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Enhancement of specific T-lymphocyte responses by monocyte-derived dendritic cells pulsed with E2 protein of human papillomavirus 16 and human p16INK4A

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Prophylactic vaccines are already available for prevention of human papillomavirus (HPV) infection. However, we still await development of therapeutic vaccines with high efficiency for stimulating specific T lymphocytes to clear HPV infection. This study investigates the potential for subunits of human p16INK4a protein and E2 protein of HPV16 to stimulate dendritic cells and enhance the specific response of T lymphocytes against HPV-infected cells. Immunogenic epitopes of HPV16 E2 and p16INK4a proteins were predicted through the common HLA class I and II alleles present in the Thai population. Then, monocyte-derived dendritic cells (MDCs) were pulsed with HPV16 E2 and/or p16INK4a proteins and their maturity assessed. MDCs pulsed with either or both of these proteins at optimal concentrations were used for activation of autologous T lymphocytes and IFN-γ production was measured for specific response function. HPV16 E2 and p16INK4a proteins contain various immunogenic epitopes which can be presented by antigen-presenting cells via both HLA class I and II molecules. The stimulation of MDCs with either HPV16 E2 or

p16INK4a proteins increased percentages and mean fluorescence intensity (MFI) of CD83$^+$ MDCs in a dose-dependent manner. An optimum concentration of 250 ng/mL and 150 ng/mL of HPV16 E2 and p16INK4a proteins, respectively, stimulated MDCs via the MAPK pathway (confirmed by use of MAPK inhibitors). T lymphocytes could be activated by MDCs

pulsed with these proteins, leading to high percentages of both CD4$^+$ IFN-γ$^+$ T lymphocytes

and CD8$^+$ IFN-γ$^+$ T lymphocytes. The production of IFN-γ was higher in co-cultures containing MDCs pulsed with HPV16 E2 protein than those pulsed with p16INK4a. Interestingly, MDCs pulsed with a combination of HPV16 E2 and p16INK4a significantly

increased IFN-γ production of T lymphocytes. The IFN-γ production was inhibited by both HLA class I and II blockade, particularly in co-cultures with MDCs pulsed with a combination of HPV16 E2 and p16INK4a. This suggests that MDCs pulsed with both proteins enhances

specific response of both CD4$^+$ and CD8$^+$ T lymphocytes. This study might provide a strategy for further *in vivo* study of stimulation of T lymphocytes for therapy of HPV-associated cancer.

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Abstract

Prophylactic vaccines are already available for prevention of human papillomavirus (HPV) infection. However, we still await development of therapeutic vaccines with high efficiency for stimulating specific T lymphocytes to clear HPV infection. This study investigates the potential for subunits of human p16INK4a protein and E2 protein of HPV16 to stimulate dendritic cells and enhance the specific response of T lymphocytes against HPV-infected cells. Immunogenic epitopes of HPV16 E2 and p16INK4a proteins were predicted through the common HLA class I and II alleles present in the Thai population. Then, monocyte-derived dendritic cells (MDCs) were pulsed with HPV16 E2 and/or p16INK4a proteins and their maturity assessed. MDCs pulsed with either or both of these proteins at optimal concentrations were used for activation of autologous T lymphocytes and IFN-γ production was measured for specific response function. HPV16 E2 and p16INK4a proteins contain various immunogenic epitopes which can be presented by antigen-presenting cells via both HLA class I and II molecules. The stimulation of MDCs with either HPV16 E2 or p16INK4a proteins increased percentages and mean fluorescence intensity (MFI) of CD83$^+$ MDCs in a dose-dependent manner. An optimum concentration of 250 ng/mL and 150 ng/mL of HPV16 E2 and p16INK4a proteins, respectively, stimulated MDCs via the MAPK pathway (confirmed by use of MAPK inhibitors). T lymphocytes could be activated by MDCs pulsed with these proteins, leading to high percentages of both CD4$^+$ IFN-γ^+ T lymphocytes and CD8$^+$ IFN-γ^+ T lymphocytes. The production of IFN-γ was higher in co-cultures containing MDCs pulsed with HPV16 E2 protein than those pulsed with p16INK4a. Interestingly, MDCs pulsed with a combination of HPV16 E2 and p16INK4a significantly increased IFN-γ production of T lymphocytes. The IFN-γ production was inhibited by both HLA class I and II blockade, particularly in co-cultures with MDCs pulsed with a

45   combination of HPV16 E2 and p16INK4a. This suggests that MDCs pulsed with both proteins

46   enhances specific response of both CD4$^+$ and CD8$^+$ T lymphocytes. This study might provide a

47   strategy for further *in vivo* study of stimulation of T lymphocytes for therapy of HPV-associated

48   cancer.

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50   **Keywords**: human papillomavirus (HPV), HPV16 E2, p16INK4a, dendritic cells, T lymphocytes

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66   **Introduction**

67   Among cancers of women in Southeast Asia, cervical cancer has the second-highest

68   incidence and is a common cause of deaths in low- and middle-income countries. There are

69   around 527,624 new cases of cervical cancer annually worldwide and 265,672 deaths. Early

70   sexual behavior and an increase in HPV infections has led to increasing incidence of cervical

71   cancer in younger women [1]. HPV infection causes genetic and epigenetic changes that can

72   promote cancer development. This virus is associated with over 90% of cervical cancer cases [2].

73   Currently, only a prophylactic vaccine is available for HPV infection: no effective

74   therapeutic vaccine has been developed [3, 4]. Immunotherapy is a strategy to promote the

75   therapeutic efficiency for HPV infection. Stimulating T lymphocytes and inducing differentiation

76   of non-protective $CD8^+$ T lymphocytes to cytotoxic T lymphocytes (CTLs) can destroy HPV-

77   infected cells and cancer cells. In addition, long-term $CD8^+$ T cells can recognize antigens and

78   can release cytotoxic molecules [5, 6]. Several mechanisms have been used to stimulate T

79   lymphocytes for vaccine development. One is stimulation by activated dendritic cells (DCs) [5].

80   Dendritic cells are antigen presenting cells (APCs) which present antigens to T lymphocytes

81   association with molecules of the major histocompatibility complex (MHC) class I and II.

82   Dendritic cells can stimulate both $CD4^+$ T lymphocytes and $CD8^+$ T lymphocytes [7]. Therefore,

83   the dendritic cells might be designed as the dendritic-based vaccine for HPV infection.

84   More than 180 types of HPV are known and can be divided into two groups: low-risk

85   HPV and high-risk HPV. HPV16, a high-risk HPV, is highly associated with various cancers [8].

86   Some proteins of HPV are immunogenic such as E2, E6, and E7. These are all "early" proteins,

87   expressed in an early stage of infection. The HPV E6 and E7 proteins are oncogenic, often

88   referred to as oncoproteins. These early proteins can stimulate the adaptive immune response,

89   but HPV can evade the immune system by modulating immune responses [9–11]. Thus, the

90 innate immune cells that play initial roles in stimulating the adaptive immune response are

91 important to study. HPV infection also results in over-expression of p16INK4a via the activity of

92 the HPV E7 oncoprotein [12]. The immune response to host p16INK4a and HPV E2 is induced

93 via specific CD8$^+$ cytotoxic T lymphocytes and antibody responses to virus antigens and

94 abnormal cells [13, 14]. Therefore, pulsing of dendritic cells with a combination of p16INK4a

95 and HPV16 E2 proteins might be a promising strategy for enhancing specific T-lymphocyte

96 responses against HPV infection.

97 Here, we aimed to develop human p16INK4a and HPV16 E2 proteins for stimulation of

98 dendritic cells, a link to the adaptive immune response, which enhances T-lymphocyte responses

99 against HPV-infected cells. The HPV16 E2 and p16INK4a proteins were expressed using a

100 bacterial system. The purified proteins were pulsed to immature monocyte-derived dendritic cells

101 (MDCs) and presented as antigens to autologous T lymphocytes. The efficiency of the HPV16

102 E2 and p16INK4a proteins was evaluated *in vitro* by examining their capacity to induce

103 immature MDCs to develop into mature MDCs and to enhance numbers of IFN-γ-producing

104 CD4$^+$ and CD8$^+$ T lymphocytes. The role of mitogen-activated protein kinase (MAPK) on

105 activation of MDCs was determined. Specific HLA blockades were used to confirm the type of

106 T-lymphocyte responses. This research reveals important information for development of

107 protein-based subunit vaccines for therapy of HPV-associated cancer.

Materials and methods

Preparation and purification of HPV16 E2 and p16INK4a proteins using *Escherichia coli* BL21 system

111   The vectors pTrcHisA-HPV16 E2 and pTrcHisA-p16INK4a were transformed using heat

112   shock into competent *Escherichia coli* BL21 cells and the resulting cells were used for protein

113   expression[15, 16]. The bacteria were cultivated for 24 hours in Luria-Bertani (LB) broth

114   containing 100 μg/ul of ampicillin at 37 °C. Expression of p16INK4a and HPV16 E2 proteins

115   was induced by adding isopropyl thiogalactoside (IPTG) (Thermo Scientific, MA, US). Proteins

116   were extracted using B-PERR Bacteria Protein Extraction Reagent (Thermo Scientific, MA, US).

117   The desired proteins were purified by using HisPur Cobalt Purification Kit (Thermo Scientific,

118   MA, US) and removed the endotoxin by using Pierce High Capacity Endotoxin Removal Resin

119   (Thermo Scientific, MA, US). Protein concentration was measured using Bradford's method

120   [17]. The identities of the purified proteins were confirmed by SDS-PAGE and western blot

121   analysis using anti-histidine antibody and anti-HPV16 E2 antibody [18].

122   Immunogenic epitope prediction of HPV16 E2 and p16INK4a

123   proteins for HLA class I and II binding

124   The common HLA class I and II molecules in the Thai population were identified at

125   http://www.allelefrequencies.net/. Possible binding epitopes of HLA class I and II for HPV16 E2

126   and p16INK4a proteins were predicted using two databases, SYFPEITHI and NetMHCpan

127   version 4.0 [19, 20]. The prediction was performed using 9-meric amino-acid residues for HLA

128   class I and 15-meric amino-acid residues for HLA class II epitopes, respectively.

129   Isolation of CD14$^+$ peripheral blood mononuclear cell (PBMC) and

130   cultivation of monocyte-derived dendritic cells (MDCs)

131   Peripheral blood was collected from 5 healthy volunteers who had given written consent.

132   The study was approved by the Human Ethics Research Committee of Khon Kaen University

133 (No. HE611307). The PBMC were separated by centrifugation with Lymphoprep (Stemcell

134 technologies, Oslo, Norway) [21]. CD14+ monocytes were isolated from PBMC with CD14

135 magnetic particles to which an anti-human CD14 monoclonal antibody was conjugated and using

136 the IMag separator (BD Biosciences, CA, USA). The 95% purity of CD14+ monocytes was

137 confirmed using flow cytometry. The CD14+ monocytes were cultured in RPMI-1640 medium

138 (Gibco, Thermo Fisher, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco,

139 Thermo Fisher, USA), 100 U/ml recombinant human GM-CSF (Peprotech, USA), 50 U/ml

140 recombinant human IL-4 (Peprotech, USA), 100 U/mL penicillin, and 100 µg/mL streptomycin

141 for 5-6 days [22–24]. This cultivation method enhanced the differentiation of monocytes into

142 immature MDCs. The CD14- cells remaining after CD14+ isolation were cultured in RPMI-1640

143 supplemented with 5% FBS, 5% autologous serum, recombinant human IL-2 (20 IU/mL)

144 (Peprotech, USA), 100 U/mL penicillin, and 100 µg/mL streptomycin for T-lymphocyte

145 proliferation [24].

146 **HPV16 E2 and p16INK4a proteins stimulate immature MDCs**

147 The immature MDCs were seeded into 96-well plates and stimulated to become mature

148 MDCs by using HPV16 E2 or p16INK4a at various concentrations; 0, 50, 100, 150, 250, and 500

149 ng/mL (added on day 5 and incubated for 48 hours). The stimulated MDCs were harvested,

150 washed and stained with FITC-HLA-DR, PE-CD80, APC-CD86 and PE-Cy7-CD83-tagged

151 monoclonal antibodies (BD Pharmingen, USA) and analyzed using flow cytometry (FACScanto

152 II, BD Biosciences, USA) [24].

153 **Evaluation the role of MAPK pathways on maturation and**

154 **activation of MDCs**

155   The role of MAPK pathways in the maturation of MDCs was determined by adding three

156   specific kinase inhibitors into immature MDCs at day 5. The specific kinase inhibitors were p38

157   MAPK inhibitor (SB203580, 50 μM), ERK inhibitor (PD98059, 50 μM), and JNK inhibitor

158   (SP600125, 20 μM). Two hours after addition of these inhibitors, HPV16 E2 and/or p16INK4a

159   were added and culture was continued for a further 48 hr. The cells were collected and examined

160   for CD83 expression by flow cytometry. The secretion of IL-12 and IL-1β was determined by

161   ELISA (Peprotech, USA) [24].

Specific protein-pulsed MDCs stimulate autologous T-lymphocyte

proliferation and IFN-γ secretion

164   Immature MDCs were seeded into 96-well U-bottom plates and cultured in RPMI-1640

165   containing 10% FBS, 100 U/ml rhGM-CSF, 50 U/ml rhIL-4, 100 U/mL penicillin, and 100

166   μg/mL streptomycin. The immature MDCs were further stimulated with HPV16 E2, p16INK4a,

167   or HPV16 E2/p16INK4a at the determined optimal concentration for 24 hr. The protein-pulsed

168   MDCs were washed and co-cultured with autologous T lymphocytes in the ratio of protein-

169   pulsed MDCs:T cells at 1:1, 1:5, and 1:10. The supernatants were harvested after 24 hr of co-

170   culture for determination of IFN-γ secretion by ELISA. The cells were harvested for staining

171   with FITC-CD3, APC-CD4, and PerCP-Cy5.5-CD8-tagged monoclonal antibodies (BD

172   Pharmingen, USA) and intracellular IFN-γ monoclonal antibodies (BD Pharmingen, USA) and

173   detected using flow cytometry [24].

Evaluation of HLA blockade on stimulation of T lymphocytes by

protein-pulsed MDCs

176   Immature MDCs were seeded into 96-well U-bottom plates and cultured in RPMI-1640

177   containing 10% FBS, 100 U/ml rhGM-CSF, 50 U/ml rhIL-4, 100 U/mL penicillin, and 100

178   μg/mL streptomycin. The immature MDCs were further stimulated with HPV16 E2, p16INK4a,

179   or HPV16 E2/p16INK4a at the determined optimal concentration for 24 hr. Then, protein-pulsed

180   MDCs were washed and blockade antibodies added. These were mouse anti-human HLA-ABC

181   monoclonal antibody (eBioscience, USA) and mouse anti-human HLA-DR, DP, DQ monoclonal

182   antibodies (BDPharmingen, USA) for 2 hr [25]. The protein-pulsed MDCs were washed and co-

183   cultured with autologous T lymphocytes in the ratio of protein-pulsed MDCs:T cells at 1:1, 1:5,

184   and 1:10. The supernatants were harvested after 24 hr of co-culture for determination of IFN-γ

185   secretion by ELISA. The cells were harvested for staining with FITC-CD3, APC-CD4, and

186   PerCP-Cy5.5-CD8-tagged monoclonal antibodies (BD Pharmingen, USA) and intracellular IFN-

187   γ monoclonal antibodies (BD Pharmingen, USA) and detected using flow cytometry [24, 25].

188   **Statistical analysis**

189   Experiments were performed independently three times. The data were analyzed using

190   analysis of variance (ANOVA) and differences among the experimental groups tested using

191   Tukey's multiple-comparison test implemented in Prism version 5. The results are shown as the

192   mean and standard deviation (SD). The symbols * and # indicate statistical significance, at $p <$

193   0.05, in comparisons with controls or between groups, respectively.

194

195   **Results**

196 **Frequency of common HLA class I and II alleles in the Thai**

197 **population and prediction of the immunogenic epitopes of HPV16**

198 **E2 and p16INK4a proteins**

199   Online database information about common HLA class I and class II alleles in Thailand

200 was based on data from 16,807 people. The three most common HLA class I alleles were HLA-

201 A*02, HLA-A*11, and HLA-A*24 with frequencies of 29.2, 27.7, and 17.3%, respectively. The

202 three most common HLA class II alleles were HLA-DRB1*15, HLA-DRB1*12, and HLA-

203 DRB1*04 with frequencies of 17.5, 16.9, and 14.4%, respectively (Table 1).

204   The prediction of HLA class I and class II epitopes for HPV16 E2 and p16INK4a

205 proteins was performed using two databases, the 9-meric amino-acid residues and 15-meric

206 amino-acid residues were predicted for HLA class I and class II alleles, respectively. The HPV16

207 E2 protein (365 amino-acid residues) has immunogenic epitopes for both types of HLA. Fig 1A

208 shows common immunogenic epitopes of HPV16 E2 protein for HLA class I alleles including

209 HLA-A*02:01, HLA-A*11:01, HLA-A*24:02, HLA-B*15:01, and HLA-B*40:01, and for HLA

210 class II alleles including

211 HLA-DRBI*04:01 and HLA-DRBI*15:01. Similarly, the p16INK4a protein (156 amino-acid

212 residues) also presents immunogenic epitopes for both types of HLA including HLA-A*02:01,

213 HLA-A*11:01, HLA-A*24:02, HLA-B*15:01, HLA-B*40:01, HLA-DRBI*04:01, and HLA-

214 DRBI*15:01 (Fig 1B). This suggests that these two proteins can be presented by antigen-

215 presenting cells via HLA class I and class II molecules.

216 **Production of HPV16 E2 and p16INK4a protein in *Escherichia coli***

217 **BL21**

218   HPV16 E2 and p16INK4a proteins expressed in *E. coli* BL21 were purified and eluted

219   twice. Identities of the proteins were confirmed by western blot using specific antibodies for

220   polyhistidine and HPV16 E2, respectively. We found the purified p16INK4a protein with a

221   polyhistidine tag was of the expected size, 18 kDa (Fig 1C). The purified HPV16 E2-

222   polyhistidine tagged protein was 43 kDa (Fig 1D). Therefore, these proteins were further used

223   for stimulating of MDCs and T lymphocytes.

224   HPV16 E2 and p16INK4a proteins induced phenotypic maturation

225   of MDCs

226   We determined the optimum concentrations of HPV16 E2 and p16INK4a proteins for

227   stimulating the maturation of MDCs. Evaluation of the expression level of CD83, which is a

228   marker of mature DCs, was done after stimulating CD14$^+$ monocytes with HPV16 E2 and/or

229   p16INK4a at various concentrations; 0, 50 100, 150, 250 and 500 ng/mL (treated on day 5 and

230   cultured for 48 hours). We observed that CD14$^+$ monocytes cultured with rhGM-CSF and rhIL-4

231   (Fig 2A) can be induced to develop from immature to mature dendritic morphology. Similarly,

232   immature dendritic cells were stimulated with HPV16 E2 and p16INK4a proteins resulting in

233   their differentiation into mature dendritic cells with more cytoplasmic protrusions (Fig 2B).

234   Both the percentage of positive cells and mean fluorescence intensity (MFI) of CD83$^+$

235   MDCs increased in a dose-dependent manner after stimulation with HPV16 E2 protein to peak at

236   a concentration of 250 ng/mL (Figs 2C and 2D). For p16INK4a, percentage of positive cells and

237   MFI of CD83$^+$ MDCs peaked at 150 ng/mL (Figs 2E and 2F). Therefore, the optimum

238   concentration of HPV16 E2 and p16INK4a proteins for further experiments were 250 ng/mL and

239   150 ng/mL, respectively.

HPV16 E2 and p16INK4a proteins stimulate the maturation and activation of MDCs via MAPK pathway

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242   To confirm whether HPV16 E2 and p16INK4a proteins stimulated immature MDCs via

243 the MAPK pathway, immature MDCs were treated with p38 MAPK, ERK and JNK inhibitors in

244 separate experiments prior to stimulation with the determined optimum concentrations of HPV16

245 E2 and/or p16INK4a. We found that the inhibitors significantly decreased the percentage of

246 CD83$^+$ MDCs in cultures stimulated by HPV16 E2 or p16INK4a proteins (Figs 3A and 3B).

247 Secretion levels of IL-1β did not significantly differ between all treatment groups (Fig 3C).

248 However, secretion of IL-1 was significantly lower in experiments where MAPK inhibitors

249 were administered in conjunction with p16INK4a relative to p16INK4a alone (Fig 3D). In

250 contrast, IL-12 levels were not suppressed when MAPK inhibitors were administered in

251 conjunction with HPV16E2 or HPV16 E2 together with p16INK4a (Fig 3D). These findings

252 suggest that the activation of MDCs by HPV16 E2 and/or p16INK4a proteins is via the MAPK

253 pathway.

MDCs pulsed with HPV16 E2 and p16INK4a stimulate CD4$^+$ and CD8$^+$ T lymphocytes to produce IFN-γ

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256   To determine the capability of MDCs to process immunogenic epitopes, the populations

257 of CD4$^+$ and CD8$^+$ T lymphocytes were examined. MDCs pulsed with HPV16 E2 and/or

258 p16INK4a were co-cultured with IL-2-cultured T lymphocytes in ratios of 1:1, 1:5, and 1:10.

259 Percentages of CD4$^+$ and CD8$^+$ T lymphocytes were not significantly different in all ratios (Figs

260 4A and 4B). Interestingly, the percentage of CD4$^+$ IFN-γ$^+$ T lymphocytes dramatically increased

261 ratio-wise after co-culture with MDCs pulsed with HPV16 E2 and/or p16INK4a (Fig 4C). The

262   percentage of CD8$^+$ IFN-γ^+ T lymphocytes did not significantly differ between treatments at

263   ratios of 1:5 and 1:10. However, at the ratio of 1:1, the percentage of these lymphocytes was

264   significantly greater when MDCs were pulsed with HPV16 E2 or p16INK4a relative to the

265   combination of HPV16 E2 and p16INK4a (Fig 4D). This indicates that HPV16 E2 might exhibit

266   higher immunogenicity than the combination of HPV16 E2 and p16INK4a. The activation of

267   these proteins can be triggered via CD4$^+$ or CD8$^+$ lymphocytes. We further confirmed the

268   secretion of IFN-γ using ELISA. Production of IFN-γ was higher in co-cultures where MDCs

269   had been pulsed with HPV16 E2 rather than p16INK4a, and varied according to the ratio. The

270   combination of HPV16 E2 and p16INK4a significantly increased the production of IFN-γ at

271   ratios of 1:5 and 1:10, while HPV16 E2 alone strongly induces the production of IFN-γ in a

272   ratio-dependent manner (Fig 4E). This suggests that the activation of T lymphocytes by HPV16

273   E2 and p16INK4a for IFN-γ production might be processed by CD4$^+$ and CD8$^+$ T lymphocytes.

CD4$^+$ and CD8$^+$ T lymphocytes co-response to combination of
274

HPV16 E2 and p16INK4a pulsed MDCs
275

276   To determine the effectiveness of HPV16 E2 and/or p16INK4a in stimulation of CD4$^+$ or

277   CD8$^+$ T lymphocytes, HLA blockade antibodies were specifically applied in co-culture

278   conditions at a ratio of 1:5. We found inhibition of IFN-γ production in both HLA class I and II

279   blockade in co-culture with MDCs pulsed with a combination of HPV16 E2 and p16INK4a (Fig

280   4F). This effect was not apparent when the MDCs were pulsed with either HPV16 E2 alone or

281   p16INK4a alone (Fig 4F). Suggesting that the stimulation with combination of HPV16 E2 and

282   p16INK4a clearly enhances response of both CD4$^+$ and CD8$^+$ T lymphocytes.

283

Discussion

284

285   Administration of protein subunits is a safe and effective strategy for HPV therapy by

286 stimulating immune responses [18]. The purpose of vaccine therapy against tumor or viral

287 infection is to stimulate T lymphocytes and differentiation of non-protective cells into functional

288 CTLs. The stimulation of T lymphocytes through dendritic cells, professional APCs, is an

289 efficient method [5]. Presentation of antigens by APCs to T lymphocytes occurs through the

290 HLA molecule: the HLA class II-bound peptide antigen is presented to $CD4^+$ T lymphocytes

291 whereas the HLA class I-bound peptide antigen is presented to $CD8^+$ T lymphocytes [7]. The

292 HPV16 E7 protein is a well-known antigen but has relatively low CTL epitopes which can only

293 be presented in association with HLA-A*33:03, an HLA class I allele [26]. The HPV16 E6

294 protein has a variety of epitopes that, in Chinese adolescents, can be presented in association

295 with HLA class II molecules such as HLA-DRB1 and HLA-DQB1 [27]. A number of HLA

296 alleles of both classes are common in Thailand (Table 1) and both HPV16 E2 and p16INK4a

297 proteins exhibit multiple epitopes that can be recognized by these alleles (Figs 1A and 1B).

298 HPV16 E2 and p16INK4a proteins are strong targets for stimulation of T lymphocytes via

299 dendritic cells.

300   The protein subunit was prepared using a popular bacterial system, *Escherichia coli*

301 BL21. This bacterial system capable provides the protein expression by regulating via T7

302 promoters which also used to produce the expression of HPV16 L1 [28] and HPV E7 [29].

303 Western blot indicated the size of our HPV16 E2-polyhistidine tagged protein to be 43 kDa (Fig

304 1C) a size comparable to that reported previously for HPV16 E2-GFP (62 kD, but including the

305 GFP of about 27 kDa) [30]. The size of our p16INK4a-polyhistidine tagged protein was 18 kDa

306 (Fig 1D), consistent with other reports.

307   The stimulation of dendritic cells by MtHSP70-FPR1 fusion protein, or Human

308   Immunodeficiency Virus (HIV)-1, can induce maturation of these by increasing the expression

309   of CD83, a marker for mature dendritic cells, and secretion of IL-1β and IL-12 [24, 31]. We

310   found that the HPV16 E2 and p16INK4a proteins also increase the expression of CD83 in MDCs

311   in a dose-dependent manner and induce cytoplasmic protrusions (Fig 2). Mature dendritic cells

312   play an important role as APCs to stimulate the differentiation of naïve T lymphocytes into

313   functional CTLs. The maturation of dendritic cells also increases their secretion of IL-1β and IL-

314   12, which are important in initiating T-lymphocyte responses and enhancing the immune

315   response. IL-12 stimulates the production of IFN-γ by T lymphocytes whereas IL-1β can

316   stimulate the growth of MDCs [24]. The MAPK-ERK signaling pathway controls the survival of

317   dendritic cells which can be stimulated by lipopolysaccharide (LPS) [32]. We found that HPV16

318   E2 and p16INK4a proteins stimulate the secretion of IL-1β and IL-12 which was partially

319   suppressed by MAPK inhibitors (Figs 3C and 3D). Interestingly, the MAPK inhibitors

320   significantly suppress the expression of CD83 in dendritic cells pulsed with HPV16 E2 or

321   p16INK4a (Figs 3A and 3B). These results suggest that the subunit proteins, HPV16 E2 and

322   p16INK4a, individually or in combination, stimulate the maturation of MDCs thus enhancing

323   their APC properties.

324   CTLs play an important role in cancer treatment based on immune responses. Among

325   other approaches, CTLs can be stimulated by tumor-specific antigens or tumor-associated

326   antigens. Tumor-specific antigens in the case of HPV infection include the non-structural

327   proteins HPV16 E7 and HPV18 E7, which can stimulate specific T lymphocytes via APCs [33].

328   The structural L1 protein of HPV16 and HPV18 has been used as prophylactic vaccine against

329   HPV in combination with adjuvant system 04 (AS04) and also stimulates the maturation of

330   dendritic cells, secretion of pro-inflammatory cytokines and stimulation of cytotoxic activity

331   against HPV-infected tumors [34]. There have also been reports about the use of a combination

332   of non-structural HPV16 E7 protein and PD-L1 blockade, an immune blockade, which exhibited

333   highly effective stimulation of dendritic cells *in vivo* and an effective CTL response [35]. A

334   modified vaccinia virus, Ankara virus encoding bovine papillomavirus E2 (MVA-E2), enhances

335   the elimination of CIN lesions in the cervix [36]. The MtHSP70-FPR1 fusion protein, a tumor-

336   associated antigen has recently been reported to stimulate dendritic cells through the p38 MAPK

337   signaling pathway and to promote the specific responses of CTLs to cervical cancer cells [24].

338   Moreover, a combination of tumor-specific antigens and tumor-associated antigens may also

339   stimulate immune response. An example is the DNA encoding HPV-16 E7/HSP70 fusion

340   protein, which can stimulate the specific antitumor responses in mice [37]. The fusion protein

341   HPV-16 E6/E7 and Fms-like tyrosine kinase-3 ligand also stimulates the response of CD8$^+$ T

342   cells specific to E6 and E7 [18]. However, a strategy to develop a therapeutic vaccine against

343   HPV infection is not well developed.

344   The tumor-specific antigen, HPV E2, is an early protein that controls the expression of

345   E6 and E7 oncoproteins and is more immunogenic than either of these oncoproteins [38]. The

346   tumor-associated antigen, human p16INK4a, is a protein that acts as a tumor suppressor and is

347   over-expressed in HPV infection [39]. Therefore, we considered HPV16 E2 and p16INK4a to be

348   important and interesting target proteins to stimulate the immune system of patients infected with

349   HPV. In this study, MDCs pulsed with HPV16 E2 and/or p16INK4a were able to stimulate

350   autologous T lymphocytes to proliferate and to secrete IFN-γ. The IFN-γ is produced by CD8$^+$ T

351   lymphocytes that eliminate infected cells and CD4$^+$ T lymphocytes that promote cell responses

352   through the presentation of HLA class I and II molecules [5, 7]. Particularly, the combination of

353 HPV16 E2 and p16INK4a proteins can promote greater T lymphocyte responses either protein

354 alone, which obviously stimulates via HLA class I and II (Figs 4E and 4F). The optimum

355 concentrations of the two proteins in combination, as used in this study to activate MDCs, were

356 250 ng/ml for HPV16 E2 and 150 ng/ml for p16INK4a. Combined subunit vaccines generally

357 use different concentrations of each antigen to elicit effective immune responses. An example is

358 the combined protein vaccine for diphtheria-tetanus-pertussis [40, 41]. Our study might provide a

359 strategy for development of HPV infection treatment using the optimum concentrations of

360 HPV16 E2 and p16INK4a proteins for stimulation of T lymphocytes.

361

Conclusions

363   In this study, we have evaluated immune-cell responses to HPV16 E2 and p16INK4a

364 proteins in blood from healthy volunteers. HPV E2 is a tumor-specific antigen and human

365 p16INK4a is a tumor-associated antigen that can stimulate MDCs cells through MAPK pathways

366 and increase the autologous T-lymphocyte response. The combination of HPV16 E2 and

367 p16INK4a proteins increases specific responses in both $CD4^+$ and $CD8^+$ T lymphocytes. Both

368 proteins can stimulate cell proliferation and IFN-γ secretion by $CD8^+$ T lymphocytes (that

369 eliminate infected cells) and $CD4^+$ T lymphocytes (that promote cell responses) through the

370 presentation of HLA class I and II molecules, respectively. We determined that the optimum

371 concentration of the proteins in combination was 250 ng/ml of HPV16 E2 and 150 ng/ml of

372 p16INK4a. This study provides strategies and optimal concentrations for developing therapies to

373 treat HPV infections using a combination of HPV16 E2 and p16INK4a proteins for stimulation

374 of T lymphocytes.

375

Acknowledgements

Conflict of interest statement

The authors declare no conflict of interest for this study.

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500

Figure Legends

501

502 **Figure 1.** Mapping of immunogenic epitopes and protein sizes of p16INK4a and HPV16 E2.

503 Possible immunogenic epitopes of (A) HPV16 E2 and (B) p16INK4a were mapped using two

504 databases, SYFPEITHI and NetMHCpan version 4.0. The prediction was performed by using 9-

505 meric amino-acid residues for HLA class I epitope binding and 15-meric amino-acid residues for

506 HLA class II, respectively. The protein sizes of (C) p16INK4a and (D) HPV16 E2 were checked

507 by western blot.

508

509 **Figure 2.** Stimulation of immature MDCs by HPV16 E2 and p16INK4a proteins. (A)

510 Characteristics of immature MDCs before stimulation. (B) Characteristics of mature MDCs after

511 stimulation by proteins. (C) Percentage and (D) Mean fluorescence intensity (MFI) of CD83$^+$

512 MDCs stimulated by HPV16 E2 protein at various concentrations. (E) Percentage and (F) MFI of

513 CD83$^+$ MDCs stimulated by p16INK4a protein at various concentrations. (*) p-value < 0.05

514 compared to control. (#) p-value < 0.05 compared between groups.

515

516 **Figure 3.** Expression of CD83 and cytokine production by MDCs stimulated by HPV16 E2

517 and/or p16INK4a proteins. (A) The expression of CD83 stimulated by HPV16 E2 and (B)

518 p16INK4a after treatment with p38 MAPK inhibitor (SB203580), ERK inhibitor (PD98059), or

519 JNK (SP600125), examined using flow cytometry. (C) The production of IL-1β (pg/mL) and (D)

520 IL-12 after blocking of MDCs with SB203580, PD98059 or SP600125, then stimulated by

521 HPV16 E2 and/or p16INK4a proteins. (*) p-value < 0.05 compared to control. (#) p-value < 0.05

522 compared between groups.

523

524   **Figure 4.** MDCs pulsed with HPV16 E2 and/or p16INK4a stimulate $CD4^+$ and $CD8^+$ T

525   lymphocytes. (A) The percentage of $CD4^+$ T lymphocytes and (B) $CD8^+$ T lymphocytes in co-

526   cultures with MDCs pulsed with HPV16 E2 and/or p16INK4a at various ratios. (C) The

527   percentage of $IFN\text{-}\gamma^+CD4^+$ T lymphocytes and (D) $IFN\text{-}\gamma^+CD8^+$ T lymphocytes in co-cultures

528   with MDCs pulsed with HPV16 E2 and/or p16INK4a at various ratios. (E) The concentration of

529     $IFN\text{-}\gamma$ (pg/mL) after co-culture of T lymphocytes with MDCs pulsed with HPV16 E2 and/or

530   p16INK4a at various ratios. (F) The concentration of $IFN\text{-}\gamma$ (pg/mL) after blockade with HLA

531   class I and II antibodies and co-culture of T lymphocytes and MDCs pulsed with HPV16 E2

532   and/or p16INK4a at a ratio of 1: 5. (*) p-value < 0.05 compared to control. (#) p-value < 0.05

533   compared between groups.

534

535   # Table

536   **Table 1.** The table showed % frequency of HLA class I and II alleles that are commonly found

537   in the Thai population.

538

Table 1(on next page)

Table 1. The table showed % frequency of HLA class I and II alleles that are commonly found in the Thai population.

PeerJ

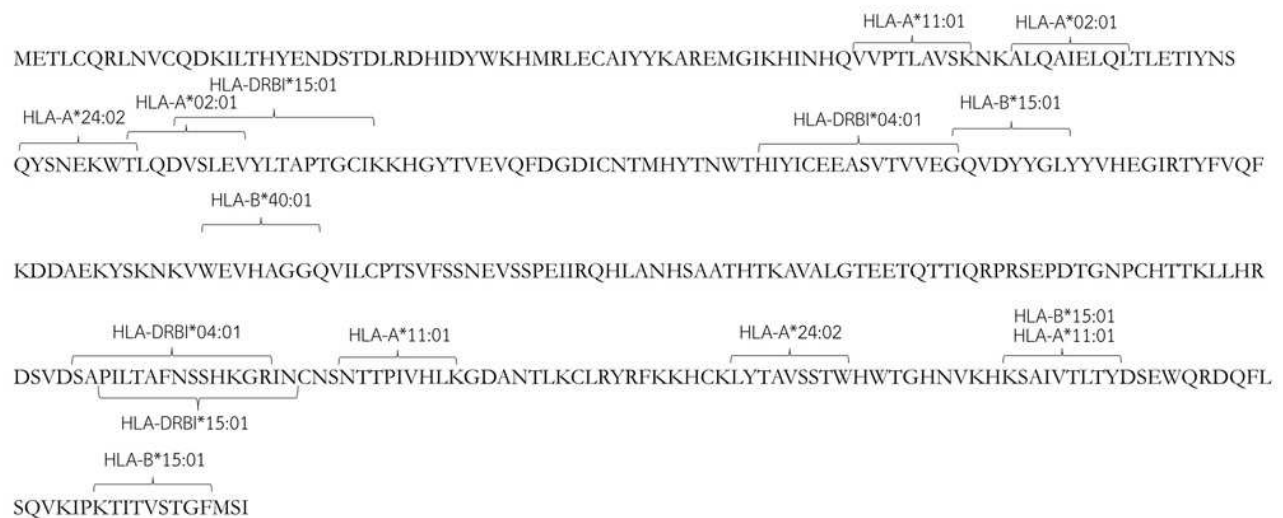1 **Table 1.** The frequency of common HLA class I and II alleles in Thai populations

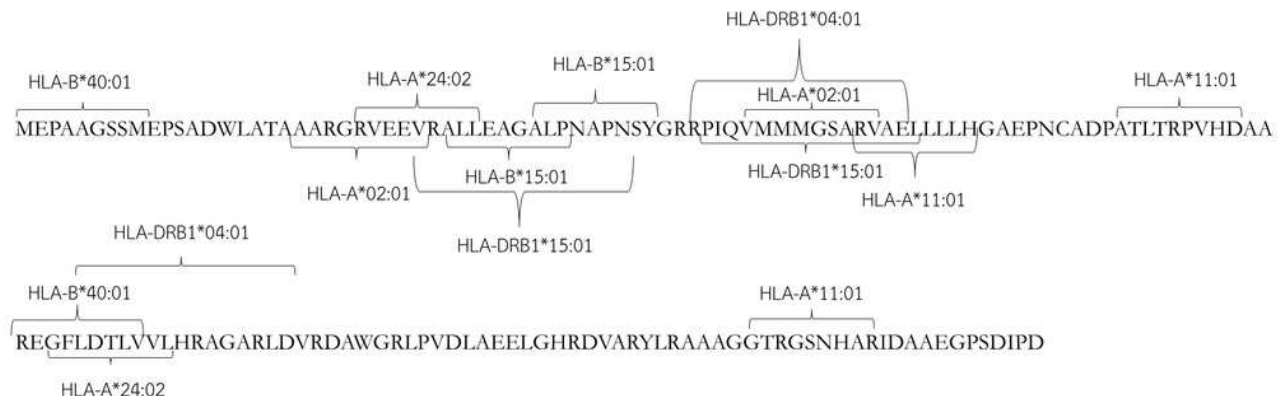| HLA class I		HLA class II	
Allele	**% Allele Frequency**	**Allele**	**% Allele Frequency**
HLA-A*02	29.2	HLA-DRB1*04	14.4
HLA-A*11	27.7	HLA-DRB1*09	11.5
HLA-A*24	17.3	HLA-DRB1*12	16.9
HLA-A*33	13.8	HLA-DRB1*15	17.5
HLA-B*15	14.8		
HLA-B*40	13.4		
HLA-B*46	13.2		

2

3

Figure 1

Figure 1. Mapping of immunogenic epitopes and protein sizes of p16INK4a and HPV16 E2.

Figure 1. Mapping of immunogenic epitopes and protein sizes of p16INK4a and HPV16 E2. Possible immunogenic epitopes of (A) HPV16 E2 and (B) p16INK4a were mapped using two databases, SYFPEITHI and NetMHCpan version 4.0. The prediction was performed by using 9-meric amino-acid residues for HLA class I epitope binding and 15-meric amino-acid residues for HLA class II, respectively. The protein sizes of (C) p16INK4a and (D) HPV16 E2 were checked by western blot.
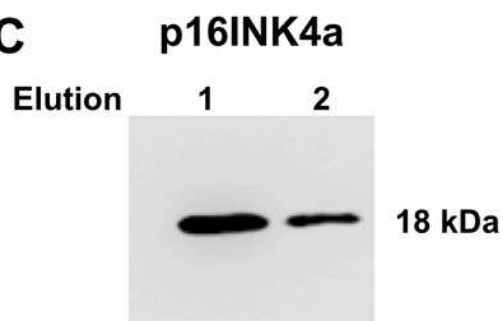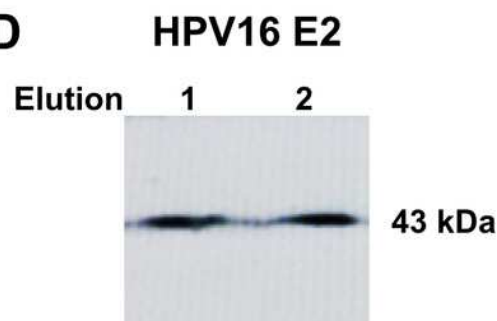
A

HLA-A*11:01   HLA-A*02:01

METLCQRLNVCQDKILTHYENDSTDLRDHIDYWKHMRLECAIYYKAREMGIKHINHQVVPTLAVSKNKALQAIELQLTLETIYNS

HLA-A*24:02   HLA-A*02:01   HLA-DRBl*15:01   HLA-DRBl*04:01   HLA-B*15:01

QYSNEKWTLQDVSLEVYLTAPTGCIKKHGYTVEVQFDGDICNTMHYTNWTHIYICEEASVTVVEGQVDYYGLYYVHEGIRTYFVQF

HLA-B*40:01

KDDAEKYSKNKVWEVHAGGQVILCPTSVFSSNEVSSPEIIRQHLANHSAATHTKAVALGTEETQTTIQRPRSEPDTGNPCHTTKLLHR

HLA-DRBl*04:01   HLA-A*11:01   HLA-A*24:02   HLA-B*15:01
                                                                              HLA-A*11:01

DSVDSAPILTAFNSSHKGRINCNSNTTPIVHLKGDANTLKCLRYRFKKHCKLYTAVSSTWHWTGHNVKHKSAIVTLTYDSEWQRDQFL

HLA-DRBl*15:01

HLA-B*15:01

SQVKIPKTITVSTGFMSI

B

HLA-DRB1*04:01

HLA-B*40:01   HLA-A*24:02   HLA-B*15:01   HLA-A*02:01   HLA-A*11:01

MEPAAGSSMEPSADWLATAAARGRVEEVRALLEAGALPNAPNSYGRRPIQVMMMGSARVAELLLLHGAEPNCADPATLTRPVHDAA

                    HLA-A*02:01   HLA-B*15:01   HLA-DRB1*15:01
                                                                              HLA-A*11:01

                              HLA-DRB1*15:01

HLA-DRB1*04:01

HLA-B*40:01   HLA-A*11:01

REGFLDTLVVLHRAGARLDVRDAWGRLPVDLAEELGHRDVARYLRAAAGGTRGSNHARIDAAEGPSDIPD

HLA-A*24:02

C   **p16INK4a**

Elution   1   2

18 kDa

D   **HPV16 E2**

Elution   1   2

43 kDa

Figure 2

Figure 2. Stimulation of immature MDCs by HPV16 E2 and p16INK4a proteins.

Figure 2. Stimulation of immature MDCs by HPV16 E2 and p16INK4a proteins. (A) Characteristics of immature MDCs before stimulation. (B) Characteristics of mature MDCs after stimulation by proteins. (C) Percentage and (D) Mean fluorescence intensity (MFI) of CD83$^+$ MDCs stimulated by HPV16 E2 protein at various concentrations. (E) Percentage and (F) MFI of CD83$^+$ MDCs stimulated by p16INK4a protein at various concentrations. (*) p-value < 0.05 compared to control. (#) p-value < 0.05 compared between groups.
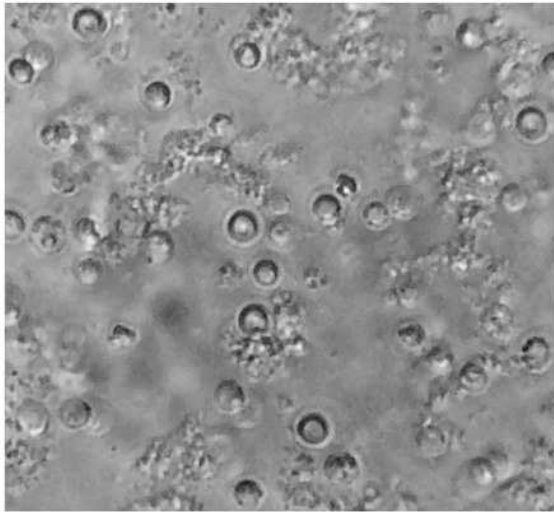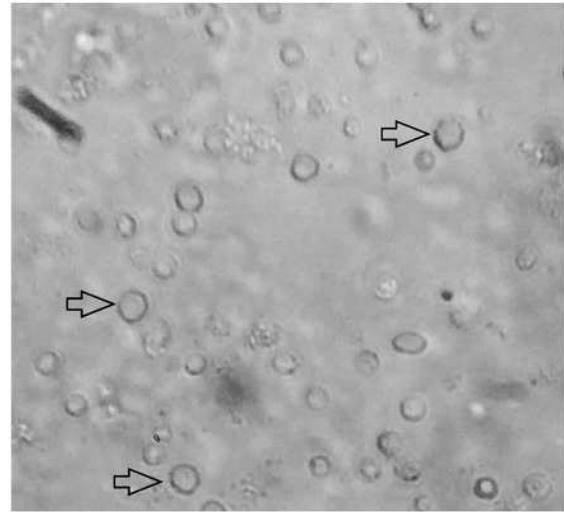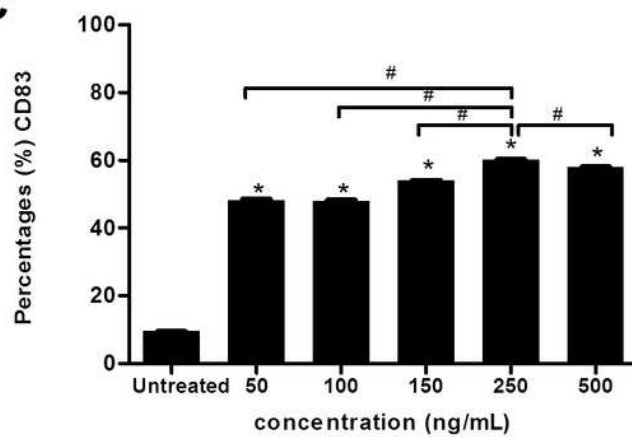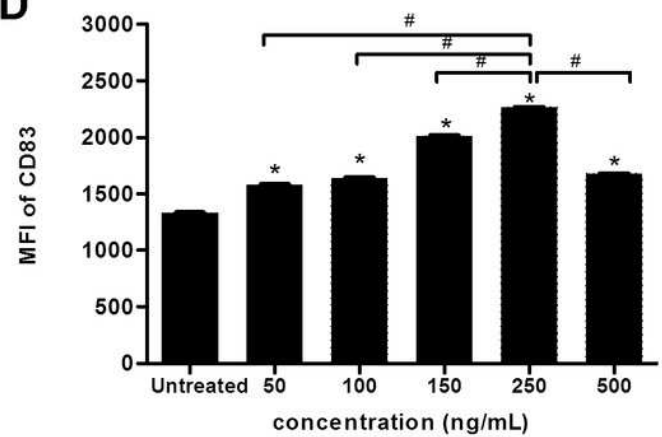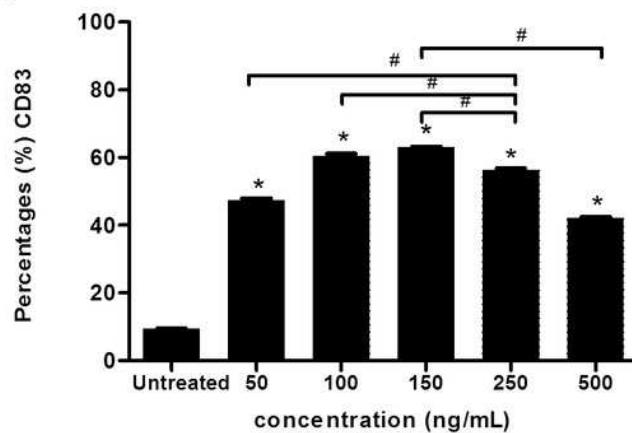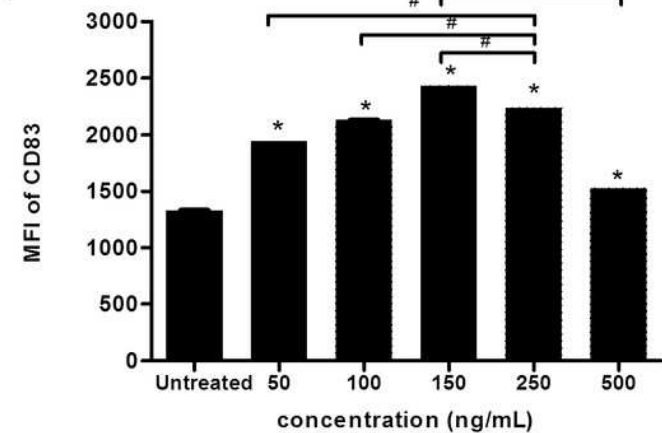
Figure 3

Figure 3. Expression of CD83 and cytokine production by MDCs stimulated by HPV16 E2 and/or p16INK4a proteins.

Figure 3. Expression of CD83 and cytokine production by MDCs stimulated by HPV16 E2 and/or p16INK4a proteins. (A) The expression of CD83 stimulated by HPV16 E2 and (B) p16INK4a after treatment with p38 MAPK inhibitor (SB203580), ERK inhibitor (PD98059), or JNK (SP600125), examined using flow cytometry. (C) The production of IL-1β (pg/mL) and (D) IL-12 after blocking of MDCs with SB203580, PD98059 or SP600125, then stimulated by HPV16 E2 and/or p16INK4a proteins. (*) p-value < 0.05 compared to control. (#) p-value < 0.05 compared between groups.
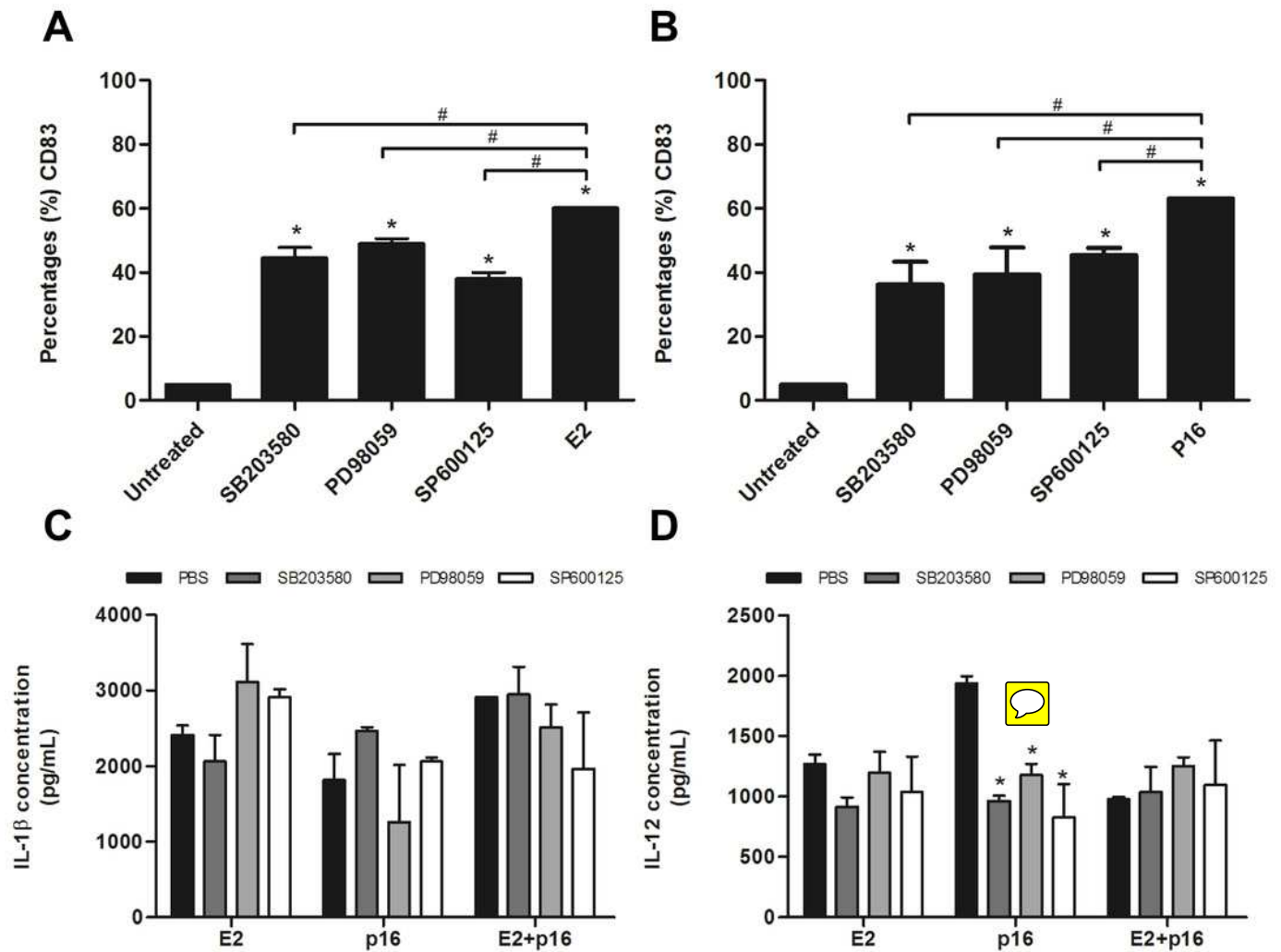
Figure 4

Figure 4. MDCs pulsed with HPV16 E2 and/or p16INK4a stimulate CD4$^+$ and CD8$^+$ T lymphocytes.

Figure 4. MDCs pulsed with HPV16 E2 and/or p16INK4a stimulate CD4$^+$ and CD8$^+$ T lymphocytes. (A) The percentage of CD4$^+$ T lymphocytes and (B) CD8$^+$ T lymphocytes in co-cultures with MDCs pulsed with HPV16 E2 and/or p16INK4a at various ratios. (C) The percentage of IFN-γ$^+$CD4$^+$ T lymphocytes and (D) IFN-γ$^+$CD8$^+$ T lymphocytes in co-cultures with MDCs pulsed with HPV16 E2 and/or p16INK4a at various ratios. (E) The concentration of IFN-γ (pg/mL) after co-culture of T lymphocytes with MDCs pulsed with HPV16 E2 and/or p16INK4a at various ratios. (F) The concentration of IFN-γ (pg/mL) after blockade with HLA class I and II antibodies and co-culture of T lymphocytes and MDCs pulsed with HPV16 E2 and/or p16INK4a at a ratio of 1: 5. (*) p-value < 0.05 compared to control. (#) p-value < 0.05 compared between groups.