confocal fluorescence microscopy (28).

Results
HPV16 VLPs induce polarized responses in different mouse splenic DC subpopulations

To address the hypothesis that specialized DC subpopulations regulate the polarization of immune responses (7, 8), we performed a global analysis of the early transcriptional response of key subpopulations of immature splenic DCs to HPV16 VLPs. Because this analysis could be confounded by contaminants, we included additional steps in the purification scheme for HPV16 VLPs. The endotoxin level was <0.058 endotoxin units/ml VLPs (Limulus assay E-Toxate; Sigma-Aldrich), insufficient to cause the effects described herein. We first evaluated the ex vivo interaction between VLPs and CD4^+CD11c^+ , CD8α^+CD11c^+ , and CD4^-CD8α^-CD11c^+ DC subsets that were directly sorted from mouse splenocytes (Fig. 1A and not shown). The phenotype of the key CD4^+CD11c^+ and CD8α^+CD11c^+ DC subpopulations was determined for the Ly6C, B220, and CD11b markers. Both subpopulations exhibited minimal Ly6C and high B220 staining, but only CD4^-CD11c^- and CD8α^-CD11c^- DC subsets were high CD11b staining (Fig. 1A, panel A6). We determined the capacity of each subpopulation of splenic DCs for binding and internalization of HPV16 VLPs. As described for in vitro-cultured BMDCs (24), HPV16 VLPs bound and entered the CD4^-CD11c^- , CD8α^-CD11c^- (Fig. 1B), and CD4^-CD8α^-CD11c^- (not shown) DC subpopulations, suggesting that VLPs can be taken up by multiple subsets of murine DCs. Because HPV16 VLPs activate in vitro-derived BMDCs (24), we performed a microarray analysis of the transcription response of CD4^-CD11c^- , CD8α^-CD11c^- DC subsets to HPV16 VLPs, which are quite different in these primary splenic DC subsets. At 6 h after exposure of CD4^-CD11c^- DCs with HPV16 L1 VLP, IFN-α transcripts were dramatically elevated, notably subtypes α1, α2, α4, α5, and α1–9, but neither IFN-β nor -γ. Furthermore, the CD4^-CD11c^- DCs also induced transcript expression for many interleukins, notably IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13, and chemokines including sacyA1 (thymus-derived chemotactic agent 3/CCL1), sacyA8 (MCP2/CCL8), sacyA7 (MCP3/CCL7), sacyA12 (MCP5/CCL12), sacyA11 (eotaxin/ CCL11), sacyB5 (LPS-induced CXC chemokine), and sacyD1 (fractalkine/CX3CL1) that are associated with Th2 responses. This suggests that CD4^-CD11c^- DCs might play an important role in type 2 immune responses, such as humoral immune induction and Th2 polarization.

Conversely, HPV16 VLP-stimulated CD8α^-CD11c^- DCs also elevated transcription of several interleukins, namely IL-1β (IL-1m), IL-7, IL-12, and IL-15, and chemokines, including sacyA3 (MIP-1α/CCL3), MIP-1β/CCL4, sacyA9 (MIP-1γ/MIP-related protein-2/CCL9), MIP-2, stromal cell-derived factor 1/CXCL12, and sacyA5 (RANTES/CCL5) that are strongly associated with Th1-like responses. Such CCR5 ligands and CXCL12 can provide T cell costimulation (29). Furthermore, we observed up-regulation of MHC class I transcripts in CD8α^-CD11c^- DCs treated with HPV16 VLPs (Table I).

CCL7 is a marker of DC maturation and has a determinant role in the accumulation of Ag-loaded mature DC in T cell-rich areas of the draining lymph node (30). Significantly, CCL7 is the most strikingly elevated (56-fold) transcript in CD8α^-CD11c^- DCs 6 h after exposure to HPV16 VLPs. CCR7 is also up-regulated 7-fold by CD4^-CD11c^- DCs, suggesting both subpopulations are activated by HPV16 VLP. TNF family members are powerful modulators of immune function: transcripts of TRAIL are strongly up-regulated in VLP-stimulated CD4^-CD11c^- DCs, but not in CD8α^-CD11c^- DCs, CD40L expression is down-regulated in CD8α^-CD11c^- DCs, whereas 4-1BB expression is up-regulated in CD4^-CD11c^- DCs (Table I).

Another impressive feature of the microarray analysis is the dichotomy of transcriptional up-regulation of bone morphogenetic protein (BMP) family members; CD4^-CD11c^- DCs up-regulate BMP-2, BMP-6, BMP-7, BMP-8a, and growth and differentiation factor-9B, whereas CD8α^-CD11c^- DCs up-regulate BMP-1, BMP-5, and BMP-8a (Table I) further confirming that CD8α^-CD11c^- DC and CD4^-CD11c^- DC subpopulations generate polarized responses to the same Ag.
Type I IFN promotes Ab isotype switching and acts as a potent adjuvant of humoral immunity to monomeric Ag (31, 32). HPV16 VLPs represent a highly repetitive microbial Ag (11) that induces high titer Ab without the need of an adjuvant (15). Significantly, our microarray analysis shows that HPV16 VLPs cause CD4⁺CD11c⁺ DCs to dramatically up-regulate expression of multiple IFN-α forms, namely IFN-α1, IFN-α2, IFN-α4, IFN-α5, and IFN-α1–9 (Table I). HPV16 VLP-induced transcription by CD4⁺CD11c⁺ DCs was verified using RT-PCR (Fig. 2A) for IFN-α1, IFN-α4, and IFN-α5, but no significant changes were observed.
HPV16 VLP ACTIVATION OF DC SUBSETS

**FIGURE 2.** IFN-α and IL-4 are produced by HPV16 VLP-stimulated CD4⁺CD11c⁺ DCs. A, Relative expression of multiple IFN-α mRNAs in CD4⁺CD11c⁺ DCs stimulated with PBS, HPV16 VLPs (25 μg/ml), or LPS (026:B6) (1 μg/ml) for 6 h, as assessed by RT-PCR. B, Production of IFN-α by CD4⁺CD11c⁺ and CD8α⁺CD11c⁺ DC subpopulations on addition of HPV16 VLP (25 μg/ml), LPS (026:B6 at 1 μg/ml), or poly(I:C) at 25 μg/ml. The poly(I:C)-stimulated samples are shown at 0.1 times their absolute value. C, Production of IL-4 by CD4⁺CD11C⁺ and CD8α⁺CD11c⁺ DC subpopulations on addition of HPV16 VLP (25 μg/ml), LPS (026:B6 at 1 μg/ml), or CpG (5 μM).

**FIGURE 3.** Type I IFN enhances the humoral immune response to HPV16 VLP vaccination. A, Type I IFNR-deficient and control mice were immunized with 10 μg of HPV16 VLPs at 0, 7, 14, and 10 days after the final immunization, sera were collected and analyzed at 1:5 by HPV16 VLP ELISA using a hybridoma isotype kit containing isotype-specific secondary Abs (Biomeda, Foster City, CA). A, A titration of the sera described in A with the HPV16 VLP ELISA using isotype-specific secondary Abs. B, Sera from mice immunized twice 1 wk apart with PBS or 10 μg of HPV16 VLP either with or without 250 U of IFN-α were tested in the HPV16 VLP ELISA using isotype-specific secondary Abs.

for IFN-α2, IFN-α6–11, IFN-α gene B, or IFN-β (not shown). Production of IFN-α and the Th2 cytokine IL-4 in the supernatant of HPV16 VLP-stimulated CD4⁺CD11c⁺ DCs was also confirmed using quantitative ELISA (Fig. 2, B and C). To ascertain the role of IFN-α in the induction of high titer Abs to HPV16 VLPs, we immunized type I IFNR-deficient mice with HPV16 VLPs. Type I IFNR-deficient mice exhibited a weakened humoral immune response to HPV16 VLPs (Fig. 3, A and B). Although specific IgG1 titers remained at a level similar to wild-type mice, the IgG2a titer was most significantly reduced in the type I IFNR-deficient mice (Fig. 3, A and B). We observed no specific IgE production (not shown).

It is possible that IFN-αβR⁻/⁻ mice have defects in DC development that could account for these effects and indeed we observed approximately half the number of CD4⁺CD11c⁺ DC in these mice (not shown). Therefore, we performed a reciprocal experiment to address the effect of IFN-α upon humoral responses to HPV16 VLPs. We immunized wild-type mice twice with 10 μg of HPV16 VLPs either with or without coadministration of 250 U of IFN-α and compared the induction of specific Ab by isotype (Fig. 3C). Similar to studies with monomeric Ags (31), coadministration of IFN-α boosted the specific IgG, but not IgM, responses to HPV16 VLPs (Fig. 3C). Although we observed enhanced IgE responses to a monomeric Ag (OVA) upon coadministration of IFN-α (not shown), we did not observe this after VLP vaccination. Thus, CD4⁺CD11c⁺ DCs not only produce Th2 cytokines and chemokines (Table I and Fig. 2C), but also express type I IFN which promotes humoral immunity (Fig. 3). Production of type I IFN by CD4⁺CD11c⁺ DCs may be important for early control of multiplication and spread of invading pathogens, but their relative in vivo contribution as compared with other DC subsets remains to be determined.

**Induction of IFN-γ expression in CD8α⁺CD11c⁺ DCs by HPV16 VLPs is not required for humoral immunity**

Interestingly, most of the IFN, cytokine, and chemokine transcripts that were elevated by CD4⁺CD11c⁺ DCs in response to HPV16...
FIGURE 4. IFN-γ is produced by HPV16 VLP-stimulated CD8α⁺CD11c⁺ DCs but do not contribute to humoral immunity. A, Relative expression of IFN-γ mRNA in CD8α⁺CD11c⁺ DCs stimulated with PBS, HPV16 VLPs (25 μg/ml), or LPS (026:B6) (1 μg/ml) for 6 h, as assessed by RT-PCR. B, ELISA for IFN-γ in the supernatant of CD8α⁺CD11c⁺ DCs 0, 3, or 6 h after addition of PBS, HPV16 VLP (25 μg/ml), or LPS (026:B6 at 1 μg/ml). C, IFN-γ-deficient and control mice were immunized with 10 μg of HPV16 VLPs at 0, 7, 14, and 10 days after the final immunization, sera were collected and analyzed by HPV16 VLP ELISA using a hybridoma isotype kit (Biomeda).

VLPs were either unchanged or down-regulated in CD8α⁺CD11c⁺ DC. Rather, CD8α⁺CD11c⁺ DCs strongly up-regulated IFN-γ transcripts, but only weakly induced type I IFN message in response to HPV16 VLPs (Table I). We verified this HPV16 VLP-induced up-regulation of IFN-γ by the CD8α⁺CD11c⁺ DCs using RT-PCR (Fig. 4A) and quantitative ELISA (Fig. 4B). LPS induced higher levels of IFN-γ transcripts and protein in CD8α⁺CD11c⁺ DCs than HPV16 VLPs (Fig. 4A and B). However, IFN-γ levels induced by HPV16 VLPs were sufficient for the CD8α⁺CD11c⁺ DCs to dramatically increase expression of many IFN-regulated transcripts (such as IFN-induced protein with tetraitropicopeptide repeats 1 and 2, IFN-induced 15-kDa protein, Mx1, and glucocorticoid attenuated response gene-49/IFN-γ responsive gene-2), presumably via autocrine signaling. Neither IFN-γ nor up-regulation of the above transcripts were observed in HPV16 VLP-stimulated CD4⁺CD11c⁺ DCs.

Production of IFN-γ, as well as IFN-α, by DCs has been linked to T cell-independent induction of Ab class switching (32). Therefore, we examined whether the humoral immune response to HPV16 VLPs was blunted in IFN-γ-deficient mice when compared with wild type. However, the difference in specific Ab response to HPV16 VLP vaccination was limited to a small increase in the specific IgG1/IgG2α ratio in the IFN-γ-deficient mice (Fig. 4C). This suggests that, in contrast to IFN-α, IFN-γ provides only a minimal contribution to the induction of humoral immunity to HPV16 VLPs.

**IFN-γ enhances IL-12 production by HPV16 VLP-stimulated CD8α⁺CD11c⁺ DCs**

IL-12 is central to Th1 responses and its production is strictly controlled by complex positive and negative regulatory mechanisms (33). We confirmed the up-regulation of IL-12b transcript expression using RT-PCR (Fig. 5A) that was indicated by our microarray analysis of HPV16 VLP-stimulated CD8α⁺CD11c⁺ DCs (Table I). Conversely, neither CD4⁺ DCs nor CD4⁻CD8⁻ DCs up-regulate IL-12b transcripts upon stimulation with HPV16 VLPs (not shown). Furthermore, production of IL-12p70 was also observed in the supernatant of CD8α⁺CD11c⁺ DCs, peaking 12 h after stimulation with HPV16 VLPs (Fig. 5B). We observed that CD8α⁺CD11c⁺ DCs rapidly generate both IL-12 and IFN in response to HPV16 VLPs (Figs. 5A, 4A, and Table I). Both IFNs and cytokines can regulate production of IL-12 (34–37), and a “jump-start” mechanism for IL-12-stimulated IFN-γ production by DCs has been proposed (38), suggesting that HPV16 VLP-induced IL-12 production by CD8α⁺CD11c⁺ DCs might be regulated through autocrine IFN signaling. To address this possibility, we included IFN-neutralizing Abs in the medium of CD8α⁺CD11c⁺ DCs and assessed the induction of IL-12 transcripts by HPV16 VLPs (Fig. 5C). The presence in the medium of neutralizing Ab to IFN-γ, but not IFN-α, inhibited HPV16 VLP-induced IL-12b mRNA expression in CD8α⁺CD11c⁺ DCs (Fig. 5C). However, raising the concentration of IFN-γ-neutralizing Ab in the medium to as high as 0.1 mg/ml failed to completely eliminate IL-12b expression by the CD8α⁺CD11c⁺ DCs (Fig. 5D). To confirm the enhancement of IL-12 production by autocrine IFN-γ signaling, we compared IL-12p70 production by IFN-γ⁺/⁺ and IFN-γ⁻/⁻ DCs 12 h after exposure to HPV16 VLP. In the absence of IFN-γ, DCs produce significantly lower levels of IL-12p70 in response to HPV16 VLP (Fig. 5E).

**Discussion**

Rational development of vaccines requires knowledge of the pathways that regulate innate and adaptive immune responses to clinically effective vaccinogens such as HPV16 L1 VLPs (17). Because vaccination with HPV16 VLP induces not only a Th1-biased response but also potent humoral immunity, we sought to determine the in vivo DC subsets that mediate these responses. We demonstrate that after HPV16 VLP stimulation, splenic CD4⁺CD11c⁺ DCs produce Th2-related cytokines and chemokines and IFN-α, which are critical in the induction and enhancement of humoral immunity; conversely, CD8α⁺CD11c⁺ splenic...
DCs produce Th1-related chemokines and cytokines, such as IFN-γ and IL-12, which play a central role in inducing Th1 responses. However, the relative contribution of CD4+ CD11c+ and CD8α+ CD11c+ splenic DCs and possibly other cell populations to these responses in vivo needs to be validated. Upon engaging a pathogen, the cytokines, chemokines, and type I IFN produced by CD4+ CD11c+ DCs may directly activate B cells to produce Abs, thus enabling the host to rapidly control the spread of the invading pathogen. Furthermore, IFN-α and IFN-γ produced by the DCs can directly inhibit viral replication in local infected cells. IFN-γ also provides an autocrine signal to promote the production of IL-12 by CD8α+ CD11c+ DCs and further strengthen type 1 immune responses to the invading pathogen. Thus, our studies suggest a very rapid (within 6 h) “DC-based emergency response” to combat pathogen infection.

Autocrine IFN-γ signaling by CD8αCD11c+ DCs on the production of IL-12

The production of IFN-γ by Th1 cells and NK cells has been widely described, but several recent studies also observed IFN-γ production by DCs (38). We demonstrated that CD8α+ CD11c+ DCs represent a significant source of IFN-γ. The production of IFN-γ by DCs could not only enhance innate immunity but also establish a link to adaptive immune responses. However, the absence of IFN-γ had minimal effect upon the humoral response to HPV16 VLPs, limited to a slight reduction in the IgG2a/IgG1 ratio. IL-12 plays a key role in polarizing Th1 responses and inducing cellular immunity. IFN-γ enhances transcription of both IL-12a and IL-12b (33), and it has a particularly marked effect on production of the heterodimer (Fig. 5). IFN-γ also plays a role by enhancing mRNA expression of the IL-12R (39, 40), and IL-12R transcripts are up-regulated in VLP-treated CD8α+ CD11c+ DCs. IFN-γ positively regulates IL-12p70 expression in CD8α+ CD11c+ DCs, but is not absolutely required for IL-12 production in response to HPV16 VLP. This may account for the small contribution of IFN-γ to the humoral immune response. However, we show that IFN-γ enhances production of IL-12 via autocrine signaling; this suggests a feed forward mechanism that amplifies innate responses and cellular immunity to HPV16 VLPs.

CD4+ CD11c+ DCs promote humoral immunity to HPV16 VLPs

The cytokines IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, and G-CSF are associated with a Th2 response (41). Transcription of all these cytokines and IL-2 is significantly up-regulated in VLP-stimulated CD4+ CD11c+ DCs. Conversely IL-4, IL-10, and IL-13 expression is down-regulated in CD8α+ CD11c+ DC. The cytokines IL-4, IL-5, IL-6, and IL-10 can directly provide B cells with a second signal that can promote and modulate Ig secretion. IL-6 activates transcription mediated by NF-AT leading to production of IL-4 by naive CD4+ T cells and IL-6 also up-regulates suppressor of cytokine signaling-1 that interferes with IFN-γ signaling and the differentiation of Th1 T cells (41). IL-10 inhibits IL-12 production by CD8α+ DCs in response to Ag (47).

Type I IFN plays a central role in the innate response against viral infections and can regulate the adaptive immune response by influencing T cell polarization (42). Intermediate type I IFN levels are associated with limited viral infection, high IL-12 production, and a Th1-biased response, whereas high level production of IFN-α is linked to Th2 polarization (42–45). Furthermore, recent studies also show that type I IFN can enhance humoral immunity and promote isotypic switching (31, 32). Indeed, the absence of type I but not IFN-γ signaling dramatically reduced Ab titers to VLPs and distinct isotype usage. Notably, the specific IgG2a response to HPV16 VLPs is significantly reduced in type I IFN-α deficient mice, suggesting that IFN-α promotes Th1 responses to HPV16 VLPs and IgG2a isotype switching. Endogenous production of type I IFN is essential to the adjuvant activity of CFA (31). Therefore, the high titer Ab response to HPV16 VLPs in the absence of adjuvant as compared with a monomeric Ag may reflect their ability to induce IFN-α. Thus, CD4+ CD11c+ DCs not only produce Th2 cytokines but also express type I IFN to promote humoral immunity.

Although virtually any cell type can be induced to express type I IFN if appropriately stimulated, our microarray and RT-PCR analyses (not shown) revealed that CD4+ CD11c+ DCs dramatically up-regulate IFN-α expression in response to HPV16 VLP. The major mouse cell population producing type I IFN in response to HPV16 VLP is a pattern of cytokine and chemokine presentation in Th1/Th2 regulation.

CCR2 and CCR4 are markers for Th2 cells (29). Ligands for CCR2 include MCP-3 and MCP-5 whose transcripts are up-regulated in VLP-stimulated CD4+ CD11c+ DCs, and strongly down-regulated in CD8α+ CD11c+ DCs. ABCD1/I-CCL22 and thymus and activation-regulated cytokine/CCL17 are ligands for the other Th2 marker, CCR4. CD4+ CD11c+ DCs up-regulate both ABCD1/I-CCL22 and thymus and activation-regulated cytokine/CCL17 transcription in response to HPV16 VLPs. Because CD4+ CD11c+ DCs produce the chemokines that are ligands of the Th2 markers CCR2 and CCR4 but not the Th1 marker CCR5, the chemokine profile implies that this DC subset is associated with a Th2 response to VLPs. HPV16 VLP-stimulated CD4+ CD11c+ DCs also up-regulate CX3CL1/fractalkine, which is critical to homing of the CX3CR1+/CCR2+Gr1+ monocyte subset, and CCR2 ligands, specifically MCP2, MCP3, MCP5, that attract the CX3CR1lowCCR2+Gr1+ monocyte population (48). Conversely, CD8α+ CD11c+ DCs up-regulate transcripts for chemokines that bind the Th1 marker CCR5, notably CCL5/RANTES, consistent with a role for CD8α+ CD11c+ DCs in inducing Th1 responses to HPV16 VLPs.

The dichotomy between the responses of CD8α+ CD11c+ DCs and CD4+ CD11c+ DCs to HPV16 VLPs is even maintained for transcriptional up-regulation of the BMPs; CD8α+ CD11c+ DCs up-regulate BMP1-like, BMP5, BMP8a transcripts, whereas CD4+ CD11c+ DCs up-regulate BMP2, BMP6, BMP7, BMP8b, and growth and differentiation factor-9B transcription. Because BMPs play an important role in early thymocyte differentiation (49), we speculate that BMPs produced by the different DC subpopulations upon HPV16 VLP stimulation regulate adaptive immunity.

A pattern emerging from the expression of cytokine and chemokine profiles together with baseline gene expression patterns suggests the existence of functionally distinct subsets of mouse splenic DCs (47, 50, 51). A single Ag, HPV16 VLP, promotes Th1-associated cytokines and chemokines in CD8α+ CD11c+ DCs whereas the CD4+ CD11c+ DCs transcriptionally up-regulate Th2 cytokines and chemokines. DC1 and DC2 subpopulations that affect adaptive immunity by polarizing CD4 cells have been proposed. The clearly polarized responses of CD4+ CD11c+ DCs and CD8α+ CD11c+ DCs to the same Ag is consistent with such a specialized lineage model in which different subsets of immature DCs diverge early in their development producing functionally distinct sublines (2, 50).
References


