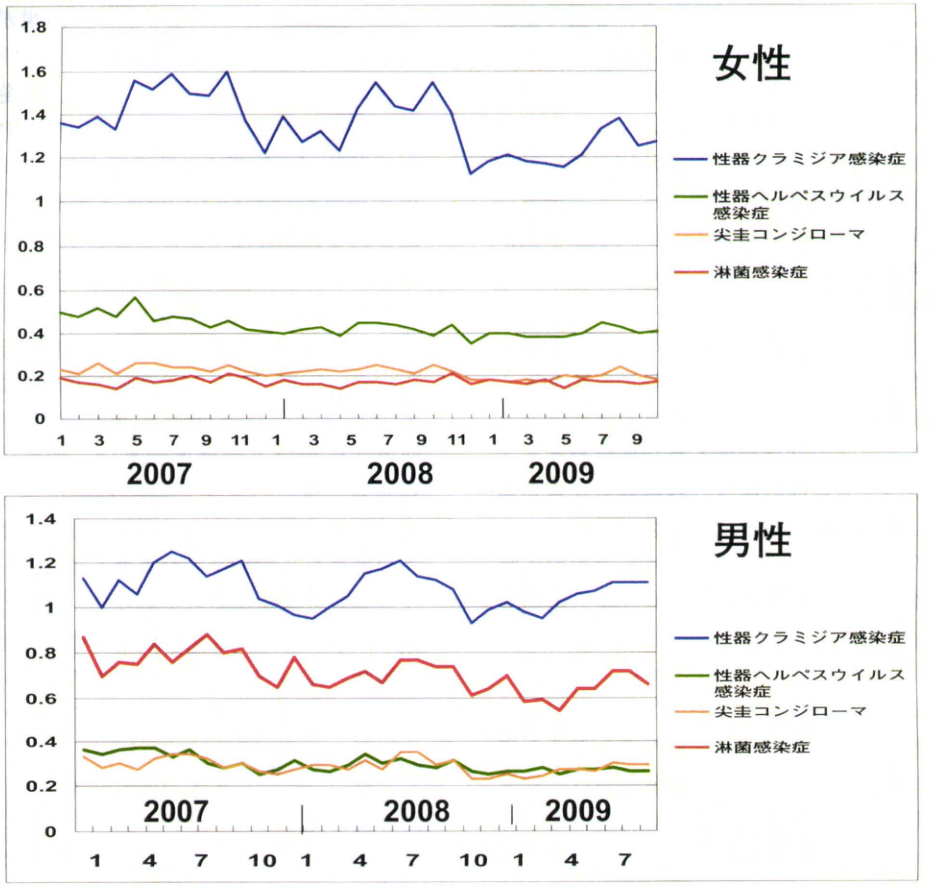

図1　STIの年次推移

●急性型（初感染）

感染後4日～10日目で発症する．初感染では，HSVに対して免疫を持っていないために，発熱，リンパ節腫脹など種々の全身症状を伴い，また，個疹は大きく，神経の走行に一致せずに外陰部の左右対称性に播種状に分布する．排尿困難，排尿痛，帯下を訴える．口内炎や咽頭炎も合併することも多く，病変部からHSVが検出される．全経過2～6週で自然治癒する．発症1～2日目は局所のかゆみや紅斑が出現し，水疱化するにつれて，疼痛が出現する．一般に女性では男性よりも重症化する．膣壁の病変は少ないが，子宮頸部は約88%に病変がみられる[8]．特にHSV-1型感染の場合は症状が重いが，50歳以降の2型初感染も尿閉（Elsberg症候群）[9]などが起こり，重症になることがある．また，頸部硬直，羞明や頭痛が生じ，無菌性髄膜炎を起こしたり[10]，ごく稀にウイルス血症を起こして全身感染を起こす．一般に初感染ではカンジダや細菌の二次感染を起こすことが多い．

●非初感染初発型（誘発型）

既にHSVに感染し，HSVの抗体を持っているものが，性器ヘルペスに初めて罹患する場合を云う．欧米では初めて性器ヘルペスに罹患した患者の約40%が初発型に入るといわれている[8]．無症候感染していたものが始めて再活性化して生じた場合や既にHSVは感染しているが異なる型の初感染の場合がある．また，不顕性感染していたものが，化学療法後，放射線治療後に始めて発症することがあるがこの場合，免疫能が低下しているために再発型よりも重症化する．

●再発型

再発型では一般に症状は軽く，個疹は小さく，

皮疹の分布も限局性である．4日～2週で治癒する．再発型の約8割のものが，腰痛，下肢のしびれ感など再発の前駆症状が数日前より起こり，再発の数時間前より局部のかゆみ，違和感や疼痛が認められる．初感染後，同じ神経節領域の殿部に再発型として発症することもある．再発頻度はHSV-1感染者では平均年1回，HSV-2感染者は平均年10回程度である．

●無症候性ウイルス排泄

HSV-2抗体陽性者の中で性器ヘルペスを自覚しているもの20%，自覚していないもの60%，無症候性のもの20%であると云われている[11]．初感染後3ヶ月の期間が最も無症候性ウイルス排泄が多い[12]．女性の場合，外陰部に症状が現れずに，子宮頸部に無症状に再発していることがあるので特に注意が必要である．新生児ヘルペスの多くは，母親が無症候性ウイルス排泄者であるといわれている．

3. 診　　断

外陰部に浅い潰瘍性や水疱性病変を認めた場合は，性器ヘルペスを疑う．病変の数は，初発では数個から多数あり，広い範囲に及ぶこともあるが，再発では一般に少なく，限局性で，大きさも小さく，ときにピンホール程度のこともあるので注意を要する．また外陰部に潰瘍性病変を形成する疾患は多くあるので，病原診断を行うことが大切である．

HSVの分離培養法が最も良いが，時間と費用がかかるので，塗抹標本を用いて蛍光抗体法によるHSV抗原の証明などによって診断するのが実際的である．ただし，感度が悪いのが欠点である．核酸増幅法（PCR法，LAMP法）が開発されつつあるが，まだ一般臨床には用いることはできない．

血清抗体による診断は，初感染では，急性期に抗体が陰性で回復期になって初めて陽転するので，回復期にならないと診断できない．再発や非初感染初発では，抗体が発症時から検出され，回復期における上昇がないことが多いので，診断には役に立たない．ただし，初感染ではIgM分画の抗体が7～10病日には出現するので，病変が治癒期で病原診断が難しいときは，診断に役立つことがある．しかし，再発型性器ヘルペスの約7%はIgM抗体の出現がみられるので注意を要する[13]．

HSVの型を調べておくことは，再発の予後を推定する上で有用である．我が国では初感染例でHSV-1が検出されることが半数であるが，再発型性器ヘルペスのほとんどはHSV-2が検出される．HSV-2は，ほとんどが性器の感染であるので，HSV-2特異抗体が検出される場合は，性器ヘルペスが疑われる．HSV-2特異抗体は，型特異的糖蛋白gGを用いて酵素免疫抗体法（enzyme-linked immunosorbent assay，ELISA）[14]またはimmunoblot法で検出する方法[15]で，ELISA法のほうが感度が高い．血清学的に型判定が出来，CDCでは疑心例や抗体保有率の統計処理，感染の有無を調べるときに有用であるために，この検査法を推薦している．残念ながら本邦では承認されていない．また，制限酵素によるウイルスDNA断片の電気泳動パターン解析[16]により型の識別だけではなく，株間の識別も可能である．

4. 治　　療

a）初　発

初発例には，アシクロビル錠200mgを1回1錠1日5回，または，バラシクロビル錠500mgを1回1錠1日2回5～10日間経口投与する．重症例では，注射用アシクロビル5mg/kg/回を1日3回，8時間ごとに1時間以上かけて，7日間点滴静注する．症状に応じて，経口，静注ともに投与期間を10日間まで延長する．脳炎や髄膜炎を合併したものではアシクロビル5～10mg/kg/回を1日3回8時間ごとに1時間以上かけて，7日間点滴静注する．

病変が出現したときには，すでにHSVは神経節に潜伏感染しているので，抗ヘルペスウイルス薬で治療しても，再発を免れることはできない．

b）再　発

アシクロビル錠200mgを1日5回，またはバラシクロビル錠500mgを1日2回，5日間経口投与する．再発の前駆症状である局所の違和感や神経痛様の疼痛があるときに本剤を服用すると，病変の出現を予防できることがある．したがって，あらかじめ薬をわたしておいて，早め

に服用させるが，前駆症状出現後6時間以降では抑制率が20%に低下する[17]．

c) 免疫不全を伴う重症例

点滴静注用アシクロビルを5 mg/kg/ 回で1日3回点滴静注，7～14日間投与する．

d) 再発の抑制

年6回以上再発を繰り返す患者や再発時の症状が重い患者に対して，患者の精神的苦痛を取り除きQOLの改善のためや，他人への感染を予防するため，抗ヘルペスウイルス薬の継続投与による抑制療法が欧米では行われている．抗ヘルペスウイルス薬としては，アシクロビル（1回400mg，1日2回）バラシクロビル（1回500mg，1日1回）が用いられ，1年間継続投与後，中断させ，再投与するかを検討することを勧めている．アシクロビルでは，6年間にわたり長期服用しても副作用はほとんどないとされている．日本では2006年9月にバラシクロビル1回500mg，1日1回の服用による抑制療法が健康保険で行えるようになった．なお，HIV感染症の成人（CD4リンパ球数100/mm にはバラシクロビル1回500mgを1日2回経口投与する．本療法により60～70%の患者では再発を抑制できるが，年10回以上も再発する患者では服用中に再発することもある．この場合一般的に症状は軽く，バラシクロビルの治療量（1回500mg，1日2回）を増量し治癒したら再びもとに戻す．この抑制療法を行う場合は，患者に薬剤を慢然と渡すのではなく，治療目標を設定し，その効果，副作用，服薬状況などを観察する必要がある．再発抑制に対してバラシクロビルを投与しているにもかかわらず頻回に再発を繰り返すような患者に対しては，症状に応じて1回250mg，1日2回または1回1,000mg，1日1回投与に変更することを考慮する．これでも再発するものは，診断を見直す意味でも，ウイルスを分離し，アシクロビル感受性を調べる．また，体重40kg以上の小児に対しても2010年承認され，バラシクロビル500mgを1日1回投与するか，ゾビラックス1回200mgを1日4回投与する．

5. 感染予防対策

性器ヘルペス患者は，性器ヘルペス発症時は治療と性行為を禁止させる．

抑制療法中の患者でも，パートナーをも含めて，コンドームの使用が勧められている．しかし，再発は，肛門，殿部，大腿部などにも起こりうるので，コンドームの使用だけでは完全に防止できない．また，抑制療法を行うことによりHIV感染予防になり，一方HIV感染患者のウイルス量を減少させることができると云われている[18]．

母子感染の予防のため，性器にヘルペス性病変がある場合は，帝王切開で胎児を分娩させることが勧められている．今までのデータによると，ヒトにおけるアシクロビルの催奇形作用はほとんどないとされており，妊娠中に性器ヘルペスに罹患した場合，アシクロビルの投与は可能である．

今後，米国では，性器ヘルペス撲滅のためにワクチンの開発を促進しており，現在gD-Alum/MPL Vaccineが10歳から30歳までのHSVの抗体を持っていない女性7,750名を対象に治験が行われている．

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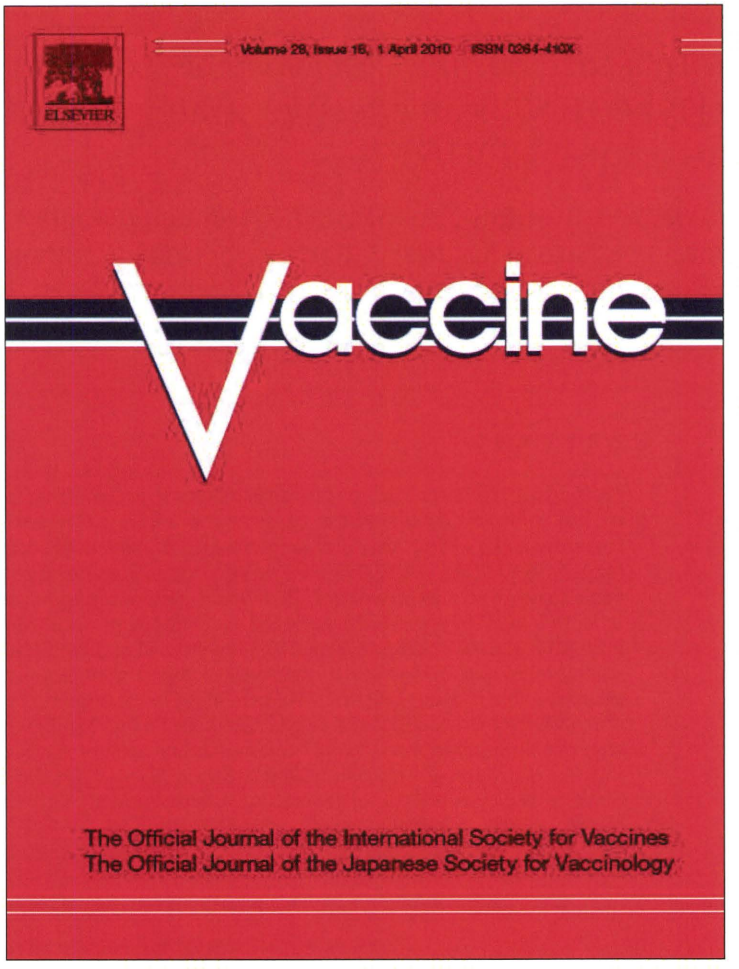

Vaccine 28 (2010) 2810–2817

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Oral immunization with a *Lactobacillus casei* vaccine expressing human papillomavirus (HPV) type 16 E7 is an effective strategy to induce mucosal cytotoxic lymphocytes against HPV16 E7

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ARTICLE INFO

Article history:
Received 27 October 2009
Received in revised form 22 January 2010
Accepted 3 February 2010
Available online 17 February 2010

Keywords:
HPV therapeutic vaccine
Mucosal cellular immune response
Lactobacillus-based vaccine
Oral immunization

ABSTRACT

Although many clinical trials on human papillomavirus (HPV) therapeutic vaccines have been performed, clinical responses have not been consistent. We have addressed mucosal cytotoxic cellular immune responses to HPV16 E7 after oral immunization of mice with recombinant *Lactobacillus casei* expressing HPV16 E7 (LacE7). C57BL/6 mice were orally exposed to 0.1–100 mg/head of attenuated LacE7 or vehicle (Lac) vaccines at weeks 1, 2, 4, and 8. Responses to subcutaneous or intramuscular injection of an HPV16 E7 fusion protein using the same timing protocol were used for comparison. Oral immunization with LacE7 elicited E7-specific IFNγ-producing cells (T cells with E7-type1 immune responses) among integrin $\alpha4\beta7^{+}$ mucosal lymphocytes collected from gut mucosa. An induction of E7-specific granzyme B-producing cells (E7-CTL) exhibiting killer responses toward HPV16 E7-positive cells was also observed. The induction of T cells with specific mucosal E7-type1 immune responses was greater after oral immunization with LacE7 when compared to subcutaneous or intramuscular antigen delivery. Oral immunization with *Lactobacillus*-based vaccines was also able to induce mucosal cytotoxic cellular immune responses. This novel approach at a therapeutic HPV vaccine may achieve more effective clinical responses through its induction of mucosal E7-specific CTL.

1. Introduction

Human papillomavirus (HPV) is a major risk factor for the development of cervical cancer which is the second most common cancer among women. An estimation of the worldwide cancer incidence and mortality for 2002 showed that persistent HPV infection had caused about 500,000 cases of cervical cancer [1]. Some 99% of cervical cancer cases are associated with genital infection with HPVs. At present, there are about 100 identified genotypes of HPVs and more than 40 subtypes are known to infect human genitalia. HPV type 16 (HPV16) infection is most commonly associated with cervical cancer. It is found in 50% of cervical cancers and cases of high-grade cervical intraepithelial neoplasia (CIN) [2–4] and in 25% of low grade CIN lesions. Since at least 50–80% of women are thought to be exposed to genital HPV in their lifetime and persistent HPV infection is associated with progression to high-grade CIN or cervical cancer [2,5] a vaccine that prevents genital infection with HPV16 should substantially reduce the incidence of cervical cancer. Still, while prophylactic vaccines composed of L1 virus-like particle are available and have been shown to prevent HPV infection with the virus types contained in the vaccine [6], they cannot help the millions of patients who have already been infected [7]. Therapeutic vaccines are also needed.

While some HPV infections may persist and progress to cervical cancer, most infections resolve spontaneously, probably as the result of host cellular immune responses to HPV viral proteins. The HPV E6 and E7 oncogenic proteins are essential to the process of carcinogenesis, and their immunogenicity has given HPV an attractive target for use in immunization strategies to treat CIN or cervical cancer. In fact, several therapeutic vaccines against HPV E6 and/or E7 oncogenic proteins have been developed and clinical vaccination trials using these reagents against CIN and cervical cancer have been completed [8–16]. Using immunohistochemistry, Ressler et al. have demonstrated that high-grade CIN lesions strongly express E7 proteins, suggesting E7 as a target molecule for immunotherapy against high-grade CIN [17].

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doi:10.1016/j.vaccine.2010.02.005

However, no studies to date have demonstrated clinical efficacy of E7-based vaccines. E7-based vaccines have been shown to elicit E7-specific systemic cellular immune responses after subcutaneous or intramuscular delivery, but few have addressed E7-specific cellular mucosal immune responses. Because CIN lesions develop in the cervical mucosa, we believe that cervical mucosal cytotoxic cellular immune responses to E7 are essential for clearance of CIN. Subcutaneous or intramuscular antigen administration may be significantly less effective delivery routes for therapeutic vaccines against HPV-associated CIN.

Poo et al. have shown that oral immunization of C57BL/6 mice with *Lactobacillus casei* (*Lb. casei*) expressing HPV16 E7 reduces tumor formation induced by TC-1 cell administration. TC-1 cells have been immortalized by HPV16 E6/E7 and c-Ha-*ras*. Immunization in these experiments elicited type1 T cell immune responses to E7 in lymphocyte from the spleen and from anogenital regional lymph nodes [9]. Using intranasal mucosal immunization, Bermúdez-Humarán et al. also demonstrated that TC-1 tumors regressed in mice exposed to a *Lactobacillus*-based E7 vaccine and that antigen-specific CTL responses in splenocytes were robust [18]. Although both studies used transmucosal immunization with *Lactobacillus*-based vaccines, they examined E7-specific systemic cellular immune response and regression of subcutaneous TC-1-induced tumors. These investigations provide no insight into mucosal cellular immune responses after immunization and the antigen specificity of mucosal lymphocytes.

Mucosal immunity serves as the first line of defense against microbial infections. These activities include the clearance of microbes from infected lesions and the production of antimicrobial neutralizing antibodies and involve a dynamic immune network that combines innate and acquired mucosal responses. All mucosal sites (lachrymal, nasal, bronchial, gastrointestinal, and genital) share a common immune network that includes induction in mucosa-associated lymphoid tissues (MALT), distinctive effector sites, and specific mucosal lymphocytes (intraepithelial and lamina propria-associated). MALT and mucosal lymphocytes are functionally and anatomically distinct from the systemic lymphoid tissues (e.g. spleen) and lymphocytes (e.g. splenocyte and peripheral white blood cells) of the peripheral immune system. Integrin $\alpha4\beta7$ is a mucosa-associated homing receptor, the expression of which is induced on mucosal lymphocytes only upon interaction with dendritic cells residing in the gut-associated lymphoid tissues (GALT) [19]. Although integrin $\alpha4\beta7^+$ lymphocytes are commonly considered to be gut-derived mucosal lymphocytes, they may be derived in and home to several other distinct mucosal sites. For instance, after binding to natural ligands, such as mucosal addressin cell adhesion molecule (MAdCAM) that is expressed in venular endothelial cells at mucosal sites, integrin $\alpha4\beta7^+$ lymphocytes can also home to nasal, urogenital and other mucosal sites [20–23]. Hawkins et al., have shown that integrin $\alpha4\beta7^+$ lymphocytes home to the chlamydia-infected murine genital tract [24]. In response to these and other findings, oral immunization has become an attractive antigen delivery system that can effectively carry antigen to inductive sites (e.g. GALT such as Peyer's patches) that elicit antigen-specific mucosal immune responses [25]. Further, oral immunization of *Lb. casei* is reported to have an adjuvant effect on type1 T cell immune responses at gut mucosa [26].

Mucosal memory and effector T cells against E7 are essential to the clearance of CIN. Investigations on mucosal immune response to mucosally administered HPV therapeutic vaccines are scarce and no groups have specifically addressed the induction of integrin $\alpha4\beta7^+$ antigen-specific mucosal T cells. In this study, we orally administered *Lb. casei* expressing HPV16 E7 to C57BL/6 mice and assessed mucosal cellular immune responses to HPV16 E7, including the induction of IFNγ-producing type1 T cells and granzyme B-producing $CD8^+$ T cells, and cytotoxic activity against TC-1 cells. Since it was difficult to isolate strictly mucosal T cells from the murine cervix, we used mucosal T cells isolated from gut mucosa as a surrogate for those isolated from the cervix. We demonstrated that oral immunization with *Lb. casei* expressing HPV16 E7 elicited E7-specific mucosal cellular immune responses in gut-derived integrin $\alpha4\beta7^+$ lymphocyte. This may accompany similar homing of primed memory/effector cells from the gut inductive site to effector sites in the cervical mucosa for clearance of high-grade CIN.

2. Materials and methods

2.1. Preparation of attenuated Lb. casei-based vaccines

Lb. casei strain 525 was isolated from sugar apple. *Lb. casei 525* was cultured in MRS medium at 30 °C. The pKV-based expression plasmid was a kind gift from Dr. Sung (Bioleaders Corporation, Korea). The plasmid has a *Lactobacillus*-derived promoter upstream of the integration site for the gene of interest. This plasmid also fuses the gene of interest to pgsA, a peptideglican that traffics the protein of interest to the cytoplasmic membrane as shown previously [9]. In this study, we modified the HPV16 E7 gene by inserting a point mutation into the Rb-binding site (the D, C, and E in the E7 aa21, aa24 and aa26, respectively, were all replaced by a G). Through this mutation, the carcinogenicity of E7 was lost its immunogenicity remained intact [10]. Two plasmid subtypes (pKV/mutated E7 and pKV alone) were created, purified and transformed into *Lb. casei* by electroporation. The recombinant *Lb. casei* expressing HPV16 mutated E7 and the vehicle, *Lb. casei* alone, were named LacE7 and Lac, respectively. LacE7 and Lac were expanded using large scale culture in MRS medium with erythromycin (16 μg/mL) at 30 °C and attenuated using heat. The attenuated *Lb. casei* were purified by washing several times with distilled water, dried to powder and stored at 4 °C until use. The powder weight of the attenuated *Lb. casei* was used to define vaccine inoculum dosage. Semi-quantitative SDS-PAGE and ELISA analyses revealed that 1 g of attenuated LacE7 contained about 7 mg of HPV16 E7 protein (data not shown).

2.2. Immunization of mice with Lactobacillus-based vaccines

Eight-week-old, female SPF C57BL/6 ($H\text{-}2^b$) mice were used for immunization experiments (CLEA Japan. Inc., Japan). Various vaccine doses (0.1, 0.3, 1.0, 3.0, 10, and 100 mg/head) of the attenuated LacE7 or Lac were administered to five mice per dose at weeks 1, 2 and 4 (priming) and at week 8 (booster). LacE7 and Lac powders were insoluble in water-based solvents. All inoculums were suspended in PBS (200 μL/head) and administered via an intra-gastric tube after 3 h of fasting, once per day for 5 days each week. For comparison, 50 μg of purified HPV16 E7-tag fusion protein (kind gift from Dr. Sung, Bioleaders Corporation, Korea) was suspended in complete Freund's adjuvant and administered to mice by intramuscular (i.m.) or subcutaneous (s.c.) injection once per week at weeks 1, 2, 4 and 8.

2.3. Isolation of murine splenocytes and intestinal mucosal lymphocytes

Intestinal mucosal lymphocytes and splenocytes were isolated from immunized mice 1 week after priming or booster inoculations (at week 5 or 9, respectively). After sacrifice, intestines or spleen were obtained from five distinct mice and washed three times in HBSS containing protease inhibitors. The intestines were opened longitudinally and shaken vigorously in RPMI1640 containing 10% FCS, 100 units/mL of penicillin and 100 μg/mL of streptomycin for 30 min at 37 °C. The resulting cell suspensions were passed through a BD Falcon Cell-strainer (BD Bioscience, USA) to remove tissue

debris and were subjected to discontinuous density gradient centrifugation in a 15 mL tube layered from the bottom with 70% and 40% Percoll (Sigma, USA). Approximately 10^{7-8} cells were layered onto the gradients and centrifuged at $600 \times g$ for 20 min at room temperature (LX-130 TOMY SEIKO Co., Ltd., Japan) The interface between the 70% and 40% layers contained lymphocytes with a cell viability of more than 95%. Splenocytes were prepared by gently teasing the spleen in the HBSS. Clumped debris was removed by centrifugation. Approximately $5–10 \times 10^6$ intestinal mucosal lymphocytes and 10^7 splenocytes were obtained from individual mice.

2.4. Flow cytometry

Murine intestinal mucosal lymphocytes were incubated with an anti-CD4 or anti-CD8 mAb conjugated to PE-Cy5 (1 μg/mL, eBioscience, USA or Beckman Coulter, USA), or with an anti-integrin α4β7 (LPAM-1) mAb conjugated to PE (1 μg/mL, Santa Cruz Biotechnology, USA) for 30 min at 4 °C. Controls were exposed to an isotype-matched irrelevant mAb (1 μg/mL, Beckman Coulter). Cells were suspended in 1% paraformaldehyde, and analyzed using a FACSCalibur flow cytometry system (BD Bioscience, USA).

2.5. ELISPOT assay

50 μL of intestinal mucosal lymphocytes or splenocytes (2×10^6 cells/mL) were incubated for 24 h at 37 °C with antigen presenting cells, 50 μL of splenocyte (1×10^6 cells/mL) treated with mitomycin C (75 μg/mL, Nakarai, Japan), and washed four times with PBS. 10 μL of a synthesized peptide (working conc. = 1 μg/mL) corresponding to amino acid 49–57 of HPV16 E7 (a reported CTL epitope for C57BL/6 (H-2^b) mice), mitogen (PMA 40 ng/mL + ionomycine 4 μg/mL), or medium alone (negative control) were added to a 96-well ELIIP plate (Millipore USA) coated to anti-mouse IFNγ or granzyme B monoclonal antibodies (15 μg/mL) according to the manufacturer's protocols for ELISPOT for Mouse Interferon-γ (MABTECH AB, Sweden) and Granzyme B Development Module (R&D systems, USA). Spot numbers of IFNγ and granzyme B-producing lymphocytes were analyzed with a fully automated computer assisted video imaging analysis system, KS ELISPOT (Carl Zeiss Vision, Germany) [10].

2.6. Cytotoxic T lymphocyte (CTL) assays

E7-specific cytotoxicity was measured using a CTL assay system and the Cellular DNA Fragmentation ELISA (Roche, USA). TC-1 cells are derived from a primary lung cell from C57BL/6 mice (allogeneic to the immunized mice) and immortalized by HPV16 E6/E7 plus c-Ha-*ras* and were used in these experiments as target cells (kind gift from Dr. T.C. Wu, Johns Hopkins University, Baltimore, MD USA) [27]. Briefly, target cells were cultured in medium containing BrdU for 12 h at 37 °C. The cells were harvested the following day, washed and plated at a concentration of 2×10^4 cells/well in a 96-well plate. Plates were incubated for 6 h at 37 °C with effecter cells at an effecter cell/target cell (E:T) ratio of 1–20. The release of BrdU from killed cells was measured at OD 450 nm according to the manufacturer's protocol.

2.7. Statistical analysis

ELISPOT data are presented as means ± standard deviations. Experiments were performed independently at least three times. ELISPOT numbers were compared between the immunization groups (5–6 mice/each group) using paired, two-tailed Student's *t*-tests. A *p*-value of <0.05 was considered significant.

3. Results

3.1. Integrin α4β7⁺ mucosal T cells

Assessment for mucosal cellular immune response requires isolation of mucosal T cells that express homing receptors, including integrins (integrin α4β7 and α4β1). A study in a chlamydia-infected murine model demonstrated that intestinal mucosal lymphocyte also home to the genital tract mucosa. Integrin α4β7 rather than α4β1 is reported to be a central homing receptor directing transit of mucosal lymphocytes to genital tract mucosae [28]. This gut/genital tract similarity allowed us to study mucosal lymphocytes isolated from murine intestinal mucosa in our investigation. These lymphocytes were isolated as described previously [29] and their phenotype was examined using flow cytometry (Fig. 1). About 90% of the lymphocyte were positive for integrin α4β7. The proportions of CD4⁺ and CD8⁺ integrin α4β7⁺ cells were 45% and 56% of the lymphocytes, respectively. A portion of CD4⁺ or CD8⁺ integrin α4β7⁺ cells may be CD4⁺ and CD8⁺ (double positive) T cells since the total percentages exceed 100%. The CD4⁺ and/or CD8⁺ T cells expressed intermediate levels of integrin α4β7 (bold lines in Fig. 1). A CD4⁻ and CD8⁻ subset of integrin α4β7⁺ cells was presumed to include integrin α4β7⁺ B, NK, or double negative (CD4⁻ and CD8⁻) T cells. The phenotypes of these intestinal mucosal lymphocytes were markedly different from those isolated from peripheral blood and confirmed our isolation of mucosal T cells. 0.3–2% of isolated mucosal lymphocytes were B cells (data not shown).

3.2. Increase in IFNγ-producing type1 T cell after oral immunization with Lb. casei

Clearance of cells infected with HPV or immortalized by HPV requires cellular cytotoxic immune responses that are provided

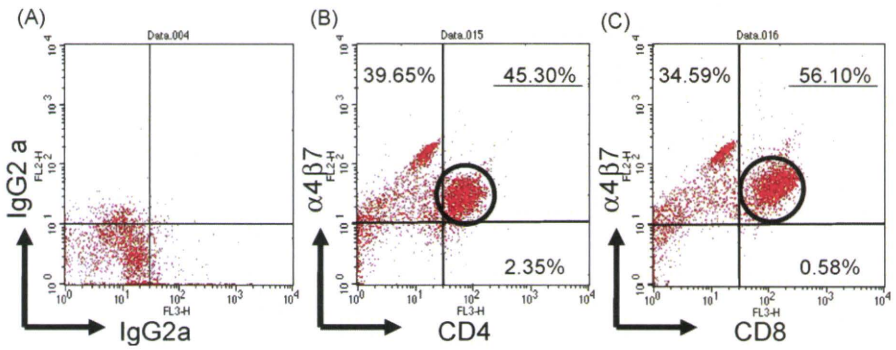

Fig. 1. Flow cytometry analysis of isolated mucosal lymphocytes. Instrument settings for separate samples were identical. The appropriate isotype profile is shown in (A). These profiles depict PE-Cy5 CD4⁺(B) or CD8⁺(C) cells on the *x*-axis and PE integrin α4β7⁺ cells on the *y*-axis. The dual-labeled CD4⁺ or CD8⁺/integrin α4β7⁺ cells cluster in the upper right quadrant (bold line).

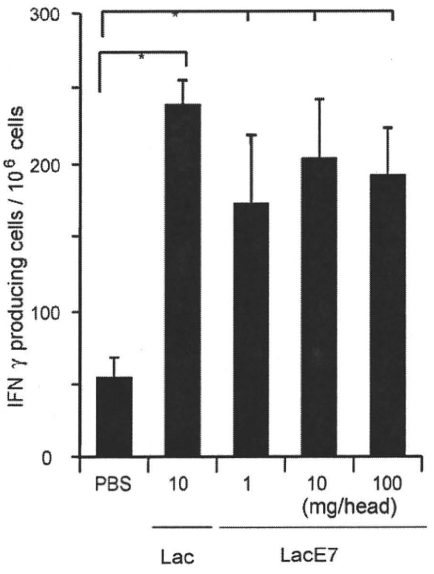

Fig. 2. To examine effect of oral immunization with a recombinant *Lactobacillus casei*-based vaccine on mucosal type1 cell immune responses, the number of non-specific IFNγ-producing cells among the mucosal lymphocytes was assessed using an ELISPOT assay after mitogen stimulation (40 ng/mL of PMA and 4 μg/mL of ionomycin). Groups of five mice received LacE7 (1, 10, and 100 mg/head) or Lac (10 mg/head) orally. Integrin $\alpha4\beta7^+$ intestinal mucosal lymphocytes were collected from each mouse 1 week after final immunization. Asterisks indicate those comparisons (PBS vs. Lac/LacE7) with statistical significance ($p < 0.05$) ($n = 5$).

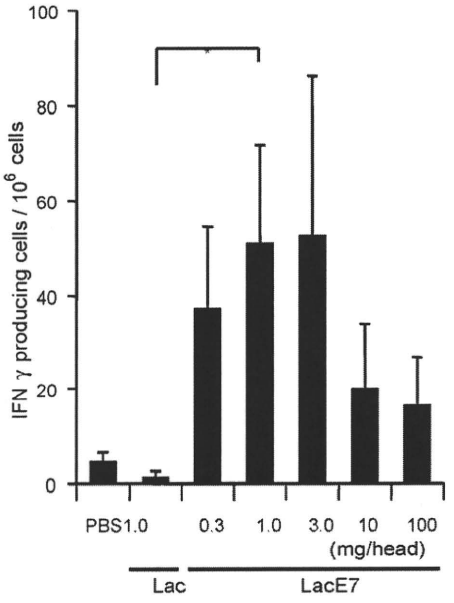

Fig. 3. Various doses of LacE7 (0.3, 1.0, 3.0, 10, and 100 mg/head) or Lac were orally administered to a group of five mice at weeks 1, 2, and 4. The same dose was used for all oral administrations to a given mouse. Integrin $\alpha4\beta7^+$ intestinal mucosal lymphocytes were collected from the immunized mice at week 5. The number of E7-specific IFNγ-producing cells among the mucosal lymphocytes was assessed using an ELISPOT assay after stimulation with 1 μg/mL of a synthetic peptide corresponding to amino HPV16 E7 acids 49–57. Asterisk indicates those comparisons (Lac vs. LacE7) with statistical significance ($p < 0.05$) ($n = 5$).

by IFNγ-producing $CD4^+$ helper T cells (type1 immune response-related T cells) and cytotoxic $CD8^+$ T (Tc) cells. To examine the effect of oral immunization with a *Lb. casei*-based vaccine on mucosal type1 immune response-related cells, the number of IFNγ-producing cells among all mucosal lymphocytes was assessed by ELISPOT assay (Fig. 2). Each group of five mice was orally exposed to *Lb. casei* bearing HPV16 E7 (LacE7) at 1 mg/head, 10 mg/head, or 100 mg/head or to vehicle (Lac) vaccines at 10 mg/head at weeks 1, 2, and 4. Oral administration occurred once per day for 5 days each week. Intestinal mucosal lymphocytes were collected from each mouse 1 week after the last immunization. For ELISPOT assays, the lymphocytes were stimulated with the mitogens, PMA and ionomycin, and the type1 immune response-related cells were detected as IFNγ positive spots. The number of IFNγ-producing type1 immune response-related cell increased by a similar amount after immunization with equal amounts of Lac and LacE7. Type1 cell increases were also dependent on the dosage of LacE7. This suggested that *Lb. casei* provides a non-specific adjuvant effect on the induction of type1 immune responses at mucosal sites.

3.3. The dose-dependent induction of E7-specific cellular immune response by oral immunization with LacE7

HPV16 E7 amino acids 49–57 are known to represent an E7-CTL epitope that is recognized by the CTL and type1 T cells of C57BL/6 mice [11]. A synthetic peptide with an amino acid sequence corresponding to this CTL epitope was used as a stimulant in E7-specific ELISPOT assays to assess type1 T cell immune responses to HPV16 E7. Various doses of LacE7 (0.3, 1.0, 3.0, 10, and 100 mg/head) or Lac were administered orally to five mice at weeks 1, 2, and 4 as in Fig. 3. Integrin $\alpha4\beta7^+$ intestinal mucosal lymphocytes were collected from the immunized mice at week 5 and examined in an E7-specific ELISPOT assay. The number of type1 immune response-related T cell producing IFNγ after stimulation by the E7 peptide increased significantly in LacE7-immunized mice but not in Lac-immunized mice. This demonstrates that oral immunization of mice with LacE7 elicits type1 mucosal immune response-related T cells that direct E7-specific CTL in the intestinal mucosa. Interestingly, the number of the E7-specific type1 immune response-related T cells peaked at exposure levels of 1.0–3.0 mg/head and decreased with dose escalation over 3.0 mg/head (Fig. 3).

To increase type1 immune response-related T cell cellular immune responses, mice immunized orally with LacE7 or Lac at weeks 1, 2, and 4 received booster oral immunization at week 8. Intestinal mucosal lymphocytes were then isolated and analyzed at week 9. In these experiments, a variety of exposures to 1.0 mg/head or less of LacE7 were used for prime and boost administrations to find the minimum dose that would elicit an optimal type1 immune response-related T cells (Fig. 4). E7-specific, IFNγ-producing type1 cells increased in number with LacE7 dose escalation. 1.0 mg/head of LacE7 oral immunization induced E7-specific type1 cellular immune responses significantly and appeared to be the optimal induction dose while the same dose of Lac alone had no effect on these responses. The booster immunization at week 8 raised E7-specific type1 immune cell numbers 4-fold when compared to the induction at week 5.

3.4. Comparison of mucosal cellular immune responses after oral, intramuscular, and subcutaneous immunizations

Previous studies on HPV therapeutic vaccines have demonstrated E6/E7-specific cellular immune responses in splenocyte or PBMCs in mice [8]. All previous clinical trials in humans have also used peripheral or systemic response read-outs (PBMCs). Prior studies on HPV therapeutic vaccines have also been limited to intramuscular or subcutaneous exposure to E6/E7-fused proteins or plasmid DNA expressing E6/E7 genes [12,13]. Few investigators have addressed specific mucosal cellular immune and few have used mucosal exposure as the route of immunization. Although some studies have analyzed post-exposure lymphocytes collected from minced genital tract tissue or its draining lymph nodes [30,31],

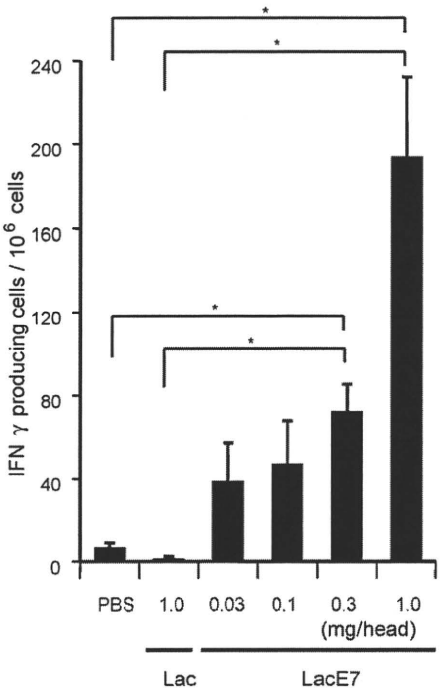

Fig. 4. Various doses of LacE7 (0.03, 0.1, 0.3, and 1.0 mg/head) or Lac were orally administered to a group of five mice at weeks 1, 2, and 4 (priming) and week 8 (boost). The same dose was used for priming and boost exposures in a given mouse. Integrin $\alpha 4\beta 7^+$ intestinal mucosal lymphocytes were collected from the immunized mice at week 9 and examined using an E7-specific ELISPOT assay as shown in Fig. 3. The boost immunization at week 8 raised E7-specific type1 immune cell numbers 4-fold more than those at week 5. Asterisks indicate those comparisons (PBS/Lac vs. LacE7) with statistical significance ($p < 0.05$) ($n = 5$).

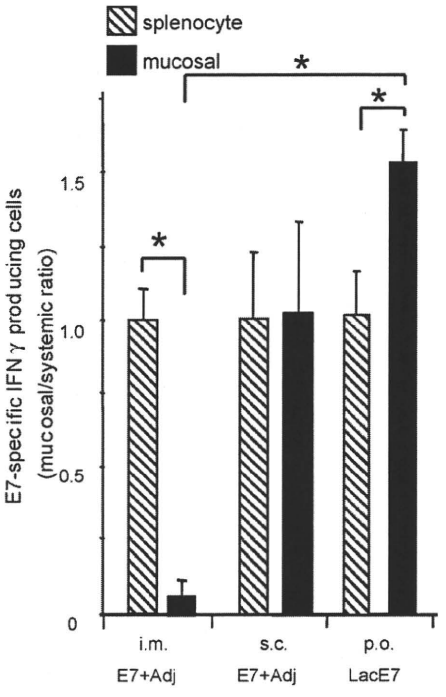

Fig. 5. Oral, intramuscular, or subcutaneous immunizations were compared for the induction of mucosal E7-specific type1 immune responses. Mice were immunized by each route at weeks 1, 2, 4 and 8. Both intestinal mucosal lymphocytes (black) and splenocytes (stripe) were collected from mice immunized by each method and assessed using an E7-specific ELISPOT assay. Each route of immunization elicited similar E7-specific IFNγ-producing type1 immune responses in splenocytes. The number of E7-specific type1 cells among mucosal lymphocytes was normalized to that among splenocytes for each immunization route. This ratio is depicted on the *y*-axis. Asterisks indicate those comparisons (oral vs. other immunization) with statistical significance ($p < 0.05$) ($n = 5$).

these cells are grossly contaminated by peripheral cells in the vessels supplying these tissues and represent local, but not specifically mucosal immune responses.

In this study, intestinal mucosal lymphocytes and splenocytes were collected from immunized mice to represent mucosal and systemic cellular immune responses, respectively. To address the role of route of vaccination on mucosal immunity, mucosal and peripheral responses to oral immunization with LacE7 was compared to intramuscular and subcutaneous immunizations with E7. For intramuscular and subcutaneous injections, a purified E7-fused protein with adjuvant was used since LacE7 was not water soluble and was difficult to use in injections. Each immunization was performed in five mice at weeks 1, 2, 4 and 8 (Fig. 5).

Oral, subcutaneous and intramuscular exposures elicited fairly consistent levels of induction of E7-specific IFNγ-producing type1 immune cells among splenocytes (about hundreds cells/10^6 splenocytes). For comparison, the number of E7-specific type1 immune cells among mucosal lymphocytes was normalized to that among isolated splenocytes for each immunization. Intramuscular immunization induced barely detectable levels of E7-specific type1 mucosal immune cells, significantly less than that found among splenocytes (about one-tenth of that in splenocytes). Subcutaneous immunization induced E7-specific type1 mucosal and peripheral immune responses equally. Oral immunization of LacE7 elicited a predominant mucosal E7-specific type1 immune response, with type1 immune response-related cell levels approximately 1.5–2.0-fold higher than those among splenocytes. The most effective immunization route for the induction of HPV-specific mucosal cellular immune response was oral immunization, followed by subcutaneous immunization. Intramuscular immunization had little effect on mucosal responses.

3.5. E7-specific cytotoxic activity of induced mucosal lymphocytes

E7-specific $CD4^+$ type1 immune response-related cells were detected among the mucosal lymphocytes collected from mice immunized orally with LacE7. To confirm the activity of E7-specific $CD8^+$ CTLs among mucosal lymphocytes, T cells producing granzyme B in response to the E7-CTL epitope (HPV16 E7 amino acids 49–57) were measured using ELISPOT assays and killer activity to HPV16 E7-expressing (TC-1) cells examined. The murine H-2^b tumor cell line, TC-1, is derived from an allogeneic mouse strain (C57BL/6) and immortalized by HPV16 E6/E7 plus c-Ha-*ras*. TC-1 cells were used as a target cells in killing assays. Mucosal lymphocyte and splenocyte were collected from mice receiving oral immunization of LacE7 at weeks 1, 2, 4, and 8 and used for CTL assays (Fig. 6). ELISPOT assays revealed an induction of E7-specific granzyme B-producing cells in isolated mucosal lymphocyte and splenocyte populations. As shown in Fig. 5, there was a trend toward higher numbers of E7-specific, granzyme B-producing cells among mucosal lymphocytes when compared to splenocytes although this difference was not significant (Fig. 6A). When mucosal lymphocyte were used as effector cells in killer assays with E7-expressing epithelial cell targets derived from C57BL/6 mice (TC-1 cells), the effector cell will recognize target cell MHC class I molecules since both cells are derived from same mouse strain. Mucosal lymphocytes isolated from mice immunized with LacE7 had increased lytic effects against TC-1 cells that were appropriately dependent on the E:T ratio; those isolated from control mice did not. This demonstrates that mucosal lymphocyte killer activity was specifically induced through recognition of the E7-CTL epitope by the mucosal $CD8^+$ T cell.

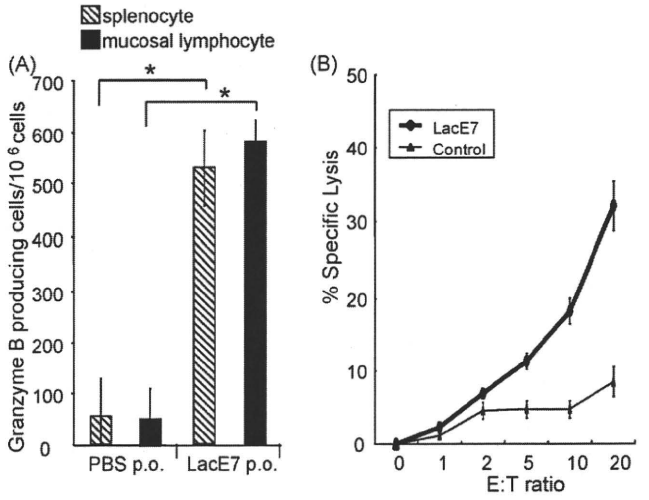

Fig. 6. T cells producing granzyme B in response to E7-CTL epitope exposure were measured using an ELISPOT assay and their killer activity toward TC-1 cells was examined. Mucosal lymphocytes and splenocytes collected from mice receiving oral immunization of LacE7 at weeks 1, 2, 4, and 8 were used for CTL assays. (A) ELISPOT assay revealed an induction of E7-specific granzyme B-producing cells in both mucosal lymphocytes (black) and splenocytes (stripe). The ELISPOT assay was examined as shown in Fig. 3 using an anti-granzyme B antibody. Asterisks indicate those comparisons (PBS vs. LacE7) with statistical significance ($p < 0.05$) ($n = 5$). (B) Mucosal lymphocytes effector cells were mixed with TC-1 target cells at various E:T ratios indicated on the *x*-axis. The cell lytic effect (%) against TC-1 cells is depicted on the *y*-axis.

4. Discussion

HPV E7 expression in cervical neoplasia has been previously verified at transcriptional and translational levels [17,38]. RT-PCR and immunohistochemical assessments have shown that high-grade CIN lesions (CIN 2–3) strongly express the E7 gene and protein but CIN1 lesion exhibit very low expression levels, suggesting that the E7 protein represents an important target molecule for immunotherapy against high-grade CIN (CIN 2–3).

In this study, we demonstrated that mucosal type1 immune response-related T cell activity against HPV16 E7 can be induced by oral immunization with a recombinant *Lactobacillus*-based vaccine (LacE7). Mucosal lymphocytes obtained from the intestinal mucosa were used for assays of cellular immune response. About 90% of the lymphocyte possessed integrin α4β7, a mucosa-associated homing receptor whose expression is induced by dendritic cells (DCs) residing in the GALT via retinoic acid. The proportions of $CD4^+$ and $CD8^+$ cells in the isolated lymphocytes were quite distinct from those in the mucosal lamina propria or among intraepithelial lymphocytes [32]. The mucosal lymphocytes appeared to be a mixture of T cells derived from GALT, mucosal lamina propria and intraepithelial compartments. The number of B cells among isolated mucosal lymphocytes was much less than that in peripheral blood. These flow cytometry data demonstrate that peripheral blood contamination was negligible using the mucosal lymphocyte isolation protocols employed in this study. It is impossible to collect pure mucosal lymphocyte from the murine genital tract mucosa because of its small size. Therefore, previous studies have substituted lymphocytes collected from the lymph nodes draining genital tract (e.g. inguinal and iliac) for the detection of local immune responses [30,31]. Rank et al. have directly demonstrated immune responses in genital tract mucosal cells isolated from guinea pigs [33], but HPV infected target cell lines, like TC-1, are not available in guinea pig models. Although we attempted to use similar methods to isolated genital tract lymphocytes from mice, these cells were massively contaminated by peripheral blood (data not shown) and we could not assure the cells were purely mucosal. In this study, intestinal mucosal lymphocytes were used to address mucosal cellular immune responses to an HPV E7 vaccine in mice. Our data make this manuscript a proof-of-concept paper until the cervix can be assessed directly in humans.

In the mucosal immune system, MALT is the crucial inductive site for adaptive immunity. The cervical mucosa, however, does not possess MALT [34], a characteristic that may help to prevent specific immune attack against sperm deposited repeatedly in the female reproductive tract. Iwasaki's group has demonstrated that DCs in the vaginal mucosa migrate to draining lymph nodes, including inguinal, iliac and sacral lymph nodes, where they present antigen to $CD4^+$ T cells [35–37]. These draining lymph nodes are critical as inductive sites for antigen-presentation after pathogen invasion. Interestingly, these lymph nodes possess endothelial cells that express MAdCAM, a natural ligand for integrin α4β7 [38]. We have shown that integrin $\alpha4\beta7^+$ lymphocytes comprise 50–70% of the cervical lymphocytes isolated from patients with HPV-associated lesions, indicating that GALT-derived cells home to the cervical mucosa in human (Yokoyama et al., unpublished data). Cervical mucosae appear to utilize GALT as alternative inductive sites for antigen-presentation of pathogens that have invaded the genital mucosa. The induced integrin $\alpha4\beta7^+$ memory/effector cells can then traffic to the cervical mucosa and provide cellular immune responses in the cervical mucosa similar to those in the intestinal mucosa. We therefore presumed that mucosal lymphocyte isolated from intestinal mucosa can be used as surrogates for those populating the cervical mucosa and we chose to study oral rather than intranasal immunization. Bermúdez-Humarán et al. demonstrated that intranasal immunization with lactic acid bacteria expressing HPV E7 and IL12 elicits antitumor effects on E7-related murine tumors and assessed CTL responses in splenocytes [18]. The antitumor effects on mucosal neoplastic lesions by mucosal lymphocytes have never been specifically addressed. Oral immunization with lactic acid bacteria should directly stimulate GALT and integrin $\alpha4\beta7^+$ memory/effector cells and this should result in strong mucosal immune responses in the gastrointestinal tract and the cervix. In our investigation, the mucosal type1 immune responses to E7 were quite different depending on the route of immunization. Oral immunization had the predicted advantage of preferential induction of mucosal rather than systemic immunity. Intramuscular immunization, in contrast, was quite suitable for the induction of systemic but not mucosal immunity. This work is the first to compare mucosal cellular immune response to HPV E7 among several routes of vaccine administration.

To induce mucosal immunity to a vaccine antigen, the antigen must be delivered to inductive sites and presented by APC to activated memory cells [39]. The intestinal mucosa possesses many inductive sites, including Peyer's patches, making it an attractive site for the stimulation of protective mucosal immunity. *Lb. casei* has been shown to act as an efficient vaccine carrier that delivers antigen across the gut to GALT as well as a vaccine adjuvant that promotes type1 T cell immune responses [9,26]. Mohamadzadeh et al. demonstrated that *Lactobacillus* species promote type1 immune response polarization through interactions with myeloid dendritic cells (MDC) [40]. *Lactobacillus* activate MDC through TLR-2 and the activated MDC stimulate the proliferation of autologous $CD4^+$ and $CD8^+$ T cells and their secretion of IFNγ [41]. Kajikawa et al. further confirmed that recombinant *Lb. casei* can induce IFNγ production at mucosal sites [26]. Our data demonstrate that oral immunization using *Lb. casei* that lack or express E7 equally elicit non-specific IFNγ-producing type1 immune cells in the intestinal mucosa, indicating that the stimulation of mucosal type1 immune responses is *Lb. casei*- but not E7-specific. The adjuvant effect of *Lb. casei* on type1 immune responses did not change over a range of 1–100 mg/head

exposures, suggesting that *Lb. casei* represents an excellent antigen delivery vehicle when cytotoxic mucosal immune responses to vaccine antigen are desired.

The E7-specific type1 immune response induced by LacE7 was directly dose-dependent over a range of 0.03–1.0 mg/head, but decreased when exposure exceeded a LacE7 level of 1.0 mg/head. These findings could be caused by an aggregation of the attenuated bacteria when levels approach 10 mg/head, resulting in interference with antigen translocation to GALT through M cells. In these investigations we chose LacE7 doses of 1.0 mg/head as optimal for oral immunization to induce E7-specific type1 immune response-related T cell activities and used this level of exposure for CTL detection and killer activity assays. E7-specific type1 immune cell numbers increased after boosting when compared to non-boost protocols, suggesting that mucosal lymphocyte populations include memory T cells that recognize E7.

The lack of an animal model with HPV E7-related mucosal neoplastic lesions hampers assessment of our therapeutic vaccination strategy for preclinical efficacy against CIN. Most previous studies on HPV therapeutic vaccines utilized murine models in which HPV16 E7-transformed TC-1 cells were injected subcutaneously to induce tumor formation [42,43]. This model can assess systemic, but not mucosal, immune responses to HPV-related tumors. In our study, TC-1 cell was used as target cells for mucosal E7-specific CTL in *in vitro* killer activity assays. The HPV-specific killer activity of mucosal lymphocytes was clearly demonstrated by the induction of granzyme B-producing $CD8^+$ T cells. Poo et al. have revealed that oral immunization of mice with *Lb. casei* expressing HPV16 E7 reduces the growth of subcutaneous TC-1 cell tumor and induces E7-specific type1 immune response-related splenic T cells [9]. We have shown that oral immunization with LacE7 preferentially elicits E7-specific type1 T cell responses in mucosal lymphocytes (2-fold higher) when compared to splenocytes. These data strongly suggest that the induced mucosal $CD4^+$ and $CD8^+$ T cells will have antitumor effects on mucosal HPV E7-related neoplastic lesions.

Oral routes of immunization offer many advantages: easy self-administration at home, reduction in hypersensitivity reactions, and decreased costs (no needles, syringes or trained personnel). Further, the production of lactic acid bacteria is also inexpensive. In this study the recombinant *Lb. casei*, LacE7, was heat-attenuated. Attenuation results in the destruction of the expression plasmid and prevention of self-replication. This negates the possibility for transfer of foreign genes to normal bacterial in the gut. The Rb-binding site of HPV16 E7 was mutated in the antigen-producing plasmid, thereby eliminating its oncogenicity but not its immunogenicity [44]. These modifications make the recombinant *Lb. casei* vaccine ensure drug safety. Unfortunately, we must await clinical trials on this promising therapeutic HPV vaccine to assess its actual antitumor effect on mucosal neoplastic lesions. Our data support the development of an initial clinical study on therapeutic vaccination of patients with CIN 2–3 patients using LacE7.

Acknowledgements

This work was supported by a grant-in-aid from the Ministry of Health, Labour and Welfare of Japan for the Third-Term Comprehensive 10-Year Strategy for Cancer Control, by a cancer research grant from the Ministry of Education, Culture, Sports, Science and Technology of Japan, by a grant from Kanzawa Medical Research Foundation and by a grant from the Okinawa New Industry Creation Project.

We are grateful to Dr. Sung in Korea for his kind gift of the expression plasmids, pKV-HPV16 E7 (Rb) and to Dr. T.C. Wu for his kind gift of the TC-1 cell line.

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JOURNAL OF VIROLOGY, Nov. 2010, p. 11614–11623 Vol. 84, No. 22
0022-538X/10/$12.00 doi:10.1128/JVI.01053-10

CD1d, a Sentinel Molecule Bridging Innate and Adaptive Immunity, Is Downregulated by the Human Papillomavirus (HPV) E5 Protein: a Possible Mechanism for Immune Evasion by HPV▽

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Received 16 May 2010/Accepted 20 August 2010

CD1d and CD1d-restricted natural killer T (NKT) cells serve as a natural bridge between innate and adaptive immune responses to microbes. CD1d downregulation is utilized by a variety of microbes to evade immune detection. We demonstrate here that CD1d is downregulated in human papillomavirus (HPV)-positive cells *in vivo* and *in vitro*. CD1d immunoreactivity was strong in HPV-negative normal cervical epithelium but absent in HPV16-positive CIN1 and HPV6-positive condyloma lesions. We used two cell lines for *in vitro* assay; one was stably CD1d-transfected cells established from an HPV-negative cervical cancer cell line, C33A (C33A/CD1d), and the other was normal human vaginal keratinocyte bearing endogenous CD1d (Vag). Flow cytometry revealed that cell surface CD1d was downregulated in both C33A/CD1d and Vag cells stably transfected with HPV6 E5 and HPV16 E5. Although the steady-state levels of CD1d protein decreased in both E5-expressing cell lines compared to empty retrovirus-infected cells, CD1d mRNA levels were not affected. Confocal microscopy demonstrated that residual CD1d was not trafficked to the E5-expressing cell surface but colocalized with E5 near the endoplasmic reticulum (ER). In the ER, E5 interacted with calnexin, an ER chaperone known to mediate folding of CD1d. CD1d protein levels were rescued by the proteasome inhibitor, MG132, indicating a role for proteasome-mediated degradation in HPV-associated CD1d downregulation. Taken together, our data suggest that E5 targets CD1d to the cytosolic proteolytic pathway by inhibiting calnexin-related CD1d trafficking. Finally, CD1d-mediated production of interleukin-12 from the C33A/CD1d cells was abrogated in both E5-expressing cell lines. Decreased CD1d expression in the presence of HPV E5 may help HPV-infected cells evade protective immunological surveillance.

There are approximately 100 identified genotypes (types) of human papillomavirus (HPV). Over 40 of these are classified as genital HPV subtypes that invade the reproductive organs, including the uterine cervix, vaginal wall, vulva, and penis. Genital HPV types are further subclassified into high-risk types that are commonly associated with cervical cancer and low-risk types that cause noninvasive condyloma acuminata. Although exact classification varies among researchers, subtypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 66, and 68 are typically classified as high risk and subtypes 6, 11, 40, 42, 43, 44, 54, 61, and 72 as low risk (44). Genital HPV infection involves short-term virus proliferation, followed by the long-term latent presence of a small number of copies of the viral genome within the basal cells of the genital epithelium (44). Infections with high-risk HPV subtypes result in progression to genital tract cancers (most commonly cervical) in only a small percentage of infected women and typically after a long latency period. A high percentage of high-risk HPV DNA-positive infected women resolve their infections during the proliferative phase and thereby clear the virus or progress to latency with undetectable HPV DNA levels. The clearance of viral DNA is often accomplished through activation of the host immune system against viral antigen (19), and chronic immune suppression represents a risk factor for viral DNA persistence and benign and/or neoplastic lesion progression (23).

Completion of the HPV life cycle requires infection of epidermal or mucosal basal cells that have the potential to proliferate and differentiate. Within these cells, overall viral gene expression is suppressed, although limited expression of specific early viral genes, including E5, E6, and E7, causes lateral expansion of infected cells within the basal layer of the epithelium (44). HPV E5 seems to be particularly important early in the course of infection. Large amounts of E5 mRNA have been found in cervical intraepithelial neoplasia (CIN) lesions (37). However, as HPV-infected lesions progress to cervical cancer, episomal viral DNA becomes integrated into host cell DNA, and a substantial part of the HPV genome, commonly including the E5 coding sequence, is deleted (16). Therefore, E5 is not obligatory in the late events of HPV-mediated carcinogenesis.

E5 is a small hydrophobic protein that can be localized within the Golgi apparatus (GA), endoplasmic reticulum

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▽ Published ahead of print on 1 September 2010.

(ER), and occasionally at the plasma membrane of the host cell. It has been proposed that binding of HPV16 E5 to a subunit of the cellular proton ATPase (15) is responsible for the lack of acidification of the GA and endolysosomes and the consequent impaired function of these organelles that is seen with HPV16 infection (32, 38). The presence of HPV16 E5 has also been linked to downregulation of antigen presentation by HLA class I molecules, a process that may aid in HPVs ability to evade immune clearance through cytotoxic-T-lymphocyte (CTL)-mediated adaptive immunity (1, 2, 3, 21, 30). Ashrafi and coworkers have demonstrated that HPV16 E5 retains HLA-A and -B molecules in the GA and interferes with their trafficking to the cell surface but does not alter the transcription of HLA class I heavy chains or the transporter associated with antigen processing (TAP) (2, 3, 4, 28). Others have shown that HPV16 E5 interacts with calnexin in the ER and thereby interferes with the modification of HLA class I heavy chains (21).

CD1d is an major histocompatibility complex (MHC) class I-like glycoprotein that presents self or microbial lipid antigen to natural killer T (NKT) cells (39). In humans, a specific subset of NKT cells expresses an invariant Vα24-JaQ/Vβ11 TCR (iTCR) and can recognize CD1d on the surface of antigen-presenting cells (APCs) through this receptor. CD1d is expressed not only in typical APCs (macrophages, dendritic cells, and B cells) but also in intestinal epithelial cells (8, 12), foreskin keratinocytes (9), and reproductive tract epithelial cells (25, 26). Like MHC class I, CD1d is synthesized, glycosylated by *N*-glycosyltransferase, modified, and assembled with β2m within the ER and then transferred to the GA (5, 24, 27). CD1d plays a role in both innate and adaptive immunity to various bacteria, viruses, fungi, and parasites (reviewed in reference 10). Activation of CD1d-restricted invariant NKT (iNKT) cells enhances host resistance to some microbes in a manner that depends on the level of CD1d expression on APCs (34, 35). In contrast, the activation of iNKT cells promotes susceptibility to some microbes (7, 33). The activation of CD1d-restricted iNKT cells in response to microbial invasion is antigen dependent, but these antigens can be derived from the invading microbe or possibly from host lipids (11, 22, 29). Intracellular signaling mediated by surface CD1d utilizes NF-κB, a well-known immune-related transcription factor (36, 43). CD1d-restricted NKT cells can modulate adaptive immune cells by altering Th1/Th2 polarization. Recognition of CD1d by iNKT cells can also result in rapid release of both interleukin-4 (IL-4) and gamma interferon (IFN-γ) from the NKT cell (6). Therefore, CD1d and CD1d-restricted NKT cells serve as a natural bridge between innate and adaptive immune responses to microbes. Not surprisingly, several microbes, including herpes simplex virus type 1, human immunodeficiency virus, Kaposi's sarcoma herpesvirus, and *Chlamydia trachomatis*, are known to downregulate cell surface expression of CD1d as an immune evasion strategy (13, 26, 31, 42). Our own lab previously demonstrated that *C. trachomatis* retains CD1d in the ER and targets CD1d to both chlamydial and cellular degradation pathways (26).

Viewing the importance of CD1d in innate immune responses to microbes, we hypothesized that HPV may alter CD1d-mediated immune pathways and thereby avoid innate immune destruction of the infected cell by the host. We demonstrate here that the presence of the E5 protein of HPV6 and HPV16 is associated with reduced CD1d cell surface expression. We describe a mechanism for this downregulation and hypothesize that decreased surface CD1d expression may help HPV-infected cells evade immune surveillance during the early stages of infection.

MATERIALS AND METHODS

HPV6 and HPV16 E5 expression constructs. HPV6 and HPV16 E5 open reading frames were amplified from the HPV6 and HPV16 complete genomes (kindly provided by Tadahito Kanda, National Institute of Infectious Diseases, Japan) by PCR using primers designed to introduce BamHI (forward) and EcoRI (reverse) restriction sites. The PCR products were digested with BamHI and EcoRI and subcloned into a retroviral expression plasmid pLPCX (Clontech, Mountain View, CA).

Cell lines and establishment of a cell line stably expressing CD1d. An HPV-negative human cervical carcinoma cell line, C33A, and a vaginal epithelial cell that was originally established from normal human primary epithelial cells that were immortalized by transduction with HPV16 E6/E7 genes (VK2/E6E7) (a generous gift from D. J. Anderson, Boston University, Boston, MA) (18) were grown in Dulbecco modified eagle medium (Invitrogen, Carlsbad, CA) without $CaCl_2$ (Invitrogen), supplemented with 10% fetal bovine serum (Invitrogen) at 37°C in 5% CO_2. The vaginal epithelial (VK2/E6E7) cells used here are known to express endogenous CD1d at the cell surface (25).

A CD1d-expressing retroviral plasmid pSRα-neo (kindly provided by R. Blumberg, Harvard Medical School, Boston, MA) was transfected into Phoenix cells, a packaging cell line for recombinant retroviruses (kindly provided by K. Oda, University of Tokyo), using Lipofectamine 2000 (Invitrogen). After 72 h of incubation in DMEM, the culture medium containing released CD1d-expressing retroviruses was collected and used to infect C33A cells and transfer the CD1d gene. CD1d-expressing C33A cells were selected in medium containing 1.0 mg of neomycin/ml to establish a stably transfected cell line (C33A/CD1d).

Establishment of HPV E5-expressing cell lines. HPV6 or HPV16 E5-expressing retroviral plasmids or their empty counterparts (pLPCX-16E5, pLPCX-6E5, or pLPCX, respectively) were transfected into Phoenix cells using Lipofectamine 2000 (Invitrogen). After 72 h of incubation, culture medium with released viruses were collected and used to infect C33A/CD1d or vaginal epithelial cells. Stable cell lines were selected in media containing 1.5 μg of piromycin/ml.

Immunohistochemistry. Immunostaining for CD1d was performed on formalin-fixed, paraffin-embedded tissue sections of normal or inflamed cervix, CIN1 to CIN3, cervical cancer, and condyloma acuminata (obtained under IRB approval through the University of Tokyo). A total of 45 tissues were examined. Optimal immunostaining required antigen retrieval via microwave exposure in 0.01 M citrate buffer. A mouse anti-CD1d MAb (NOR3.2, 1:100; Abcam, Inc., Cambridge, MA) or an irrelevant, isotype-matched mouse monoclonal antibody (DakoCytomation, Glostrup, Denmark) were used as primary reagents. Immunostaining was amplified and detected by using the EnVision+System-HRP (DakoCytomation). Nuclei were counterstained by using standard hematoxylin protocols (Sigma-Aldrich, Inc., St. Louis, MO). Analyses were performed at a magnification of ×200.

Flow cytometry. C33A/CD1d cells were grown in 175-cm^2 flasks until confluent, harvested using trypsin-EDTA, and pelleted at 500 × *g* for 5 min at room temperature. The cells were then washed and resuspended in PBS-B (phosphate-buffered saline [PBS] with 1% bovine serum albumin; Invitrogen) at a concentration of 10^6 cells/ml. For detection of cell surface CD1d, 100 μl of cell suspension was incubated with an anti-CD1d NOR3.2 monoclonal antibody (MAb; Abcam) at 1:100 for 30 min at 4°C. Cells were then washed three times in PBS-B, incubated with a goat anti-mouse immunoglobulin secondary antibody conjugated to phycoerythrin (PE; BD Bioscience, San Jose, CA) for 30 min at 4°C, suspended in 1% paraformaldehyde, and analyzed by using a FACSCalibur flow cytometry system (BD Bioscience).

Proteasome inhibitor treatment. C33A/CD1d cells harboring an empty vector (C33A/CD1d-empty) or expressing HPV6 E5 (C33A/CD1d-6E5) or HPV16 E5 (C33A/CD1d-16E5) were cultured for up to 24 h in the presence or absence of the cytosolic proteasome inhibitor MG132 (10 μM in dimethyl sulfoxide [DMSO]; Sigma-Aldrich, Inc.). Control wells included vehicle alone.

HPV genotyping. DNA was extracted from cervical smear samples by using the DNeasy blood minikit (Qiagen, United Kingdom). HPV genotyping was performed by using the PGMY-CHUV assay method (20). Briefly, standard PCR was conducted using the PGMY09/11 L1 consensus primer set and HLA-dQ primer sets (20). Reverse blotting hybridization was performed as described

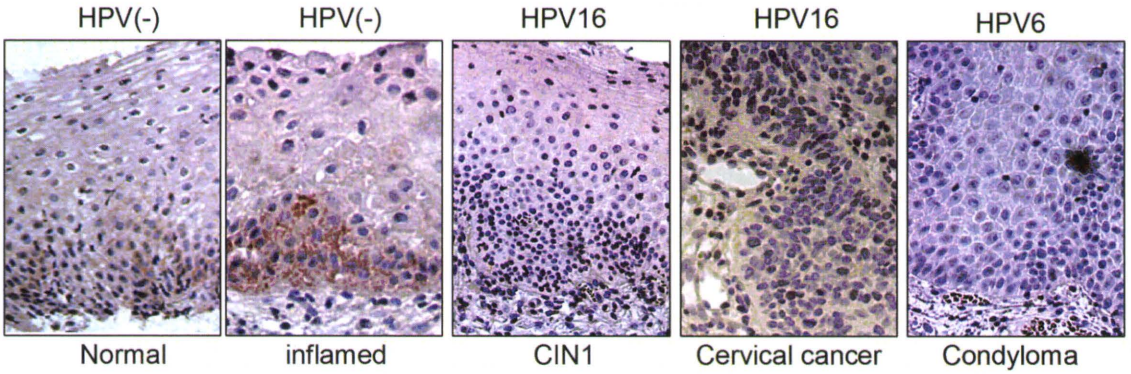

FIG. 1. Immunostaining of HPV-associated lesions for CD1d. Immunostaining for CD1d was performed after antigen retrieval on formalin-fixed, paraffin-embedded tissue sections of HPV-negative normal and inflamed ectocervical epithelium, HPV16-positive CIN1, HPV16-positive cervical cancer, and HPV6-positive condyloma acuminata. CD1d was detected with NOR3.2, a CD1d-specific MAb (1:100).

previously (20). Heat-denatured PCR amplicons were hybridized to a negatively charged nylon membrane containing specific probes for 32 HPV genotypes and HLA-dQ reference samples. Chemiluminescence detection used enhanced chemiluminescence (ECL) detection reagents for nucleic acids (GE Healthcare). Films were interpreted using the HPV reference guide provided.

RT-PCR and quantitative PCR. Portions (1 μg) of total RNA and oligo(dT)s were used for reverse transcriptase (RT) reactions (RNA PCR kit; Applied Biosystems, Foster City, CA). Total cDNA reaction samples were used as templates for amplification of each gene fragment using a PCR Core kit (Applied Biosystems). Primer pair sets for CD1d were synthesized by Invitrogen (CD1d, 453 bp; 5′-GCTGCAACCAGGACAAGTGGACGAG-3′ [forward] and 5′-AG GAACAGCAAGCACGCCAGGACT-3′ [reverse]). Those for IL-12 p40 were commercially available (Sigma-Aldrich, Inc.). For quantitative PCR, cDNA were produced via RT of 1 μg of total RNA extracted from the cells as described above by using an OmniScript RT kit (Qiagen, Inc., Valencia, CA). Portions (2 μl) of 5-fold-diluted cDNA aliquots were amplified in a thermal cycler (7300 Real-Time PCR System; Applied Biosystems) by using a QuantiTect SYBR green PCR kit (Qiagen, Inc.) and a primer pair set for β-actin (5′-GAAATCG TGCGTGACATTAAGG-3′[forward] and 5′-TCAGGCAGCTCGTAGCTTC T-3′ [reverse]). The mRNA levels for IL-12 were normalized to those of β-actin, the internal control.

Fluorescent deconvolution microscopy and confocal microscopy. C33A/CD1d cells were seeded onto coverslips. The ER was visualized using the ER tracker Blue-White DPX (Invitrogen) for 30 min at 37°C. All coverslips were fixed in 4% paraformaldehyde, permeabilized with 0.1% Tween 20. They were then incubated for 1 h at 37°C with either an anti-CD1d NOR3.2 MAb labeled with Zenon Alexa Fluor 555 using a mouse IgG labeling kit (Invitrogen) or an anti-FLAG MAb labeled with Zenon Alexa Fluor 488 using a mouse IgG labeling kit (Invitrogen) singly or in combination. With the exception of ER tracker-treated coverslips, the cells were then counterstained with a DAPI (4′,6′-diamidino-2-phenylindole) nucleic acid stain (Invitrogen). Images were obtained with a LSM 700, flexible confocal microscope (Carl Zeiss, Oberkochen, Germany). Filter sets were optimized for Alexa 488, Alexa 555, and DAPI. Z-axis plane capture, deconvolution, and analyses were performed with ZEN 2009 Software (Carl Zeiss).

Western blotting. Portions (50 μg) of total cell lysates from C33A/CD1d-empty, C33A/CD1d-6E5, or C33A/CD1d-16E5 cells in a modified TNF buffer (1 M Tris-HCl [pH 7.8], 10% NP-40, 5 M NaCl, 0.5 M EDTA [pH 8.0], aprotinin, 0.1 M phenylmethylsulfonyl fluoride) were electrophoresed and transferred to nitrocellulose membranes. Membranes were blocked with 10% milk and incubated with a peroxidase-labeled anti-CD1d NOR3.2 MAb (1:200; Abcam) or an anti-FLAG MAb (1:500; Sigma-Aldrich, Inc.) using a peroxidase labeling kit (Roche, Basel, Switzerland) for 1 h. Membranes were washed and bound antibody was detected using an ECL Western blotting analysis system (GE Healthcare Buckinghamshire, United Kingdom).

Immunoprecipitation and Western immunoblotting. Harvested C33A/CD1d-empty, C33A/CD1d-6E5, or C33A/CD1d-16E5 cells were lysed in modified radioimmunoprecipitation assay buffer (1% NP-40, 1% deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 10 mM Tris, 150 mM NaCl, 2 mM EDTA) with protease inhibitors (Amersham Biosciences, Piscataway, NJ). Equivalent aliquots of total cell lysates were incubated overnight at 4C with 5 μg of mouse anti-FLAG MAbs (Sigma-Aldrich, Inc.)/ml and 5 μl of protein A-Sepharose (GE Healthcare). Precipitated proteins were separated by SDS-PAGE using 7.5% acrylamide gels and transferred to polyvinylidene difluoride membranes. Mouse anti-calnexin or rabbit anti-β-actin polyclonal antibodies (Abcam) were used as primary reagents for immunoblotting, and anti-mouse IgG-HRP (1:100,000; GE Healthcare) was used as a secondary reagent. Products in Western immunoblotting experiments were visualized by using an ECL Western blotting analysis system (GE Healthcare). Molecular masses were confirmed by comparison to standard size markers (GE Healthcare).

Statistical analysis. Quantitative PCR data were presented as means ± the standard deviations. Experiments were performed independently at least three times. The Cochran-Armitage Trend test was computed to show trends in immune reactivity with NOR3.2 MAb in clinical samples. IL-12 mRNA levels were compared to those before or after cross-linking by using paired, two-tailed Student *t* tests. A *P* value of <0.05 was considered significant.

RESULTS

CD1d downregulation in HPV-related lesions and cancer cell lines. Since CD1d expression in human mucosa and skin has been demonstrated by immunohistochemistry using the anti-CD1d NOR3.2 MAb (2, 9, 12, 26), we examined immunostaining of human normal ectocervix or HPV-related lesions with NOR3.2 (Fig. 1). Immunostaining for CD1d was performed on formalin-fixed, paraffin-embedded tissue sections of normal or inflamed ectocervical epithelium, cervical intraepithelial neoplasia 1 (CIN1), cervical cancer, and cervical condyloma (obtained under IRB approval through the University of Tokyo, Faculty of Medicine). To examine alterations in CD1d expression in the presence of high-risk HPV and low-risk HPV subtypes, HPV16-positive CIN1 or cancer lesions and HPV6-positive condyloma acuminata specimens were compared to each other and to HPV-negative normal and inflamed ectocervical epithelial controls. Immunoreactivity with the NOR3.2 MAb was noted in the basal and parabasal epithelial cells of normal and inflamed ectocervical epithelia that are known to express early HPV genes (E5, E6, and E7; Fig. 1) (44). In inflamed epithelium, the immunoreactivity appeared to be intensified compared to normal epithelium. CD1d expression is known to be enhanced by inflammatory cytokines (10). NOR3.2 immunoreactivity is essentially absent in HPV16-positive CIN1, HPV16-positive cancer, and HPV6-positive condyloma lesions (Fig. 1). To statistically analyze alterations in CD1d expression, a total of 45 clinical specimens from normal controls and HPV-related lesions were immunostained with NOR3.2 (Table 1). NOR3.2 immunoreactivity was

TABLE 1. Immunoreactivity with NOR3.2 anti-CD1d MAb in cervical epithelium of various lesions

Histological status	HPV status[a]	CD1d (no. of cases)		% Positive[b]
		Positive	Negative	
Normal/inflamed		9	1	90.0
CIN1 and CIN2	HR-HPV(+)	0	7	0
CIN3	HR-HPV(+)	2	16	11.1
Cancer	HR-HPV(+)	0	7	0
Condyloma	HPV6(+)	0	3	0

[a] HR-HPV(+), any high-risk HPV positive.
[b] $P = 0.0001$ (exact Cochran-Armitage Trend test).

mostly limited to the HPV-negative normal or inflamed ectocervical epithelial samples similar to those represented in the first two panels of Fig. 1. NOR3.2 immunoreactivity was absent in all CIN1 and CIN2, cervical cancer, and condyloma lesions. Among CIN3 samples, two lesions showed NOR3.2 immunoreactivity, whereas 16 lesions did not. Using trend analysis, we were able to demonstrate an association between decreased CD1d immunoreactivity and progression of cervical neoplastic lesions with statistical significance ($P = 0.0001$).

Although HPV E5 is not expressed in cervical cancer cells (16), immunohistochemical data demonstrated that CD1d expression was also abrogated in cervical cancer lesions. To address the mechanisms underlying CD1d downregulation in cervical cancers, we examined the level of CD1d transcription and CD1d expression at the cell surface in several cervical cancer cell lines (Fig. 2). As a positive control, we created cell transfectants that stably expressed CD1d. To avoid the potential influence of endogenous HPV protein expression, an HPV-negative cervical cancer cell line, C33A, was used for our CD1d transfectants. We used a retrovirus vector to transduce the CD1d gene into these cells and established the stable cell line, C33A/CD1d via neomycin selection. Flow cytometry revealed strong expression of CD1d on the cell surface of C33A/CD1d cells. Cd1d was not expressed on the cell surface of C33A control cells or in other cancer cell lines (Fig. 2A). To examine the level of CD1d transcription in these same cells, cDNA was produced via RT of total RNA from each cell line and subjected to PCR using primer pairs specific for CD1d. The expected single band representing CD1d was observed on agarose gels only in C33A/CD1d cells (Fig. 2B). These data indicated that CD1d expression was abrogated prior to or during transcription the tested cervical cancer cell lines.

Cell surface expression of CD1d decreases in HPV E5-expressing epithelial cells. HPV E5 has been reported to inhibit cell surface expression of HLA class I molecules by interfering with their trafficking to the cell surface (1, 2, 3, 21, 30). Since CD1d and HLA class I heavy chains utilize an identical intracellular pathway to traffic from the ER to the cell surface, we hypothesized that HPV E5 may also interfere with surface CD1d expression at a posttranscriptional level. To verify our immunohistochemical data and study CD1d trafficking in the presence of E5 *in vitro*, we created HPV6 and HPV16 E5 stably transfected cell lines using C33A/CD1d cells. Since the E5 protein is less than 10 kDa in size, the production of an anti-E5 antibody would be difficult. Instead, E5 proteins were tagged with FLAG and detected by Western blotting or immunostaining with an anti-FLAG antibody. FLAG-tagged HPV6 or HPV16 E5 genes were transduced into the C33A/CD1d cells by using retrovirus vectors. To control for the influence of retrovirus infection and the presence of the expression vector, C33A/CD1d cells were infected with empty retrovirus vectors. Retrovirus-infected cells were exposed to puromycin, and E5-expressing C33A/CD1d cells were established (C33A/CD1d-6E5, -16E5, and -empty). In Fig. 3A, lanes 5 and 6, show PCR products derived from cDNA generated by RT of total RNA from C33A/CD1d-6E5 and -16E5 cells. Lanes 2 and 3 in the same figure show PCR products derived from corresponding expression plasmid DNA. FLAG-6E5 and -16E5 were transcribed in C33A/CD1d-6E5 and -16E5 cells, respectively. Using Western immunoblotting and an anti-FLAG MAb, FLAG-6E5 and -16E5 proteins were detected as immunoreactive bands at an approximate size of 10 kDa in lanes 1 and 2, respectively (Fig. 3B).

We next examined the expression of CD1d at both mRNA and protein levels in the presence or absence of HPV E5. CD1d transcription levels in C33A/CD1d cells were unaffected by the presence of E5 or of empty vector compared to naive

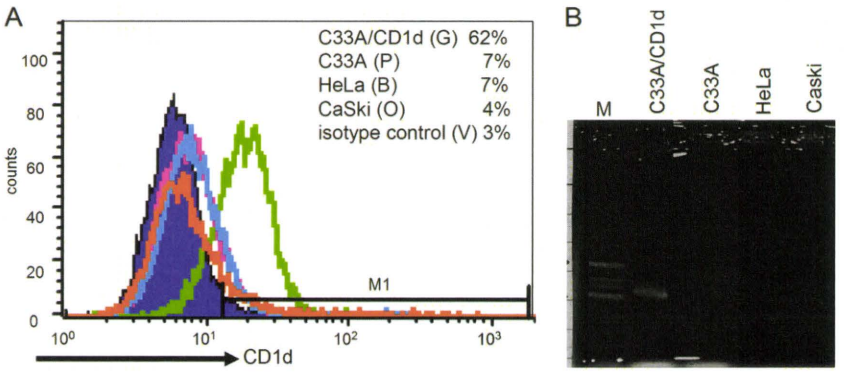

FIG. 2. CD1d alterations in cancer cell lines. (A) Cell surface expression of CD1d in C33A (pink line), HeLa (blue line), CaSki (orange line), and C33A/CD1d (green line) cells. All cells were stained with an anti-CD1d primary MAb (NOR3.2; 1:100 dilution) and a PE-conjugated goat anti-mouse immunoglobulin secondary antibody (1:20 dilution). Background staining of the cells using an isotype-matched control antibody is also shown (filled region). Cells were suspended in 1% paraformaldehyde and analyzed by using a FACSCalibur flow cytometry system. (B) Transcription of CD1d. cDNA was produced via RT of 1 μg of total RNA from each cell line and amplified by PCR with primer pairs specific for CD1d. PCR products were separated over an agarose gel containing ethidium bromide.

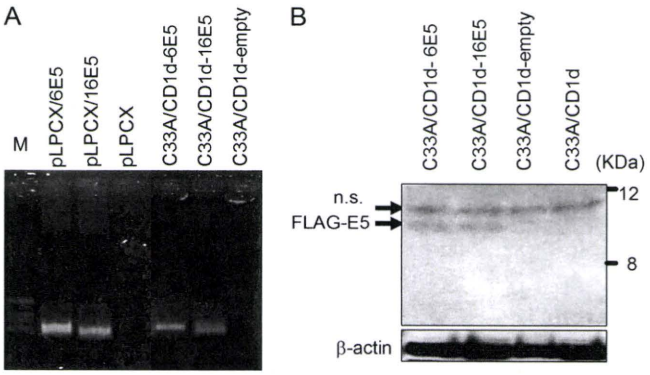

FIG. 3. HPV E5 detection in HPV E5-transformed C33A/CD1d cells. (A) Transcription of HPV E5. cDNA was produced via RT of 1 μg of total RNA from each cell line and amplified by PCR with primer pairs specific for HPV16 E5 and HPV6 E5. PCR products were separated over an agarose gel containing ethidium bromide. Lanes 5 and 6 display PCR products derived from C33A/CD1d-6E5 and -16E5 cDNA, respectively, while lanes 2 and 3 show PCR products derived from corresponding expression plasmid DNA. Lanes 4 and 7 represent negative control plasmid and cell lines lacking E5, respectively. (B) Translation of HPV E5. Fifty-microgram aliquots of protein lysates from each cell line were analyzed by Western immunoblotting with antibodies against the FLAG tag (1:500 dilution) and β-actin (loading control).

C33A/CD1d cells (Fig. 4A). In contrast, the 48-kDa, mature glycosylated form of the CD1d heavy chain (HC) that was detected in naive C33A/CD1d and C33A/CD1d-empty cells was completely abrogated in C33A/CD1d-6E5 and barely detectable in the C33A/CD1d-16E5 cells (Fig. 4B, lanes 1, 4, 2, and 3, respectively). The presence of HPV6 and HPV16 E5 drastically inhibited the maturation of CD1d HCs. Flow cytometry was used to analyze the effect of HPV E5 on cell surface expression of CD1d in the C33A/CD1d cells harboring E5-expressing or empty vector (Fig. 5). CD1d was expressed by

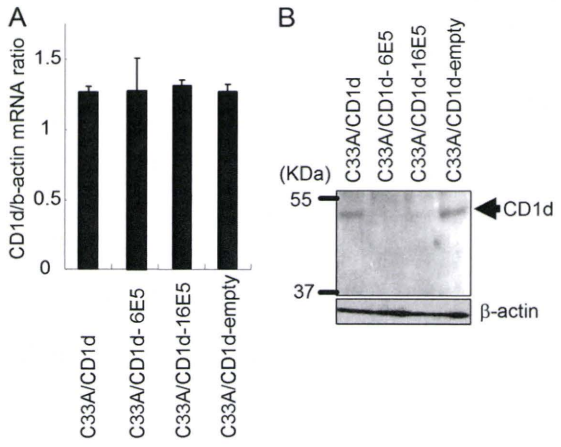

FIG. 4. CD1d heavy-chain transcription and translation in C33A/CD1d, C33A/CD1d-empty, C33A/CD1d-6E5, and C33A/CD1d-16E5 cells. (A) Transcription of CD1d HC. The mRNA levels of CD1d were analyzed by quantitative RT-PCR using SYBR green methodology. CD1d mRNA levels were normalized to β-actin. (B) Fifty-microgram aliquots of protein lysates from each cell line were analyzed by Western immunoblotting with a peroxidase-labeled anti-CD1d NOR3.2 MAb (1:200 dilution) and a β-actin loading control.

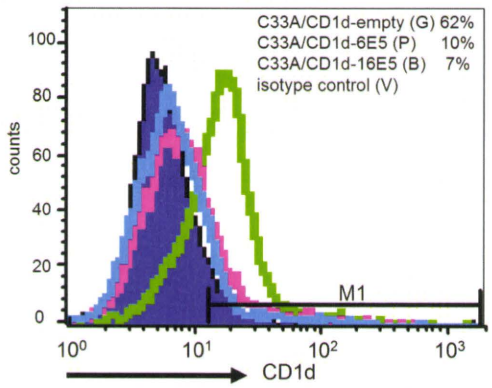

FIG. 5. Cell surface expression of CD1d in C33A/CD1d-empty (green line), C33A/CD1d-6E5 (pink line), and -16E5 (blue line) cells. All cells were stained with an anti-CD1d primary MAb (NOR3.2; 1:100 dilution) and a PE-conjugated goat anti-mouse immunoglobulin secondary antibody (1:20 dilution). Background staining of the cells with an isotype-matched control antibody is also shown (filled region). Cells were suspended in 1% paraformaldehyde and analyzed using a FACSCalibur flow cytometry system.

the majority of C33A/CD1d-empty cells but absent in >70% of C33A/CD1d-6E5 or -16E5 cells (Fig. 5).

To confirm the effect of E5 on endogenous CD1d, we used a vaginal epithelial cell line immortalized via HPV16 E6/E7 transduction of primary cells collected from normal human vaginal epithelium and subsequently well characterized as possessing histological and immunological characteristics identical to those of primary epithelial cells (18). We have previously reported the endogenous expression of functional CD1d molecules on the surface of these cells (25). Since vaginal epithelial cells are well-known targets of genital HPV, these cells were considered to be useful as an *in vitro* model for *in vivo* HPV infections. FLAG-tagged HPV6 or HPV16 E5 genes were transduced into these vaginal cells by using retrovirus vectors (Vag-6E5 and -16E5). We then examined the expression of CD1d at various levels in the presence or absence of HPV E5 (Fig. 6). RT-PCR and Western blotting revealed that CD1d transcription was unaffected by the presence of E5, but the 48-kDa CD1d HC product clearly decreased in Vag-6E5 and -16E5 cells compared to naive and Vag-empty cells (Fig. 6A). Flow cytometry confirmed the decreased cell surface expression of CD1d in 6E5-expressing vaginal epithelial cells (Fig. 6B).

E5-expressing epithelial cells retain CD1d in the ER. To demonstrate the intracellular localization of CD1d heavy chains in C33A/CD1d cells harboring HPV-6E5 and -16E5, immunofluorescence confocal microscopy was performed with an anti-CD1d MAb (NOR3.2) combined with either an anti-FLAG MAb that detects FLAG-E5 proteins, an ER-specific marker (ER tracker) or DAPI (Fig. 7). In C33A/CD1d-empty control cells, dual labeling for CD1d and the nucleus (DAPI) verified that CD1d could be detected in a diffuse pattern throughout the intracellular space, with increased accumulation near the cell surface but not in the perinuclear area (Fig. 7, upper image). In contrast, decreased amounts of CD1d could be detected in C33A/CD1d-6E5 and -16E5 cells and CD1d proteins were localized to perinuclear areas near the ER. CD1d and ER signals merged in perinuclear areas (pink

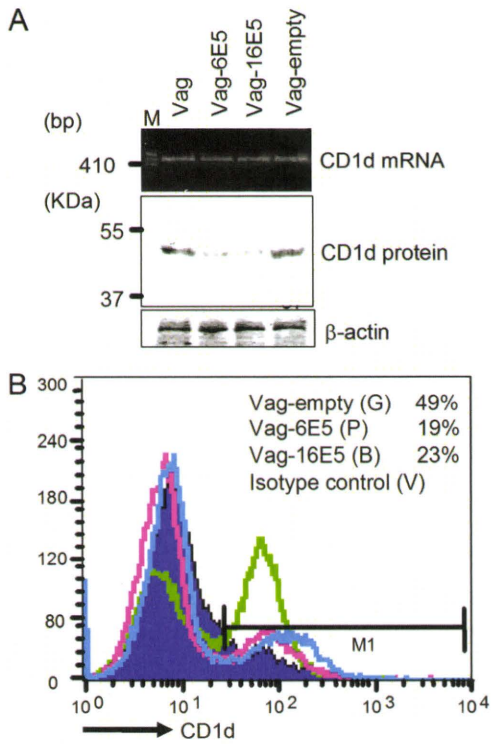

FIG. 6. CD1d downregulation in alternate genital keratinocytes in the presence of 6E5 and 16E5. (A) HPV6 and HPV16 E5 genes were transduced into vaginal epithelial cells established from normal human vaginal epithelium (17) and named Vag-6E5 and Vag-16E5, respectively. PCR products derived from cDNA generated by reverse transcription using 1 μg of total RNA from each of the vaginal cell lines were separated over an ethidium bromide-containing agarose gel. Fifty-microgram aliquots of protein lysates from each vaginal cell line were analyzed by Western immunoblotting with a peroxidase labeled anti-CD1d NOR3.2 MAb (1:200 dilution) and a β-actin loading control. (B) Vag-empty (green line), Vag-6E5 (pink line), and Vag-16E5 (blue line) were stained with an anti-CD1d primary MAb (NOR3.2; 1:100 dilution) and a PE-conjugated goat anti-mouse immunoglobulin secondary antibody (1:20 dilution). Background staining of the cells using an isotype-matched control antibody is also shown (filled region). Cells were suspended in 1% paraformaldehyde and analyzed by using a FACSCalibur flow cytometry system.

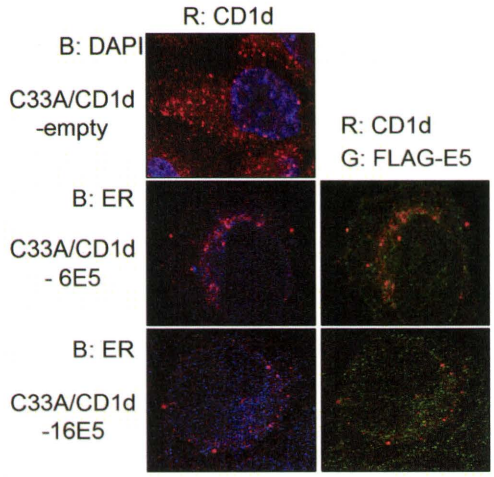

FIG. 7. CD1d trafficking in the presence or absence of E5. C33A/CD1d-empty, C33A/CD1d-6E5, or C33A/CD1d-16E5 cells were seeded onto coverslips. All of the cells were immunostained with an anti-CD1d MAb (NOR3.2, red). C33A/CD1d-empty were also exposed to DAPI (blue), and C33A/CD1d-6E5 or -16E5 cells were exposed to ER tracker (blue) and an anti-FLAG MAb (green). Cells were then visualized by using fluorescence confocal microscopy. Orange to yellow signals represent colocalization of CD1d and E5 within the ER.

signals), suggesting that the majority of CD1d is within the ER (Fig. 7, images on the left). Dual labeling for CD1d and FLAG-E5 verified the colocalization of CD1d and E5 within the ER (orange to yellow signals), while nonmerged FLAG-E5 signals were present in the perinuclear area (pure green), suggesting the presence of E5 in the GA in the absence of CD1d (Fig. 7, images on the right). The results of immunofluorescence microscopy support our biochemical and flow cytometry data showing that mature CD1d protein levels decrease and CD1d fails to traffic to the cell surface in HPV E5-expressing cells.

HPV E5 interacts with calnexin in the ER. Previous biochemical studies have reported that HPV16 E5 interacts with calnexin and that these interactions interfere with the modification of HLA class I heavy chains that typically occurs in the ER (21). The role of calnexin and/or calreticulin in the formation of the second disulfide bond of CD1d HCs in the ER is well described (24). We therefore hypothesized that E5 interacts with calnexin in the ER and may impair calnexin-mediated CD1d folding. This, in turn, could interrupt appropriate trafficking of CD1d to the surface of HPV-infected cells. To address the hypothesis, we examined the interaction of E5 with calnexin using immunoprecipitation. Total cell lysates obtained from C33A/CD1d-empty, -6E5, and -16 E5 cells were incubated with anti-FLAG MAb conjugated beads. FLAG-E5-bound proteins were immunoprecipitated and analyzed by immunoblotting with an anti-calnexin MAb. A band with an apparent molecular mass of 90 kDa and corresponding to calnexin was detected in C33A/CD1d-6E5 and -16 E5 cells, but not C33A/CD1d-empty cells, biochemically demonstrating interaction between E5 with calnexin (Fig. 8A).

To visually demonstrate the colocalization of CD1d and calnexin, C33A/CD1d-empty, -6E5, and -16E5 cells were dually stained with anti-CD1d NOR3.2 and anti-calnexin MAbs and examined by using confocal microscopy. Again, NOR3.2-reactive CD1d was detected throughout the intracellular space in C33A/CD1d-empty cells. In contrast, the majority of CD1d molecules in C33A/CD1d-6E5 or -16E5 cells localized to the perinuclear area (Fig. 8B, images on left). Calnexin detection was rendered as green signals. These mostly localized to perinuclear areas in E5-expressing cells and correspond to the location of ER (Fig. 8B, center images). Although some merge images (yellow signals) could be detected in each cell line, the merge patterns differed between C33A/CD1d-empty and E5-expressing cells (Fig. 8B, images on the right). In C33A/CD1d-empty cells, the calnexin and CD1d signals were mostly distinct and but those that did colocalize appeared to follow the synthetic pathway for type I proteins. In contrast, CD1d in the E5-expressing cells completely colocalized with calnexin, confirming our biochemical data demonstrating physiologic interaction between calnexin and CD1d in the C33A/CD1d-6E5 and -16E5 cells.

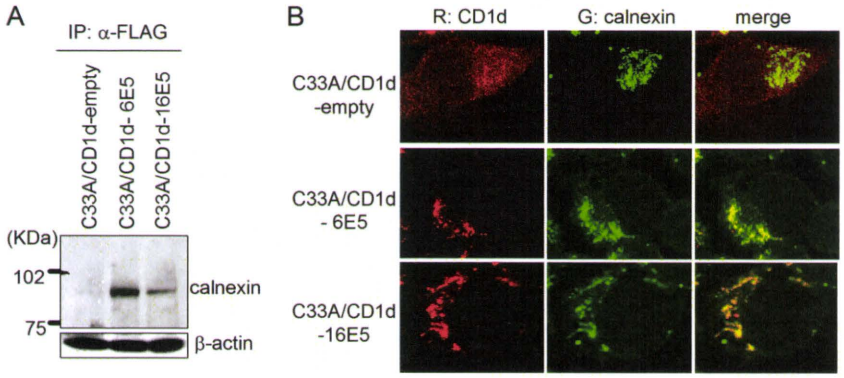

FIG. 8. CD1d and calnexin have direct interactions and colocalize in the perinuclear area in the presence of HPV E5. (A) Protein lysates from C33A/CD1d-empty, C33A/CD1d-6E5, and C33A/CD1d-16E5 cells were immunoprecipitated with an anti-FLAG MAb. Immunoprecipitants were then separated by SDS-PAGE and immunoblotted with an anti-calnexin antibody. (B) C33A/CD1d-empty, C33A/CD1d-6E5, or C33A/CD1d-16E5 cells were seeded onto coverslips. All cells were exposed to an anti-CD1d MAb (NOR3.2, red) and to an anti-calnexin MAb (green) labeled with Zenon Alexa Fluor 488 using a mouse IgG labeling kit. Cells were then visualized by using fluorescence confocal microscopy. Yellow images represent colocalization of CD1d and calnexin.

CD1d was rescued by treatment of proteasome inhibitor. We have previously demonstrated that surface expression of CD1d in human genital epithelial cells is downregulated by *C. trachomatis* infection and that downregulation involves chlamydial protein-mediated proteasomal pathways (26). We hypothesized that HPV infection could utilize posttranslational cellular proteasomal degradation to inhibit cell surface expression of CD1d HC. To address the role of the cellular proteasome in E5-associated CD1d degradation, C33A/CD1d-empty, -6E5, or -16E5 cells were exposed to the proteasome inhibitor, MG132, and CD1d HC levels in cell lysates compared to those in unexposed cells (Fig. 9). Using the NOR3.2 MAb for immunoblotting, the reduced or abrogated expression of the 48-kDa mature CD1d HC in E5-expressing cells could be rescued by the presence of MG132 (Fig. 9A). To visually replicate this effect, immunofluorescence microscopy was performed with the NOR3.2 MAb and DAPI in MG132 exposed and unexposed E5-expressing and control cells (Fig. 9B). In C33A/CD1d-empty cells, NOR3.2-reactive CD1d was detected throughout the intracellular space (Fig. 9B, upper left image). In contrast, NOR3.2-reactive CD1d was barely detected or undetectable in the majority of unexposed C33A/CD1d-6E5 or -16E5 cells (Fig. 9B, upper, right two images). In the presence of proteasomal inhibition with MG132, E5-expressing cells again show CD1d signals throughout the intracellular space (Fig. 9B, lower panels). HPV E5-expressing cells completely recover their expression mature CD1d molecules upon inhibition of cellular proteasomal degradation.

HPV E5 abrogates CD1d-mediated cytokine production in the epithelial cells. Surface CD1d interacts specifically with iNKT cells bearing an iTCR. The interaction not only activates NKT cells but also induces phosphorylation of CD1d, intracellular signaling, and the release of cytokines from the CD1d-bearing cell. We have previously demonstrated that human reproductive tract epithelial cells expressing CD1d on their cell surfaces have the capacity to produce cytokines, especially IL-12, after CD1d ligation (25). IL-12 is a central mediator in both innate and adaptive immunity and is crucial in the prevention of many infectious diseases and tumors (40). IL-12 induces IFN-γ-producing NK, NKT, T helper, and cytotoxic T cells. Since our investigations had demonstrated a decrease in cell surface expression of CD1d in the presence of HPV and specifically of HPV E5, we next examined whether CD1d-mediated IL-12 production was abrogated in E5-expressing epithelial cells (Fig. 10). An anti-CD1d 51.1 MAb can be used for CD1d cross-linking and represents an *in vitro* model for

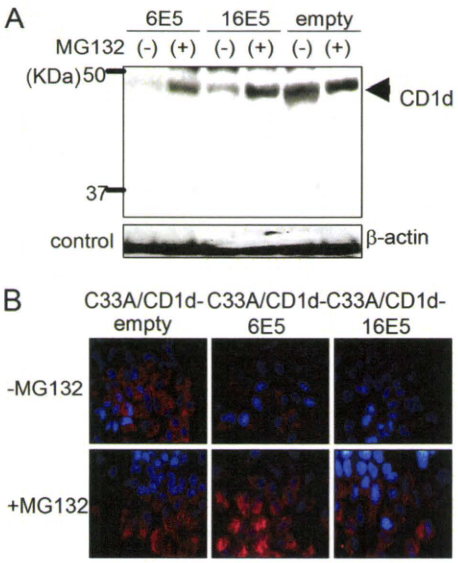

FIG. 9. Proteasome inhibition rescues CD1d from E5-mediated degradation. (A) C33A/CD1d-empty, C33A/CD1d-6E5, or C33A/CD1d-16E5 cells were cultured for up to 24 h in the presence or absence of the cytosolic proteasome inhibitors MG132 (10 μM) in DMSO. Fifty micrograms of protein lysates from each cell line were analyzed by Western immunoblotting with a peroxidase-labeled anti-CD1d MAb (NOR3.2; 1:200 dilution) and a β-actin loading control. (B) C33A/CD1d-empty, C33A/CD1d-6E5, or C33A/CD1d-16E5 cells were seeded onto coverslips and cultured for up to 24 h in the presence (lower) or absence (upper) of MG132 (10 μM) in DMSO. All coverslips were fixed in 4% paraformaldehyde, permeabilized with 0.2% Triton-X, blocked with 6% BSA, and incubated for 1 h at room temperature with an anti-CD1d NOR3.2 MAb (red) directly conjugated with Zenon Alexa Fluor 555 using a mouse IgG1 labeling kit. Cells were then counterstained with a DAPI (blue) nucleic acid stain.