Decoy receptor 3, upregulated by Epstein-Barr virus latent membrane protein 1, enhances nasopharyngeal carcinoma cell migration and invasion

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Decoy receptor 3 (DcR3), a member of tumor necrosis factor receptor superfamily, has been implicated in tumorigenesis through its abilities to modulate immune responses and induce angiogenesis. Epstein-Barr virus (EBV), a ubiquitous γ-herpesvirus, is associated with malignancies including nasopharyngeal carcinoma (NPC). Previous studies show that DcR3 is overexpressed in EBV-positive lymphomas and Rta, an EBV transcription activator, can upregulate DcR3 in Burkitt lymphoma cell lines. However, DcR3 expression has not been demonstrated in EBV-associated NPC nor have there been any EBV latent genes linked to DcR3 upregulation. Here, we showed DcR3 was overexpressed in NPC. Higher DcR3 expression score and DcR3-positive rate were found in metastatic NPC than in primary NPC tissues, suggesting DcR3 may enhance cell metastatic potential. This hypothesis is supported by our observation that NPC HONE-1 cells overexpressing DcR3 exhibited significant higher migration and invasion abilities *in vitro*. We found besides Rta, EBV latent membrane protein (LMP) 1 can upregulate DcR3 via nuclear factor-kappaB and phosphatidylinositol 3-kinase-signaling events. Approximate 75% of LMP1-positive NPC tissues overexpressed DcR3, suggesting LMP1 may enhance DcR3 expression *in vivo*. Data herein suggested that increasing DcR3 expression by LMP1 not only helps EBV-associated cancer cells gain survival advantage by preventing host immune detection but also increases the chance of cancer metastasis by enhancing cell migration and invasion. All these DcR3-mediated events facilitate normal cells to gain cancer hallmarks.

Introduction

Decoy receptor 3 (DcR3) is a soluble tumor necrosis factor receptor (TNFR), which lacks a transmembrane domain and can be secreted (1). Its overexpression was found in various malignant tumor types including lung cancers (1), gastrointestinal tract tumors (2), gliomas (3), pancreatic cancers (4) and adenocarcinomas of esophagus (5). Furthermore, DcR3 has been suggested to be a parameter for diagnosis, treatment and prognosis of cancers (5–10). DcR3 overexpression has been postulated to help tumor cells gain survival advantage by inhibiting FasL- and LIGHT (lymphotoxins-like that exhibits inducible expression and competes with HSV glycoprotein D for the HVEM, a receptor

Abbreviations: CTAR, C-terminal activation region; DcR3, Decoy receptor 3; EBER, EBV-encoded RNA; EBNA, Epstein-Barr nuclear antigen; EBV, Epstein-Barr virus; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular-regulated kinase; IHC, immunohistochemistry; JNK, c-Jun N-terminal kinase; LCL, lymphoblastoid cell line; LMP, latent membrane protein; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-kappaB; NPC, nasopharyngeal carcinoma; PI3K, phosphatidylinositol 3-kinase; TNF, tumor necrosis factor; TNFR, tumor necrosis factor receptor.

expressed by T lymphocytes)-induced apoptosis and interfering with immune surveillance (1,11,12). By neutralizing tumor necrosis factor (TNF)-like molecule 1A, DcR3 induces angiogenesis in human umbilical vein endothelial cells (13). In addition to neutralizing the cytotoxic and immunomodulatory effects of Fas ligand, LIGHT and tumor necrosis factor-like molecule 1A, DcR3 functions as an effector molecule, which transduces signaling pathways by cross-linking of heparan sulfate proteoglycan. By doing so, DcR3 modulates activation and differentiation of dendritic cell (14) and macrophage (15,16), as well as increases the expression of adhesion molecules in endothelial cells (17). Recently, DcR3 was found to induce apoptosis in dendritic cells (18) and downregulate MHC-II expression in tumor-associated macrophages by way of epigenetic control (19).

Epstein-Barr virus (EBV) is a ubiquitous γ-herpesvirus that typically establishes a lifelong infection in its hosts. EBV infects B-lymphocytes and stratified epithelia *in vivo*. B lymphotropism of EBV can be demonstrated by its ability to infect B cells and transform them into lymphoblastoid cell lines (LCLs). As a result, EBV is indicated as an etiologic factor in development of lymphoid malignancies including Burkitt lymphoma, Hodgkin's disease and lymphoproliferative disorders in the immune-compromised patients (20). Infection of epithelia can result in malignant transformation as shown by the consistent association of EBV with nasopharyngeal carcinoma (NPC) (21) and some gastric cancers (22). Monoclonality of resident EBV genomes indicates that EBV infection is an early event of tumorigenesis (23,24).

EBV uses several transcription programs to establish latent infection. A broad set of latent EBV proteins is expressed in LCLs and cancers linked to immunosuppression, including six nuclear antigens [Epstein-Barr nuclear antigens (EBNAs) 1, 2, 3A, 3B, 3C and leader protein], three latent membrane proteins (LMP1, 2A and 2B) and two RNAs [EBV-encoded RNA (EBER)-1 and EBER2]. A more restricted pattern of latent gene expression is found in NPC and EBV-positive gastric adenocarcinomas in which only EBNA1, LMP1, LMP2 and EBERs are expressed (20,25). Type I latency program, typically found in Burkitt lymphoma, does not express LMP1. LMP1 is the major oncoprotein of EBV; its expression leads to loss of contact inhibition and anchorage-independent growth in soft agar in Rat-1 cells (26), induces epidermal hyperplasia when expressed in mouse epidermis (27) and enhances B-cell lymphoma development in LMP1 transgenic mice (28). The oncogenic abilities of LMP1 include increase of cell proliferation and invasion, as well as inhibition of apoptosis, senescence and differentiation. LMP1 functions as a constitutively activated member of TNFR superfamily and activates several cellular signaling pathways in a ligand-independent manner. Two intracellular regions, the membrane-proximal C-terminal activation region (CTAR) 1 and CTAR2, are most important for induction of downstream signaling pathways. Effects of LMP1 on multiple cellular signaling pathways result from the ability of TNFR-associated factors to interact directly with CTAR1 and indirectly with CTAR2 mediated by TNFR-associated death domain protein (29). The associated TNFR-associated factor complexes allow LMP1 to engage and activate various signaling cascades mediated by nuclear factor-kappaB (NF-κB), phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinases (MAPKs) including extracellular-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 MAPK (30).

The potential link between EBV infection and DcR3 expression was first implicated in a study by Ohshima *et al.* (31) in which DcR3 expression was found to be associated with EBV-positive B-cell/nature killer cell lymphomas, whereas its expression can be rarely found in non-EBV-positive lymphomas. In their study, however, no specific EBV gene was linked to DcR3 upregulation. We have previously reported that Rta, an EBV lytic infection protein, increases DcR3 expression by direct binding to DcR3 promoter region (32). Nevertheless, EBV infection in

the associated tumors is predominantly at latent stage. Therefore, we searched for EBV latent genes that can regulate DcR3 expression. We demonstrated LMP1 can upregulate DcR3 expression in both epithelial cells and lymphocytes. DcR3 overexpression was found in >75% of LMP1-positive NPC biopsy samples. Cellular signaling pathways involved in LMP1-dependent DcR3 expression were identified in this study. We showed higher percentage of metastatic NPC tissues were DcR3 positive, with a higher DcR3 expression average, than primary NPC. Clonal HONE-1 cell lines overexpressing DcR3 had significant higher migratory and invasive potential, suggesting that DcR3 not only provides survival advantage to cells by inhibiting apoptosis (1,11,12) and induces angiogenesis (13) but also stimulates cell migration and invasion. All these DcR3-mediated events facilitate normal cells to gain hallmarks of cancers (33). This is the first report providing evidence that DcR3 can enhance cellular motility and invasiveness.

Materials and methods

Cell lines and culture

Primary B cells, EBV-transformed LCLs, Ramos, Ramos-HRIK, Ramos-B95.8 and HONE-1 cells were cultured in RPMI1640 medium (SAFC Biosciences, Lenexa, KA). SW480, a human colon adenocarcinoma cell line, was grown in Leibovitz-15 culture medium (Invitrogen, Carlsbad, CA). All cultures were supplemented with 10% fetal calf serum, streptomycin (100 µg/ml) and penicillin (100 U/ml) and incubated at 37°C with 5% CO_2 with the exception that SW480 was incubated without CO_2. Ramos-HRIK and Ramos-B95.8 were generated as described previously (30). Primary B-lymphocytes were purified from human peripheral blood mononuclear cells by using CD19 Beads (Miltenyi Biotech, Bergisch Gladbach, Germany). Peripheral blood mononuclear cell was transformed by EBV infection to generate LCL.

Generation of stable DcR3 expressing HONE-1 cell lines

DcR3 complementary DNA fragment was cloned into pIRESpuro2 (Clontech, Mountain View, CA) at EcoRI site to generate pIRESpuro2-DcR3. Clonal DcR3 cell lines were established by stable transfection of HONE-1 cells with pIRESpuro2-DcR3 and cultured in growth medium supplemented with 1 µg/ml puromycin. Individual cell clones were tested by western blotting for their DcR3 expression levels.

Antibodies and reagents

Anti-LMP1 (1A4) (DAKO, Glostrup, Denmark), anti-Rta antibody (clone 8C12) (Argene, North Massapequa, NY), anti-DcR3 antibody (3H5) (BioLegend, San Diego, CA), anti-p38 MAPK/anti-phosphorylated p38 MAPK (Thr180/Tyr182)/anti-Akt/anti-phosphorylated Akt (Ser473) antibodies (Cell Signaling Technologies, Beverly, MA), anti-RelA (06-418)/anti-ERK (06-182)/anti-phosphorylated ERK (05-481)/anti-JNK (06-748)/anti-phosphorylated JNK (07-175) antibodies (Upstate Biotechnology, Lake Placid, NY) and anti-β-actin antibody (AC-15) (Sigma–Aldrich, St Louis, MO) were from indicated companies. Anti-EBNA1, anti-EBNA2 and anti-nucleolin antibodies were gifts from J.C.Huang (National Yang-Ming University), Dr C.H.Tsai (National Taiwan University) and Dr N.H.Yeh (National Yang-Ming University), respectively. 12-*O*-tetradecanoylphorbol 13-acetate, sodium butyrate, BAY11-7082, LY294002, PD98059, SP600125 and SB203580 were from purchased Sigma.

Plasmids and siRNA duplex

Plasmids encoding wild-type LMP1 (1-386) or LMP1 mutants containing CTAR1 deletion (1-386Δ189-222), CTAR2 deletion (1-349) and CTAR1- and 2-double deletion (1-350Δ189-222) were gifts from Dr J.Y.Chen (National Health Research Institutes) and subcloned into pCMV-FLAG2 vector (Sigma) at BamHI and EcoRI sites to generate pLMP1-wt, pLMP-ΔCTAR1, pLMP1-ΔCTAR2 and pLMP1-ΔCTAR1/2. Rta expression plasmid pEGFP-Rta, DcR3 expression plasmid pCMV-Tag4a (pCMV-DcR3) and RelA expression plasmid pMT2i-RelA were gifts from Dr T.Y.Hsu (National Taiwan University), Dr S.L.Hsieh and L.P.Ting (National Yang-Ming University), respectively. Sequences of RNA duplex targeting LMP1 (siLMP1-wt) and mutant RNA duplex control (siLMP1-mut) were adapted from Mei *et al.* (34). Sequences are as follows: siLMP1-wt, 5′-GGAAUUUGCACGGACAGGCdTdT-3′ (sense) and 5′-GCCUGUCCGUG-CAAAUUCCdTdT-3′ (antisense) and siLMP1-mut, 5′-GGAA<u>U</u>GUGCACA-GACAGGCdTdT-3′ (sense) and 5′-GCCUGUC<u>U</u>GUGCA<u>C</u>AUU<u>C</u>CdTdT-3′ (antisense) (Underlined nucleotides were mutated sequences).

Transient transfection

Transfection of HONE-1 and SW480 cells was performed using Lipofectamine 2000 (Invitrogen). Electroporation of LCLs was performed by using Micro-

Porator MP-100 (Promega, Madison, WI) based on manufacturer's instructions. Amount of the plasmids used was indicated in the figure legends.

Isolation of cell nuclei

Nuclei of SW480 or HONE-1 cells were isolated by using Nuclei EZ Prep kit (Sigma). Briefly, 4×10^5 SW480 or HONE-1 cells were treated with 250 µl ice-cold Nuclei EZ lysis buffer and kept on ice for 15 min twice. Nuclei were harvested by centrifugation at 500*g* for 5 min at 4°C. Purified nuclei were used for detecting nuclear proteins in western analysis.

Western blot and DcR3 enzyme-linked immunosorbent assay

Western blot were performed as described previously (32). To detect phosphorylated forms of Akt, JNK, ERK or p38 MAPK membranes were blocked in Tris-buffered saline Tween-20 (50 mM Tris–HCl, 0.2 M NaCl, 0.1% Tween 20) and 5% bovine serum albumin. DcR3 concentration in cell-cultured medium was analyzed by DcR3 enzyme-linked immunosorbent assay (ELISA) kit (BioVendor, Brno, Czech Republic). The detecting limitation is 33 pg/ml.

Immunohistochemistry staining

Immunohistochemistry (IHC) for DcR3 and LMP1 was performed by using Histomouse SP Broad Spectrum DAB kit (Invitrogen–Zymed). Formalin fixed, paraffin-embedded primary and metastatic NPC sections were de-paraffinized and rehydrated. Samples were heated in 10 mM citrate buffer (pH 6.0) for 20 min for antigen retrieval. To block endogenous peroxidases activities, sections were incubated with 3% hydrogen peroxide. Sections were then incubated with anti-DcR3 antibody (Biolegend, 1:25) or anti-LMP1 antibody (DAKO, 1:20) and then biotinylated secondary antibody. The signal was detected with diaminobenzidine solution. All slides were counterstained with hematoxylin.

Transwell migration assay and invasion assay

The migration assay was performed by using 24-well transwell insert (8 µm pore size) (Corning, Corning, NY) and the invasion assay was done with the use of BioCoat Matrigel Invasion Chamber (BD Biosciences, Franklin Lake, NJ). A total of 5×10^4 cells in 0.2 ml of serum-free RPMI1640 medium seeded to a membrane in the upper well of a transwell apparatus and allowed to migration for 12 h and invade for 24 h toward 10% serum-containing medium. Cells were fixed with 4% formaldehyde at 37°C for 20 min and permeablized by 0.5% Triton X-100. After removing the non-migrated or non-invasive cells, hematoxylin staining was performed to visualize the cells passing through the membrane. The average number of migrated cells was calculated from three independent experiments with three inserts used each time.

Quantitative reverse transcription–polymerase chain reaction

Total RNA purification and reverse transcription process were described previously (32). Quantitative polymerase chain reaction detecting DcR3 or β-actin in complementary DNA was performed by using LightCycler TaqMan Master Kit (Roche, Mannheim, Germany). Primers for detecting DcR3 were 5′-TCTCAGCCAGCAGCTCCA-3′ (forward) and 5′-CACACTCCT-CAGCTCCTGGTAC-3′ (reverse). The sequence of DcR3-specific fluorogenic probe was 5′-TGCACAGGGTGTCATGGGAGGAAGA-3′. Primers for detecting β-actin were 5′-AGCCTCGCCTTTGCCGA-3′ (forward) and 5′-CTGGTGCCTGGGGCG-3′ (reverse). The sequence of β-actin-specific fluorogenic probe was 5′-CCGCCGCCCGTCCACACCCGCC-3′.

Chromatin immunoprecipitation assay

Experiments were carried out as described previously (32). Briefly, 1.5×10^7 HONE-1 cells were transfected with 36 µg of pCMV-Flag2, pCMV-LMP1-wt or 12 µg pMT2T-RelA plasmid DNA for 24 h. DNA–protein complexes were immunoprecipitated with anti-RelA antibody or with rabbit IgG isotypic antibody (Abcam, Cambridge, MA). The sequences of primer pair 1 were 5′-AGTTGGCAGAGGCCCC-3′ (forward) and 5′-ACCCACCTGGTAC-CATCCC-3′ (reverse). The sequences of primer pair 2 were 5′-AACCCACC-CAACAGAATAGG-3′ (forward) and 5′-TGTCCCTCAACTCCCACC-3′ (reverse).

Data analysis and statistics

Data are expressed as means ± SDs of at least three experiments. Statistical comparisons between groups were performed using two-tailed Student's *t*-test. *P* value used for statistic significance was stated in the figure legends.

Results

DcR3 overexpression can be detected in primary and metastasized NPC

Since enhanced DcR3 expression was detected in EBV-positive lymphomas and cell lines (31), we hypothesized that DcR3 overexpression

can also be found in EBV-associated NPC tissues. We analyzed DcR3 expression by IHC in 26 samples of paraffin-embedded primary non-keratinizing NPC and 31 metastatic NPC tissues from Taipei Veterans General Hospital and Taipei Medical University Hospital. Samples were scored for the positive rate of staining and results were summarized in Table I. Among them, 58% of primary (15 of 26) and 74% (23 of 31) of metastatic NPC cases were DcR3 positive. DcR3 IHC staining of two primary samples (Figure 1A–C) and one metastatic sample were shown (Figure 1D). Notably, non-cancerous squamous epithelial cells (N) were weakly stained or not stained (Figure 1H), suggesting that DcR3 is expressed specifically in cancerous cells.

DcR3 enhances cell migration and invasion

It is important to address the function of DcR3 in NPC tumorigenesis. Our IHC data show that metastatic NPC has a higher DcR3-positive rate and a higher average DcR3 expression score, suggesting DcR3 plays a role in NPC metastasis (Table I). Thus, we examined if DcR3 overexpression enhances cell motility and invasiveness. HONE-1 was used for its NPC origin and low endogenous DcR3 expression. pIR-ESpuro2-DcR3, a DcR3-expressing plasmid, was stably transfected into HONE-1 cells. Two stable HONE-1 cell lines overexpressing DcR3 (D1 and D2) and a vector control line (V1) were selected after puromycin treatment and tested for their DcR3 expression by western blotting. Both D1 and D2 expressed much higher levels of DcR3 compared with V1 and parental HONE-1 cells (P) (Figure 2A). Both parental HONE-1 and V1 express low amount of endogenous DcR3, which cannot be detected by western analysis. Transwell migration

assay was used to compare migratory abilities of HONE1 P, V1, D1 and D2 cell lines. Cells were seeded onto upper wells of transwells and allowed to migrate for 12 h. Cells adherent on the lower surface of the filters were counted after staining. The average number was 129 ± 6 and 101 ± 12 for DcR3 overexpressing D1 and D2, respectively. Either parental HONE-1 or the vector control V1 showed low migratory ability. A representative cell staining under microscopy was photographed (Figure 2B, left panel), and the quantitation from three independent studies was shown (right panel). Next, we studied cell invasiveness by using a Matrigel invasion chamber system. DcR3-expressing HONE-1 cells (D1 and D2) were more invasive than HONE-1 with only endogenous DcR3 (P and V1). The average cell number on the lower surface of the filters was 48 ± 3 and 51 ± 9 for DcR3-overexpressing D1 and D2, respectively, 24 h post-seeding. A representative cell staining under microscopy (Figure 2C, left panel) and the quantitation from three independent studies (right panel) were shown. Therefore, our hypothesis that DcR3 overexpression may enhance cell metastatic potential *in vivo* is supported by showing the increased motility and invasiveness seen in DcR3-overexpressing HONE-1 cells.

LMP1 increases DcR3 expression

EBV is an etiologic factor in NPC development. Since EBV type II latency, in which LMP1, LMP2 and EBNA1 are expressed, is constantly associated with non-keratinizing NPC (35), we studied if any of these viral proteins affect DcR3 expression. To better analyze which EBV latent gene can stimulate DcR3 expression, we used SW480, an EBV-negative cell line with relatively high DcR3 expression, as our model system. We transfected expression plasmids of LMP1, LMP2A and EBNA1 into SW480 cells and measured DcR3 protein 24 h posttransfection. Cell-associated DcR3 was measured by western analysis and secreted DcR3 was determined by ELISA. While EBNA1 had no effect and LMP2A increased <20% of DcR3 expression in SW480 (data not shown), LMP1 increased both cell-associated (Figure 3A, upper panel) and secreted DcR3 (Figure 3A, middle panel) expression up to 3-fold in a dosage-dependent manner. To examine if LMP1 upregulates DcR3 expression at transcription level, we measured DcR3 transcripts by quantitative reverse transcription–polymerase chain reaction. The values were reported as fold over the internal control of β-actin. The results demonstrated that DcR3

Table I. Expression of DcR3 in primary and metastatic NPC tissues

	DcR3 expression score					Score, (mean ± SD)	DcR3 positive, (score ≥2)
	N	1	2	3	4		
Primary NPC	26	11	7	3	5	2.08 ± 1.16	58%
Metastatic NPC	31	8	4	11	8	2.61 ± 1.15	74%

Score 1: <5%; score 2: 5–25%; score 3: 26–50%; score 4: >51% of tumor cells were positive for DcR3 expression.
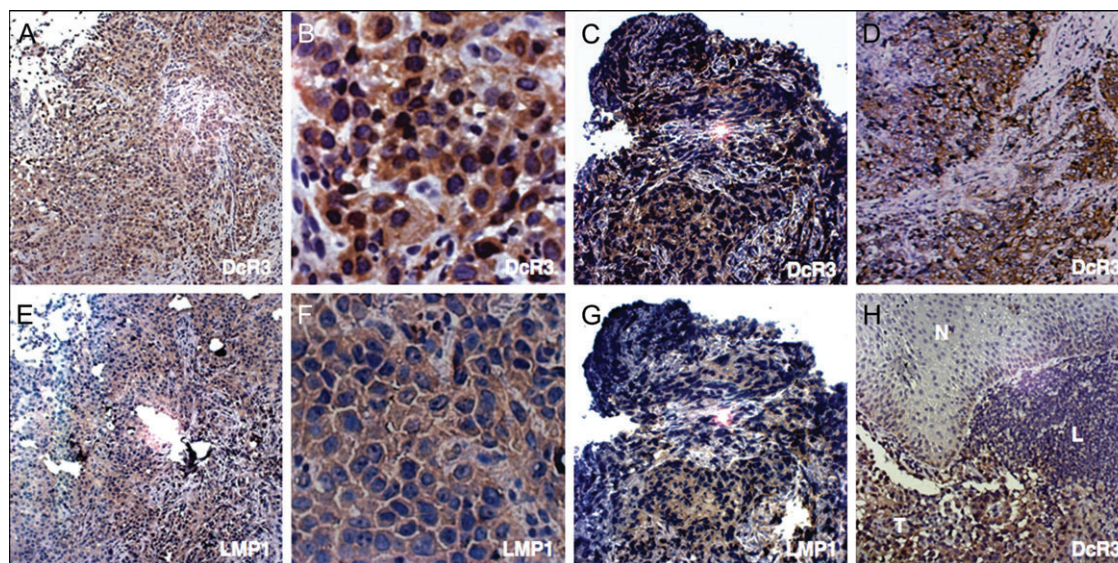

Fig. 1. Expression of DcR3 and LMP1 in NPC tissues. IHC using anti-DcR3 antibody shows the expression of DcR3 in two primary NPC tissues (**A–C**) and one metastasized NPC tissue (**D**). Anti-LMP1 antibody was used to show LMP1 expression in those two primary tissues (**E–G**). The third primary NPC containing normal epithelial cells was only stained with anti-DcR3 antibody (**H**). The specimens are shown at ×100 (A, E and H) and ×200 (C, D and G) magnification. A and E at higher magnification (×400) are shown in B and F, respectively. Positive staining is indicated by brownish coloration. Antibody isotype staining was negative in all cases. L, infiltrated lymphocytes; N, normal squamous epithelial cells and T, tumor cells.
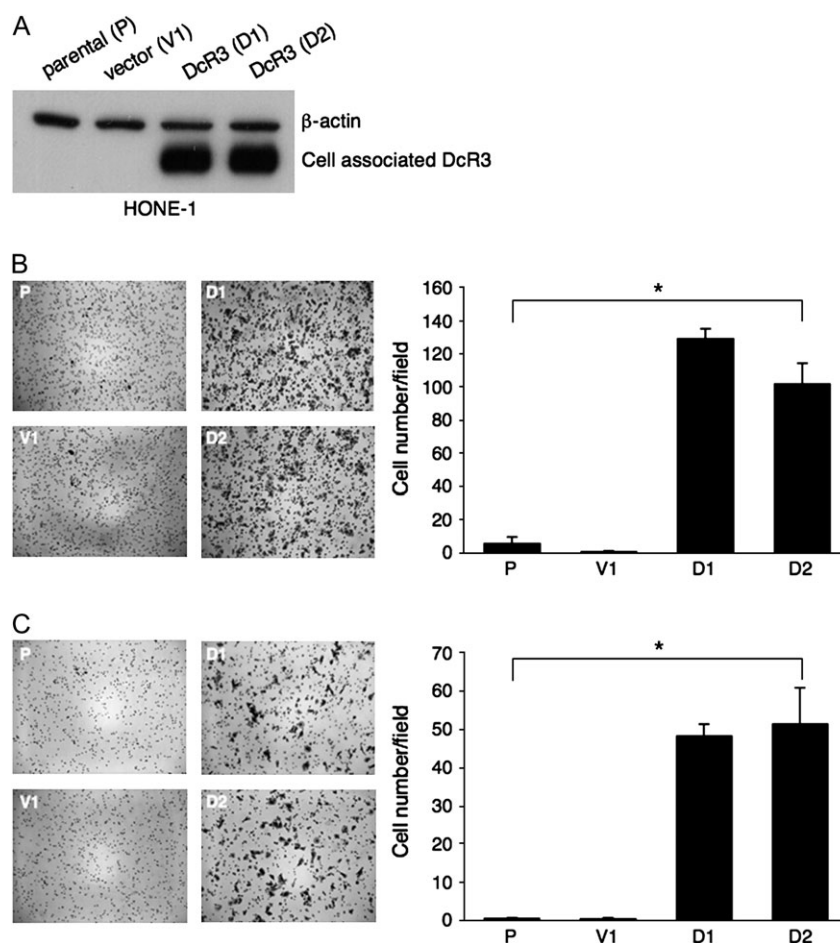
Fig. 2. DcR3 enhances cell migration and invasion. (**A**) DcR3 expression was compared by western analysis among two stable DcR3-expressing HONE-1 cell lines (D1 and D2), a vector control clone (V1) and the parental HONE-1 cell line (P). Western analysis using antibodies to DcR3 and the control cellular protein β-actin was shown. (**B**) Transwell migration assay. HONE1 P, V1, D1 or D2 were seeded onto upper wells of transwells and cells adherent on lower surface of the filters were stained and counted 12 h after seeding. A representative cell staining under microscopy was photographed (×100, left panel), and the quantitation from three independent studies was shown (right panel). (**C**) Matrigel invasion assay. Same four HONE1 lines as used in (B) were seeded onto matrigel invasion chamber and cells adherent on lower surface of filters were stained and counted 24 h after seeding. A representative cell staining under microscopy was photographed (×100, left panel), and the quantitation from three independent studies was shown (right panel). Bar graphs in (B) and (C) were expressed as mean ± SD and two-tailed Student's t-tests were performed ($*P < 0.001$).

transcripts increased in the presence of LMP1 (Figure 3A, bottom panel). To show LMP1 can enhance DcR3 expression in NPC-derived cells, we transfected HONE-1 cells with a LMP1 expression plasmid. Although endogenous DcR3 expression in HONE-1 cells cannot be detected by western blotting due to sensitivity limitation, we still found LMP1 enhanced both DcR3 protein and transcript expression in a dosage-dependent manner as demonstrated by ELISA and quantitative reverse transcription–polymerase chain reaction (Figure 3B). Overexpressing LMP1 in EBV-negative Akata cells (DcR3 low) and HCT-116 cells (colorectal carcinoma cells, DcR3 high) enhanced DcR3 expression (data not shown), suggesting LMP1-mediated DcR3 expression is not a cell line-specific effect.

We have shown DcR3 overexpression in NPC tissues (Figure 1 and Table I) and LMP1 can upregulate DcR3 expression in cell lines (Figure 3); therefore, we looked for the co-expression LMP1 and DcR3 in NPC tissues. Among 13 primary and 12 metastasized LMP1-positive NPC tissues we examined by IHC, 76.9% (10 of 13 samples) and 75% (nine of 12 samples), respectively, were found to be DcR3 positive. IHC staining demonstrating the co-expression of DcR3 and LMP1 in two representative primary NPC samples were shown. Presence of LMP1 (Figure 1E–G) and DcR3 (Figure 1A–C) could be found in the same biopsy sample, suggesting LMP1 can upregulate DcR3 expression in NPC cells.

LMP1 promotes DcR3 expression mainly through activation of NF-κB- and PI3K-signaling pathways

To find out how LMP1 upregulates DcR3, we first determined the contribution of two primary domains of LMP1 in DcR3 overexpression. We transfected pCMV vector-based expression plasmids of LMP1-wt, LMP1-ΔCTAR1, LMP1-ΔCTAR2 and LMP1-ΔCTAR1/2 into SW480 and HONE-1 cells. Amount of secreted DcR3 was determined using ELISA. While LMP1-wt increased ∼3-fold of DcR3 expression in both SW480 and HONE-1, LMP1 with deletion of CTAR1 or CTAR2 reduced LMP1-mediated DcR3 expression to 2-fold. Deletion of both CTAR1 and CTAR2 reduced DcR3 expression in both cell lines to the level slightly higher, but without statistic significance, than the background, suggesting both CTAR1 and CTAR2 domains contribute the increase of DcR3 expression (Figure 4A).

LMP1 initiates many cellular signaling events (30), among which NF-κB signaling is the well-known signaling pathway that can be induced by both CTAR1 and CTAR2. Therefore, we first investigated the effect of LMP1-induced NF-κB signaling on DcR3 expression in SW480 and HONE-1. SW480 cells were serum starved for 24 h before transfection to reduce serum-induced DcR3 expression. Cells were transfected with pCMV-LMP1 or control vector 6 h before adding BAY11-7082, the NF-κB-inhibitor, or dimethyl sulfoxide, the solvent control. The nuclear RelA was detected for the effect of
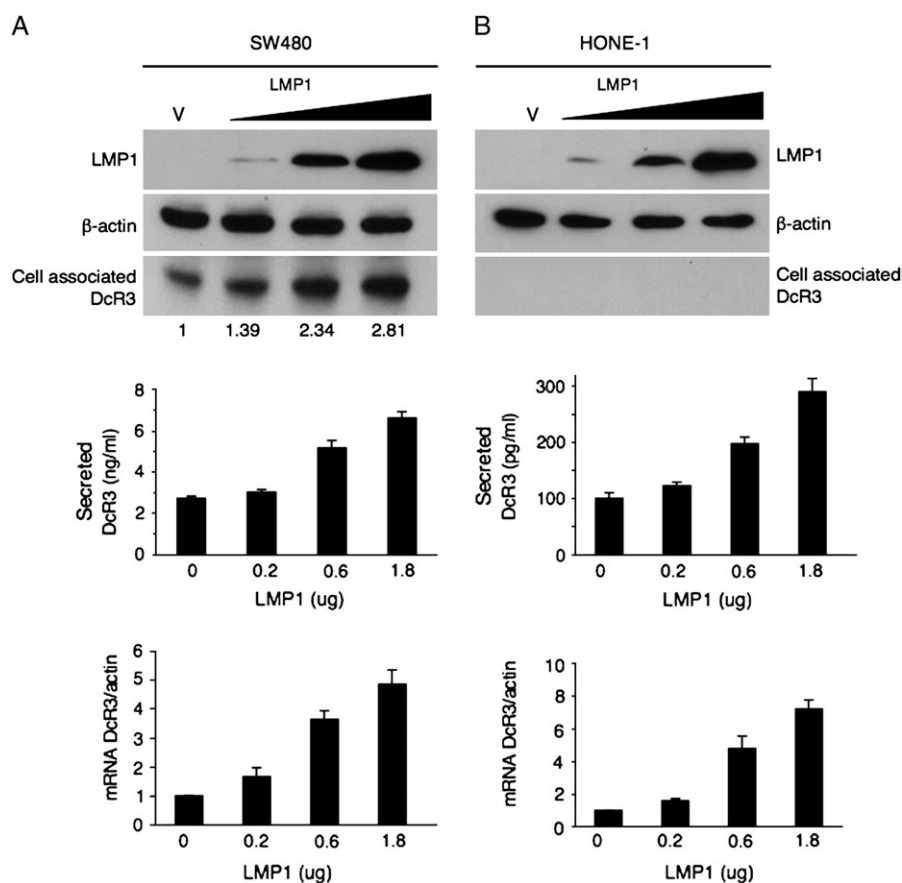
Fig. 3. LMP1 enhances DcR3 expression in SW480 and HONE-1 cells. (**A**) 0.2, 0.6 or 1.8 μg of LMP1-expressing plasmid, pCMV-LMP1, was transfected into 4×10^5 SW480 cells to demonstrate the dosage effect of LMP1 on DcR3 expression. pCMV-FLAG2 was used as the vector control. The expression levels of DcR3 were measured by western analysis for cell-associated DcR3 (upper panel), ELISA for secreted DcR3 in the culture media (middle panel) and quantitative reverse transcription–polymerase chain reaction for transcripts of DcR3 (bottom panel). Changes of cell-associated DcR3 expression were quantified by ImgeQuant software, normalized to β-actin expression and reported as fold over vector control lane. The values of quantitative reverse transcription–polymerase chain reaction were reported as fold over the internal control of β-actin. Western blots were probed with antibodies to LMP1, DcR3 and the control cellular protein β-actin. All the measurements in triplicates were taken 24 h posttransfection. (**B**) Same experiment was repeated on HONE-1 cells. ELISA (middle panel) and quantitative reverse transcription–polymerase chain reaction (bottom panel) were used to detect DcR3 expression.

LMP1-induced NF-κB signaling, as well as the inhibitory effect of BAY11-7082. BAY11-7082 clearly reduced LMP1-mediated cell-associated DcR3 as shown by western analysis and secreted DcR3 measured by ELISA with statistic significance (2.9- to 1.6-fold in SW480; 2.7- to 1.7-fold in HONE-1). The difference between fold inductions was examined by Student's *t*-test and the *P* value is indicated in each panel (Figure 4B). This inhibitor, however, did not reduce DcR3 expression to the background level, suggesting other signaling pathways are involved in LMP1-mediated DcR3 induction. Since LMP1 also activates PI3K-, ERK-, JNK- and p38 MAPK-mediated signaling pathways, we explored the contribution of individual pathway to LMP1-enhanced DcR3 expression by using pathway-specific inhibitors. While LY294002, a PI3K signaling inhibitor, reduced LMP1-induced DcR3 (Figure 4C), PD98059, SP600125 and SB203580, inhibitors for the ERK, JNK and p38 MAPK signaling, respectively, posed minor or no effect on DcR3 expression in vector or pCMV-LMP1-transfected cells (supplementary Figure 1A–C is available at *Carcinogenesis* Online). Secreted DcR3 reduced from 2.9- to 2-fold in SW480 and 2.8- to 2.4-fold in HONE-1 as measured by ELISA when cells were treated with LY294002 (Figure 4C). Similar fold reduction in DcR3 expression was observed between PD98059-, SP600125- or SB203580-treated cells and non-treated cells, suggesting ERK-, JNK- and p38 MAPK-signaling pathways played insignificant roles in LMP1-mediated DcR3 expression. This observation is further supported by the results of combinatory effects of inhibitors on LMP1-induced DcR3 expression showing when cells were treated

with both BAY11-7082 plus LY294002; LMP1-mediated DcR3 was reduced to almost background level (supplementary Figure 1D is available at *Carcinogenesis* Online, lane NP). Addition of SP600125 or PD98059 did not further reduce LMP1-induced DcR3 in cells treated with BAY11-7082 (lanes NE and NJ). Notably, all the inhibitors except SB203580 decreased endogenous DcR3 expression in SW480 cells, suggesting endogenous DcR3 is regulated by multiple signaling pathways. A similar observation was recently reported using AsPC-1, a human pancreatic adenocarcinoma cell line, as a model (36). Effects of PD98059, SP600125 and SB203580 on LMP1-induced DcR3 were examined in HONE-1 cells and the results were similar to those of SW480 (Data not shown).

LMP1 is responsible for DcR3 overexpression during EBV latency
Finally, we tested if EBV infection upregulates DcR3 expression and whether LMP1 is important for DcR3 overexpression during EBV infection. Because EBV does not efficiently or stably infect epithelial cells *in vitro*, we used B-lymphocytes to address this question. We infected primary B cells from an anonymous individual with EBV and allowed a LCL-B to establish. EBV latent infection was monitored by western blotting of LMP1 and EBNA2 expression. Amount of DcR3 secreted in culture media was compared between primary B cells and EBV-transformed autologous LCL-B by ELISA 48 h after seeding. More than 4-fold of DcR3 was secreted by LCL-B than its primary B-cell counterpart (Figure 5A), suggesting EBV latent infection stimulates DcR3 expression in LCLs. Similar amount of DcR3 (130 pg/ml)
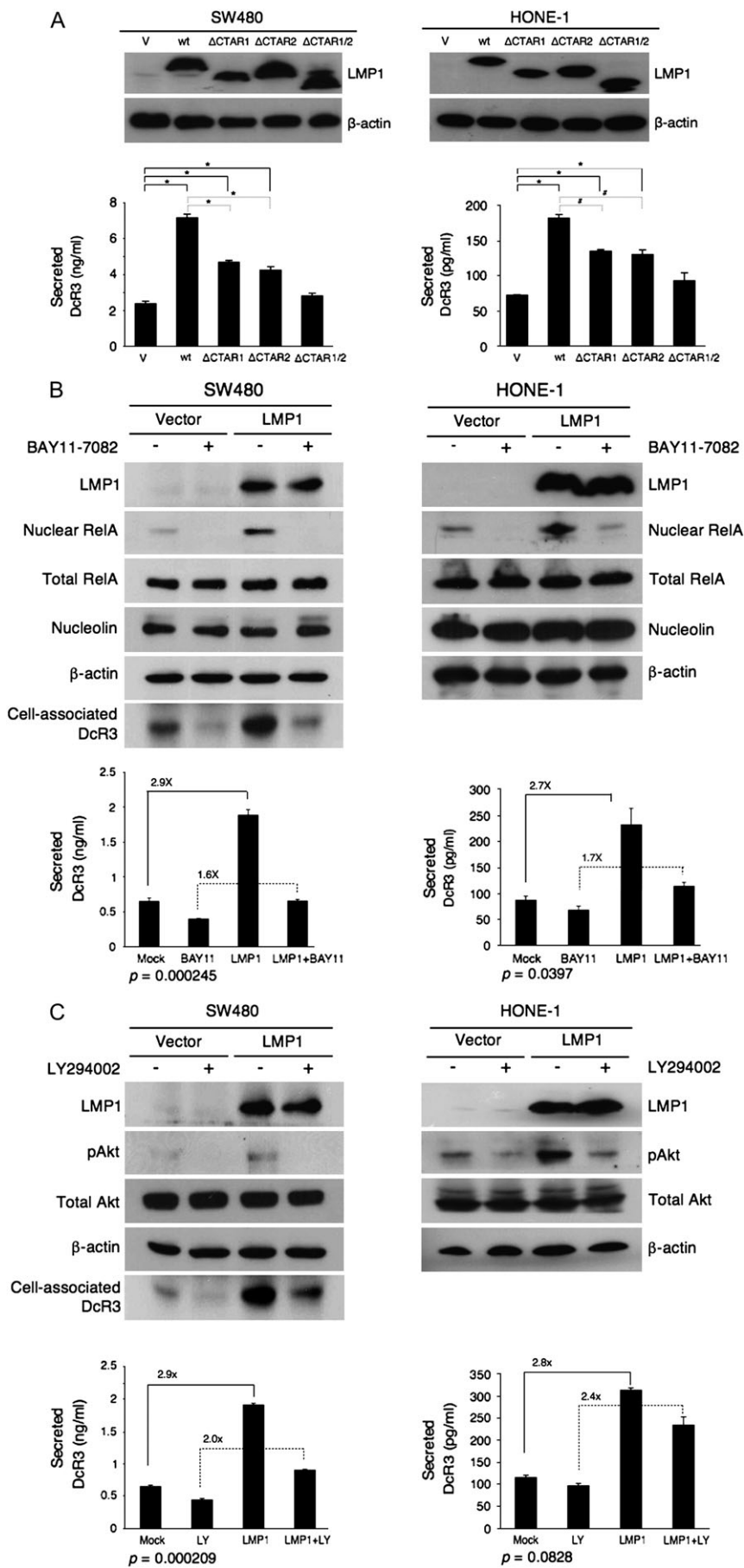
was secreted by 10^6 cells of both Akata EBV-positive and EBV-negative cells, suggesting EBNA1 and EBERs are not responsible for the DcR3 upregulation. DcR3 expression was also compared among cells with different EBV protein profile. Ramos, a Burkitt lymphoma cell line, was infected with EBV P3HR1 strain to generate Ramos–HRIK or infected with EBV B95.8 strain to generate Ramos-B95.8. Ramos-B95.8 expresses all nine EBV latent gene products (type III latency), whereas Ramos–HRIK does not express LMP1, LMP2 and EBNA2 (37). While Ramos-HRIK expressed similar levels of DcR3 as Ramos, Ramos-B95.8 expressed a higher amount of DcR3 (supplementary Figure 2 is available at *Carcinogenesis* Online), suggesting LMP1, LMP2 or EBNA2 may upregulate DcR3 in EBV type III latency. To further demonstrate that LMP1 is responsible for DcR3 upregulation during EBV latent infection in lymphocytes, we knocked down LMP1 expression by siRNA targeting LMP1 (siLMP1-wt) in two LCLs (LCL-B and LCL-S5) generated from different origin. Transfection of siLMP1-wt effectively decreased LMP1 expression and decreased DcR3 expression in both LCLs with statistical significance ($P < 0.01$), indicating the LMP1 is responsible for DcR3 overexpression in these EBV-transformed LCLs (Figure 5B). We also studied if transient overexpression of LMP1 can enhance DcR3 expression in LCLs. The result indicated overexpression of LMP1 further enhanced ~2-fold of DcR3 expression in both LCLs, confirming the positive effect of LMP1 on DcR3 expression (Figure 5C). As a comparison, we overexpressed Rta, an EBV transactivator effectively enhances DcR3 expression (32), in the LCLs and found Rta also gave ~2-fold increase of DcR3 (Figure 5D). In all the experiments shown in Figure 5, β-actin expression was measured to ensure even loadings.

LMP1 knockdown decreased, whereas LMP1 overexpression enhanced DcR3 expression level, demonstrating the positive effect of

LMP1 on DcR3 expression. These results together with our studies conducted in epithelial cells indicate LMP1, rather than other EBV latent genes, was the major factor upregulating DcR3 expression *in vivo* during EBV latency.

Discussion

Ohshima *et al.* (31) demonstrated that DcR3, a soluble TNFR, is overexpressed in EBV-associated lymphomas. We found in this study that DcR3 is overexpressed in NPC. *In vitro* studies demonstrated EBV LMP1, besides previously found EBV transactivator Rta, can upregulate DcR3 expression. We showed DcR3 enhances cell migration and invasion, which is a previous unknown function of DcR3. Current study together with previously established functions of DcR3, including antiapoptosis, immune modulation and enhancing angiogenesis, suggest EBV-induced DcR3 may be a contributory factor of NPC tumorigenesis and metastasis.

Persistent EBV infection is continuously controlled by cytotoxic T-lymphocytes and EBV-specific antibodies (38,39). EBV, on the other hand, has developed multiple counter measurements to escape host immune responses. As a result, stable numbers of latently infected B cells are maintained in the blood. Suppression of immune responses has been shown to contribute to tumor pathogenesis and malignant cells develop escaping mechanisms to evade immune recognition. It is speculated that failure of EBV-specific immune control contributes to the development of EBV-associated cancers (40). Among strategies for immune evasion, interfering FasL binding to its receptor may help malignant cell gain survival advantage by escaping immune surveillance of antigen-specific T lymphocytes. Adenovirus E3 downmodulates the apoptosis receptor Fas by inducing its internalization, and as
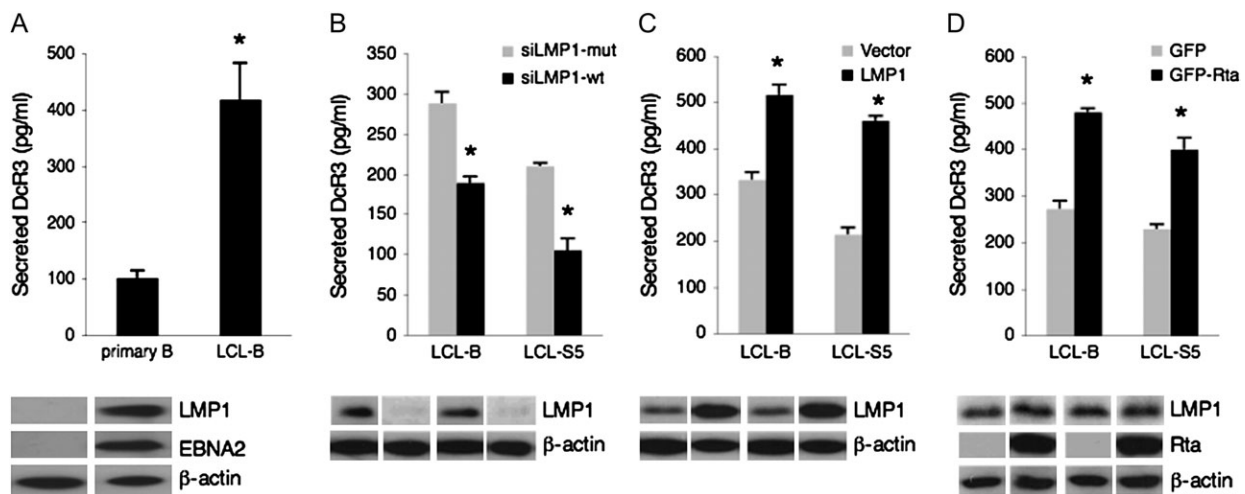
Fig. 5. LMP1 is responsible for DcR3 overexpression during EBV latency. (**A**) CD19-positive primary B-lymphocytes were purified from an anonymous individual and infected with EBV B95.8 strain to establish LCL-B. Amount of secreted DcR3 by 1×10^6 cells was compared between primary B cells and LCL-B by ELISA 48 h after seeding. Western blots were probed with antibodies to EBV latent genes, LMP1 and EBNA2. (**B**) A total of 2.5 μl LMP1-wt siRNA (siLMP1-wt, 50 μM) and its mutant control (siLMP1-mut, 50 μM), (**C**) 0.5 μg pCMV-LMP1 and control vector, (**D**) 0.5 μg pEGFP-Rta and control vector were electroporated into LCL-B and LCL-S5 (5×10^5), two LCLs generated from different origin. DcR3 expression was measured by ELISA 48 h post-electroporation. Western analysis using antibodies to LMP1 and Rta was shown. Expression of cellular protein β-actin served as a loading control for all western analyses. In all experiments, mean and SD were from measurements taken in triplicate and two-tailed, Student's *t*-tests were performed (*$P < 0.005$).

Fig. 4. LMP1 upregulates DcR3 via NF-κB- and PI3K-signaling pathways. (**A**) Map LMP1 domains required for LMP1-dependent DcR3 expression. A total of 4×10^5 SW480 or HONE-1 cells were transfected with either 1.6 μg of pCMV-LMP1, pCMV-LMP1-ΔCTAR1, pCMV-LMP1-ΔCTAR2, pCMV-LMP1-ΔCTAR1/2 or control vector (v) as indicated above the lanes. Mean and SD were from measurements taken in triplicate 24 h posttransfection and two-tailed, Student's *t*-tests were performed (#$P < 0.01$; *$P < 0.001$). (**B**) NF-κB inhibitor, BAY11-7082 (10 μM) and (**C**) PI3K inhibitor, LY294002 (50 μM), were added to the serum-starved SW480 and HONE-1 cells 6 h posttransfection. Cells were harvested 18 h after drug treatment and analyzed for DcR3 expression. The inhibitory effects of BAY11-7082 and LY294002 were demonstrated by the reduction of RelA nuclear translocation and phophorylated Akt (pAkt), respectively. Fold induction of LMP-1-mediated DcR3 was compared between inhibitor-treated cells and dimethyl sulfoxide-treated cells. The difference between fold inductions was examined by Student's *t*-test and the P value is indicated in each panel. The expression levels of DcR3 of SW480 and HONE-1 were measured by western analysis and/or ELISA. Western blots were also probed with antibodies to LMP1 and the control nuclear protein nucleolin and cellular protein β-actin.

a consequence, adenovirus infected cells are protected from Fas-mediated apoptosis (41). EBV also has developed several antiapoptosis strategies. For example, EBV BHRF1 and BALF1 encode bcl-2 homologs, which inhibit apoptosis (42,43); Zta downregulates TNFR1 expression and leads to protection of apoptosis induced by TNF-α (44); LMP1 protects LCLs from apoptosis by inducing the expression of cellular bcl-2 (45). DcR3 is known to compete FasL binding to Fas (1). It is possible that by interfering FasL binding to its receptor, EBV-induced DcR3 expression helps malignant cells escape immune surveillance exerted by infiltrated lymphocytes. In addition, DcR3 may decrease the chances that Fas and FasL double-expressing NPC cells kill each other since 60% of NPC express Fas and 90% express FasL.

Other than competing binding to FasL, DcR3 functions as an effector molecule to interfere with the immune responses. The raise of effective humoral or cell-mediated immune responses depends on the polarization of virus-specific CD4$^+$ T cells. Th2 responses are thought to be supportive of antibody-mediated immunity. In contrast, Th1 responses are more protective against viral infections and lead to the generation of virus-specific CD8$^+$ T cells. Anti-EBV Th1 immunity is thought to keep most EBV carriers free of EBV-associated malignancies (40). Overexpression of DcR3 was shown to regulate dendritic cell differentiation and downregulate several costimulatory molecules, leading to Th2 polarization (14,46). It is possible that LMP1-induced DcR3 overexpression skews a normal protective Th1 phenotype of EBV-specific immune to a less protective Th2 phenotype. EBV-infected tumor cells may outgrow as a result.

A surprising finding was that DcR3 enhanced cellular motility and invasiveness. The mechanism used by LMP1 in mediating cell migration is not totally clear. LMP1 decreases E-cadherin expression by activating DNA methyltransferase or inducing Twist expression in MDCK cells and this reduction is correlated with increased cell migration activity (47–49). Recently, ERK–MAPK pathway was identified to be required for LMP1-enhanced cell migration in the same cell line (47–49). By reducing the expression of the junction protein plakoglobin, LMP1 enhanced cell migration in C666-1 NPC cell line (50). Whether LMP1 can induce the cell migration by the same mechanism in other cell lines, such as HONE-1 and SW480, requires further investigation. Currently, we do not know how DcR3 enhances cell migration and invasion in our system. We have preliminary evidence indicating that intracellular DcR3 may contribute to DcR3-dependent cell migration. We also have tested if DcR3 induces epithelial-mesenchymal transition, which increases cellular migratory phenotype (51) and found several markers were changed by DcR3 overexpression in HONE-1 cells (data not shown).

LMP1 product can only be detected in ∼50% of NPC tissues (52), suggesting factors other than LMP1 regulate DcR3 expression. The EBV BRLF1 gene products, Rta, could be another DcR3 inducer in NPC since messenger RNA of BRLF1 (Rta) is frequently expressed in NPC tumors and Rta was shown to induce DcR3 expression (32). Other than EBV genes, inflammatory cytokines are possible candidates to induce DcR3 expression. DcR3 is overexpressed in inflamed epithelia of patients with acute appendicitis (53) and patients with chronic kidney disease (54). Expression of inflammatory cytokines, such as TNF-α, IL-1β and IL-6, was found in ∼70% primary NPC samples. The possibility that inflammatory cytokines induce DcR3 expression in NPC is supported by our preliminary data showing that TNF-α and IL-6 can upregulate DcR3 expression *in vitro* (data not shown). Chronic inflammation is one of the factors linked to human cancer (55). Inflammation cytokine-induced DcR3 expression may be a contributory factor in inflammation-induced tumorigenesis. How these cytokines regulating DcR3 expression is currently under investigation.

Recently, PI3K/Akt/NF-κB pathway was shown to regulate insulin-like growth factor-1-induced DcR3 expression in AsPC-1 cells (36). In their study, DcR3 expression was inhibited by PI3K inhibitor and NF-κB inhibitor alike, suggesting PI3K pathway may act on NF-κB to regulate DcR3 expression. We, however, found NF-κB inhibitor (BAY11-7082) has stronger inhibitory effect than PI3K inhibitor (LY294002) on LMP1-mediated DcR3 expression in both SW480 and HONE-1 cells (Figure 4B and C), suggesting LMP1-induced NF-κB activity is partially dependent on PI3K at most in our system. This is supported by the result that BAY11-7082 and LY294002 together inhibited DcR3 expression at a much higher level than individual inhibitor alone (supplementary Figure 1D is available at *Carcinogenesis* Online). It is conceivable that DcR3 is regulated distinctly in different cell backgrounds or by different inducers; nevertheless, regulation of DcR3 is still mostly unclear. To examine the direct effect of LMP1-induced NF-κB activity on DcR3 expression, we performed chromatin immunoprecipitation assays using RelA-specific antibody. Our preliminary data indicated that RelA binds to putative NF-κB-binding sites between −358 to −106 bp of DcR3 promoter region (data not shown). Further test is needed to confirm if LMP1 enhances RelA binding to DcR3 promoter.

We showed in additional to EBV-associated lymphoma (31), DcR3 is also overexpressed in NPC. Using EBV as a model system, we demonstrated DcR3 expression can be enhanced by viral infection. We suggest here that LMP1 may contribute to viral survival and turmorigenesis by activating DcR3, which provides a survival advantage to cancer cells by interfering with the immune response to EBV-infected cells. DcR3 may also be a contributory factor to cancer metastatic potential by enhancing cell migration. This result added to a new dimension to possible roles of DcR3 in tumorigenesis. With more and more functions and regulation of DcR3 revealed, DcR3 may become a target in treating cancers.

Supplementary material

Supplementary Figures 1 and 2 can be found at http://carcin.oxfordjournals.org/

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