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Fetal exposure to oncoantigen elicited antigen-specific adaptive immunity against tumorigenesis

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ABSTRACT

Background Envisioned as a similar process to tumorigenesis in terms of biological behaviors and molecular basis, embryogenesis necessitates an immune surveillance system to eliminate erratically transformed cells. Our previous study demonstrated that fetal macrophage-like phagocytes triggered Th2-skewed immunity following endocytosing prenatally administered ovalbumin to facilitate postnatal allergic airway responses, highlighting the critical role fetal phagocytes played in dealing with antigens present in developing fetuses and shaping subsequent immune responses. It prompted us to examine whether fetuses could mount Th1 tumoricidal immunity against tumorigenesis following in utero exposure to tumor antigens.

Methods Gestational day 14 murine fetuses underwent in utero injection of Th1-promoting human papilloma virus (HPV) E7 peptides. Postnatally, recipients were examined for immunological consequences and the resistance to TC-1 tumorigenesis.

Results Fetal exposure to HPV E7 did not cause tolerance but rather immunization in the recipients, characterized by proinflammatory Th1 polarization of their lymphocytes. Fetal macrophage-like phagocytes were responsible for taking up HPV E7 and triggering HPV E7-specific T-cell cytotoxicity and humoral immunity that rendered recipients resistant to TC-1 tumorigenesis in postnatal life. Adoptive transfer of HPV E7-loaded fetal phagocytes also elicited Th1 immunity with rapid expansion of HPV E7-specific cytotoxic $CD8^+$ T-cell clones in response to TC-1 cell challenge so as to protect the recipients from TC-1 tumorigenesis, but failed to completely eliminate pre-existing TC-1 cells despite perceptible attenuation of local TC-1 tumor growth.

Conclusions Our study revealed that Th2-biasing fetus was not immune-privileged to foreign peptides, but competent to mount Th1 cytotoxic immunity and generate immunoglobulins against tumorigenesis following in utero exposure to Th1-promoting oncoantigen. It shed light on the role of fetal macrophage-like phagocytes in bridging toward tumor antigen-specific cellular and humoral immunity potentially as an immune surveillance system to eliminate transformed cells that might be egressing during embryogenesis and leftover until postnatal life.

INTRODUCTION

Given the similarities in biological behaviors and molecular basis between embryogenesis and tumorigenesis,[1] embryo development can be envisioned as a process of tumor initiation and progression. Developing cells maintain a fine balance between division and differentiation to properly generate enough cells and perform specified functions in the embryo. Within the normal repertoire of embryogenesis, there must exist developing cells that differentiate improperly, potentially turn into uncontrolled growth and obtain malignant transformation.[2] At the early stage of cell transformation, the microenvironment provides a physical barrier against tumorigenesis.[3] Immune cells including lymphocytes, natural killer (NK) cells or macrophages actively contribute to the tumor microenvironment[4] as a surveillance system to eliminate transformed tumor cells. However, their functionality has to take into consideration the ontogeny of individual cells types. T-cell immunity is an important and sophisticated component of tumor immune surveillance, whereas T-cells mature late during fetal development and functionally depend on antigen presenting cells. NK cells destroy cancer cells with decreased major histocompatibility complex class I (MHC-I) expression known as a condition of 'missing self'.[5] However, their development lags far behind that of B or T-cells.[6] Macrophages emerge early on gestational day 9.5 – 10.5 ($GD_{9.5\text{-}10.5}$) in mice[7 8] and week 5 in humans[9] as the first immune cells capable of surveying their surroundings and eliminating nonself antigens, particles or dead cells in fetal life.[7 10] They might play a role in tumor immune surveillance during embryo development.

In our previous studies, prenatal exposure to ovalbumin (OVA) came to the development of Th2-skewed immunity.[9] This event could be attributed to fetal macrophage-like phagocytes (MPs) that sequestered the endocytosed OVA and differentiated toward dendritic cells to later instruct T-cells as they

fully developed to more efficiently deal with antigens. It implied that MPs were functionally important for shaping fetal immune responses. As we know, pregnancy is characterized by Th2 immune biasing that downregulates Th1 immunity to benefit pregnancy maintenance.[11] Thus, it was tempting to rationalize the induction of Th2 immunity following fetal exposure to OVA. However, whether fetuses are able to mount Th1 tumoricidal immunity is crucial to their capability of eliminating transformed cells derived from embryogenesis. In this study, murine fetus was exposed to Th1-promoting human papilloma virus (HPV) E7 peptides.[12 13] Fetal MPs endocytosed HPV E7 and triggered E7-specific humoral and Th1 cellular immunity to preclude TC-1 tumorigenesis in postnatal life. Our results unveiled the capacity of fetal MPs to shape fetal immune deviations relying on the antigen nature and prevent developmental tumorigenesis following the ingestion of tumor antigens.

MATERIALS AND METHODS

Mouse husbandry

Inbred FVB/N and C57BL/6 mice were purchased from National Laboratory Animal Center (Taipei, Taiwan) at the age of 6–8 weeks, and housed in the Animal Care Facility at Chang Gung Memorial Hospital (CGMH) under the standard guidelines from 'Guide for the Care and Use of Laboratory Animals' and with the approval of the CGMH Committee on Animal Research.

In utero injection of HPV E7 peptides

FVB/N or C57BL/6 females were caged with males of the same strain in the afternoon and checked for vaginal plugs the following morning. The day when the plug was observed was designated as day 0 of the pregnancy. Under anesthesia with ketamine (100 mg/kg) and xylazine (10 mg/kg), the uteri of GD_{14} pregnant mice were exposed through a vertical laparotomy. A 60 µm glass micropipette with beveled tip was used to inject 0.125, 0.25 and 0.5 µg endotoxin-free HPV E7 peptides (full-length sequence of 98 amino acids: MHGDTPTLHE YMLDLQPETT DLYCYEQLND SSEEEDEIDG PAGQAEPDRA HYNIVTFCCK CDSTLRLCVQ STHVDIRTLE DLLMGTLGIV CPICSQKP, MyBioSource, San Diego, California, USA) in 5 µL saline into peritoneal cavities of all fetuses at a litter via transuterine approach. Then, the abdomen of the pregnant mice was closed in two layers of 5–0 silk suture. After the operation, all mice were housed in an undisturbed room without bedding changes until the pups were 1 week old. Pups were weaned at 3 weeks of age. Control mice received saline injection at the same gestation age.

Intracellular staining of endocytosed HPV E7

Cells from GD_{14} fetal liver or peritoneum[9] were pulsed with HPV E7 peptides (25 µg/2 $\times 10^7$ cells) overnight. After vigorous washes with phosphate buffered saline (PBS), cells were fixed with cold methanol and permeabilized with 0.5% Tween-20 for 30 min. Then, they were subjected to the treatment of mouse anti-E7 monoclonal IgG_1 (ED17, Santa Cruz Biotechnology), followed by fluorophore-conjugated anti-mouse IgG antibody and 4',6-diamidino-2-phenylindole (DAPI) nuclear counterstain. Finally, cells were analyzed by flow-cytometry and cytospun on slides for confocal microscopic examinations.

Immunophenotyping of fetal MPs using flow cytometry

Cells from GD_{14} fetal liver or peritoneum were adjusted at a concentration of 4 $\times 10^6$ cells/mL RPMI 1640 medium and incubated with foreign antigen of 20 µg Alexa Fluor 647-labeled OVA (OVA-647, Molecular Probes) overnight. After being washed vigorously with PBS, cells were then stained with fluorophore-conjugated anti-CD45, F4/80, CD11b and Gr1 monoclonal antibodies (eBioscience). Samples were acquired by BD FACSCanto II flow cytometry system. To the exclusion of dead cells using propidium iodide staining, fetal MPs defined as OVA-laden cells were analyzed by BD FACSDiva software.

Adoptive transfer of HPV E7-loaded fetal MPs

Pooled GD_{14} fetal cells, collected from peritoneum by saline lavage and dissociated livers by passage through 70 µm cell strainers,[9] were subjected to Ficoll density gradient centrifugation for the enrichment of fetal MPs. Enriched MPs (>80%, 5×10^6 cells/mL) were pulsed with HPV E7 peptides (25 µg/2×10^7 cells) overnight in RPMI 1640 medium and washed vigorously with PBS. Cells were intraperitoneally injected into adult FVB/N or C57BL/6 mice (8–12 weeks old) at a dose of 2×10^7 cells/mouse in 200 µL saline. Control mice were injected with fetal MPs maintained overnight in HPV E7-free medium. Four to 6 weeks later, recipients were examined for serum anti-E7 IgG_1 levels, T-cell polarization and immune response to HPV E7. Additionally, C57BL/6 recipients were examined for HPV E7-specific cytotoxic $CD8^+$ T-cells in peripheral blood and spleen 1 week after intraperitoneal challenge of 10^5 TC-1 cells. Leukocytes enriched by Ficoll density gradient centrifugation were stained using biotin-labeled Pro5® MHC Class I pentamers of H-$2D^b$/RAHYNIVTF (HPV16 E7 49–57, Peptide code: 502H, ProImmune) in combination with fluorophore-conjugated streptavidin and anti-CD8α monoclonal antibodies (eBioscience). Cells were acquired and analyzed by flow cytometry.

Examinations of anti-E7 IgG_1

Within 5–7 weeks after in utero HPV E7 injection or 4 weeks after adoptive transfer of HPV E7 loaded-fetal MPs, the recipients were subjected to blood sampling for measuring serum anti-E7 IgG_1 levels. ELISA microtiter plates were coated with 100 ng/mL HPV E7 peptides overnight, blocked with 3% bovine serum albumin (BSA, Sigma) in PBS and incubated with 100 µL of diluted samples at room temperature for 60 min. After washing, biotinylated anti-mouse IgG_1 (RMG1-1, BioLegend) was added in each well, followed by streptavidin-horseradish peroxidase (Sigma). The reaction was developed by

adding 100 µL NeA-blue tetramethylbenzidine substrate (Clinical Science Products) and stopped with 2 M H_2SO_4. The optical density at 450 nm was measured in an automatic ELISA reader. Serum anti-E7 IgG_1 levels were determined by the standard curve of mouse anti-E7 IgG_1 mAb (ED17, Santa Cruz Biotechnology).

Activation and proliferation of lymphocytes in response to HPV E7

Splenic lymphocytes were enriched by density gradient centrifugation and then cultured in triplicate each with 2×10^5 cells in 200 µL RPMI 1640 medium containing 10% fetal calf serum in 96-well plates. Responder lymphocytes were stimulated with HPV E7 (100 ng/mL), BSA (100 ng/mL) or Con-A (1 µg/mL). IL2 secretion marks a critical landmark in the process of T-cell activation by antigen stimulation and a T-cell commitment to further proliferation and differentiation programs.[14] On day 3, T-cell activation was quantified by monitoring the release of IL2 within culture supernatants using ELISA.[15 16] For the measurement of lymphocyte proliferation, day 5 cells were first subjected to 16 hours incubation with tritiated thymidine (ICN Biomedicals) at a final concentration of 1 µCi per well and then harvested for counting incorporated tritium in a liquid scintillation counter (1450 Microbeta Plus counter). Lymphocyte proliferation was determined by the readout of incorporated tritium as counts per minute.

Examination of lymphocyte polarization by cytokines of IL-4, IL-5 and IFNγ

Enriched splenic lymphocytes were cultured in triplicate at a density of 5×10^6 cells/mL in 500 µL RPMI 1640 with 10% fetal calf serum under the stimulation of HPV E7 (100 ng/mL). Cytokines of IL-4, IL-5 and IFNγ in supernatants of 5-day cell cultures were quantified using ELISA assay kits according to the manufacturer's protocols (BioLegend, San Diego, California, USA).

In vitro cytotoxic T lymphocyte assay

Splenic lymphocytes of C57BL/6 recipients were harvested by density gradient centrifugation 4 weeks after adoptive transfer of C57BL/6 fetal MPs with or without HPV E7 loading. The lymphocytes were restimulated with HPV E7 (1 µg/2×10^7 cells) for 24 hours in RPMI 1640. Then, 2×10^5 lymphocytes were transferred to 96-well culture plates containing 4×10^4 viable TC-1 cells in 100 µL phenol-red free RPMI medium per well. After overnight incubation, cell-mediated cytotoxicity to TC-1 cells was examined under a fluorescence microscope following the treatment of DAPI viability dye and then further quantified by the supernatant lactate dehydrogenase (LDH) released from lysed cells using the CytoTox 96® Non-Radioactive Cytotoxicity Assay kit (Promega). The percentage of specific release of LDH was determined by the following equation of (experimental release – spontaneous T-cell release – spontaneous TC-1 cell release)/(maximal TC-1 cell release – spontaneous TC-1 cell release) × 100%.

Assessment for the resistance to TC-1 tumor tumorigenesis

C57BL/6 recipients were subjected to subcutaneous 10^5 TC-1 cell inoculation on their backs or abdominal walls 6 weeks after birth (HPV E7 fetal recipients) or 4 weeks following cellular transfer of HPV E7-loaded fetal MPs. TC-1 tumor growth was monitored in two perpendicular directions of the long dimension (length) and the short dimension (width) using a vernier caliper once per 2–3 days for 1 month and then weekly till excessive tumor burden (>3 cm). Tumor volumes were calculated according to the formula: $(\text{length}\times\text{width}^2)/2$.[17] The data were then used to plot a graph of tumor growth kinetics. As for survival analyses, the survival time of mice was defined by estimating the length of time from the date of TC-1 tumor cell inoculation to the date of recipients' death. Cases were considered as censored if the animals remained alive at the end (day 63) of the survival studies or were ethically euthanized before day 63 due to excessive tumor burden (>3 cm). At sacrifice, recipients that were cutaneous tumor-free would undergo histological examinations of the lung, liver and spleen following H&E staining to rule out TC-1 metastatic spread. Cutaneous TC-1 tumors, if available in the recipients, were also harvested for histological examinations at the end of the study. Controls were mice subjected to in utero saline injection or the injection of fetal MPs without HPV E7 pulse.

Alternatively, C57BL/6 mice were first subcutaneously inoculated with 10^5 TC-1 cells. Two or 4 days later, mice were subjected to the adoptive transfer of 2×10^7 HPV E7-pulsed GD_{14} C57BL/6 fetal MPs as the strategy of cellular therapy against pre-existing TC-1 tumor cells. Tumor growth and recipients' survivals were monitored as described above.

Histological examination of macrophages by immunofluorescence staining

Sections of paraffin-embedded tumor specimens were deparaffinized, rehydrated in xylene and a series of graded ethanol. Sections were subjected to antigen retrieval with citrate buffer (pH 6.0) and then incubated with fluorophore-conjugated anti-F4/80 (1 :25, RM8, eBioscience) and anti-CD11c (1:50, N418, eBioscience) monoclonal antibodies. Visualization of the nuclei was achieved by Hoechst 33342 staining (1: 20,000, Invitrogen). Sections were mounted with Dako fluorescence mounting medium. Images were taken using a confocal microscope.

Statistical analyses

The equality of means was examined by Student's *t*-test between two independent or paired groups, or one-way analysis of variance among three or more groups with post hoc multiple comparisons by Fisher's least significant difference. Plots of survival time were constructed

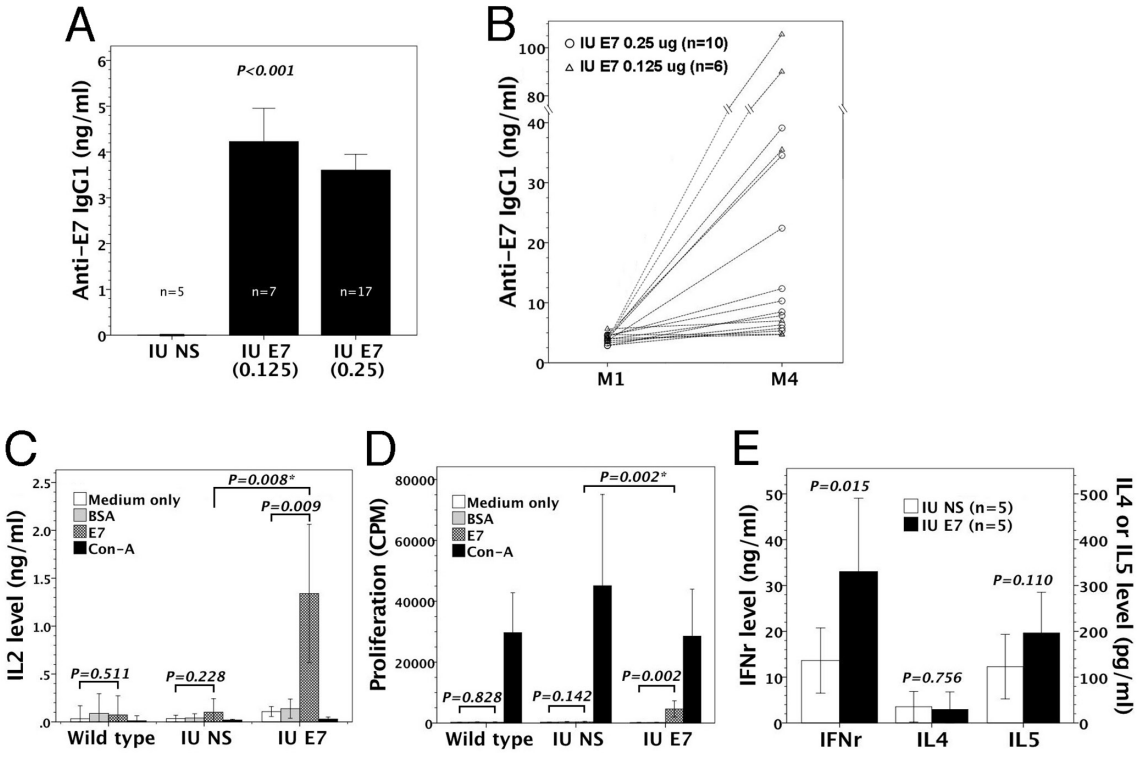

Figure 1 Th1 immunization following in utero exposure to HPV E7. GD_{14} FVB/N fetuses were subjected to intraperitoneal injection of HPV E7 peptides (IU E7) or saline (IU NS). Postnatally, recipients were examined for serum anti-E7 IgG_1 levels by ELISA at 1 month old (M1). (A) In utero injection of 0.125 (IU E7 0.125) and 0.25 µg HPV E7 (IU E7 0.25) caused the secretion of anti-E7 IgG_1 ($p<0.001$, ANOVA), as compared with in utero saline injection (IU NS). It made no difference in anti-E7 IgG_1 levels between IU E7 0.125 and IU E7 0.25. The bar data are shown as 95% CIs for the means. (B) Rechecked at the age of 4 months (M4), serum anti-E7 IgG_1 was significantly higher than that at M1 ($p=0.016$, paired comparison, *t*-test). Coupled circles or triangles represent paired data of anti-E7 IgG_1 levels at M1 and M4 from an individual mouse. (C) Splenic lymphocytes of recipients were examined for their activation, proliferation and polarization in culture systems under HPV E7 stimulation. Medium only was used as background controls, BSA as third-party stimulators and Con-A as a mitogen to stimulate T-cell population. T-cell activation was measured by IL2 secretion (n=5). IU E7 significantly secreted IL2 specifically in response to HPV E7, whereas wild type mice and IU NS failed to generate IL2 under HPV E7 stimulation. (D) Lymphocyte proliferation was measured by the readout of incorporated tritium (n=4) as CPM. The patterns of lymphocyte proliferation was similar to those of IL2 secretion except for the Con-A stimulus that elicited a vigorous proliferation of lymphocytes in sharp contrast to the background levels of IL2 secretion. It could be ascribed to the fact that the exaggerated T-cell proliferation responses by Con-A bypassed antigen presenting cell-mediated antigen recognition. All bar data are shown as 95% CIs for the means. All p values were obtained from ANOVA multiple comparisons except for p* that derived from *t*-tests between IU E7 and IU NS for IL2 secretion and lymphocyte proliferation under HPV E7 stimulation. (E) Lymphocyte polarization was examined by IFNγ, IL4 and IL5 secretions in lymphocyte cultures under HPV E7 stimulation. IU E7 compared favorably in the levels of IFNγ but not IL4 and IL5 with IU NS, indicating a Th1-skewed phenotype. All p values derived from *t*-tests. CI, confidence interval; ANOVA, analysis of variance; CPM, counts per minute; HPV, human papilloma virus.

by Kaplan-Meier method. The log-rank test was employed to compare survival curves. Differences were regarded as significant in all tests at $p<0.05$.

RESULTS

Immunological consequences after in utero exposure to HPV E7

GD_{14} FVB/N murine fetuses were subjected to intraperitoneal injection of 0.125, 0.25 or 0.5 µg endotoxin-free HPV E7. None of fetal recipients survived the dose of 0.5 µg HPV E7. Serum anti-E7 IgG_1 was first examined at the age of 4–6 weeks and significantly generated for the doses of 0.125 and 0.25 µg (figure 1A). However, its levels did not differ between the two doses. At follow-up of 4 months old, serum anti-E7 IgG_1 persisted and even notably increased (figure 1B). The results supported the induction of B-cell humoral immunity following in utero HPV E7 exposure.

In HPV E7 fetal recipients, their lymphocyte responses to HPV E7 were further evaluated in vitro at 6–8 weeks old. The activation and proliferation of recipient lymphocytes were quantified by IL2 secretion (figure 1C) and tritium incorporation (figure 1D) in cell culture systems under HPV E7 and third-party antigen stimulation. Recipient lymphocytes significantly secreted IL2 and proliferated specifically in response to HPV E7 stimulation as compared with wild type mice and saline controls. It supported the occurrence of T-cell immunization to HPV E7 antigen following in utero HPV E7 exposure.

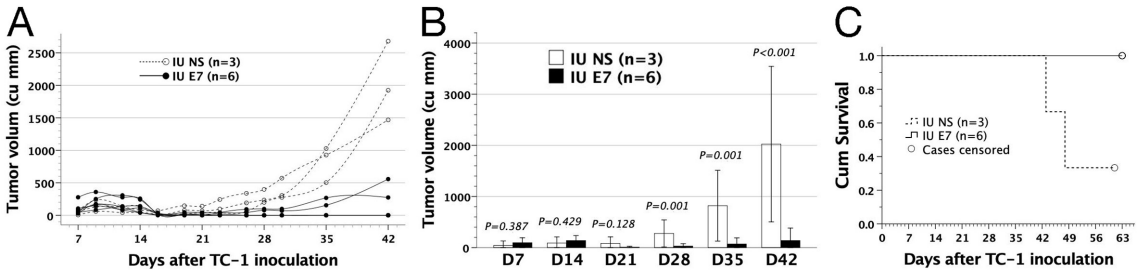

Figure 2 TC-1 tumorigenesis and survival analyses following TC-1 cell challenge in recipients with in utero HPV E7 exposure. C57BL/6 mice were subjected to in utero injection of HPV E7 (IU E7) on GD_{14}, postnatally followed by subcutaneous TC-1 cell challenge at their age of 6 weeks. Tumor growth was recorded from days 7 to 42 after TC-1 inoculation. (A) TC-1 tumors slowly grew by day 14. Within days 14–21, the tumor burden in IU E7 started to decline (paired comparison, p=0.023 for D21–D14, *t*-test), as compared with that in the controls subjected to in utero saline injection (IU NS) (paired comparison, p=0.890 for D21–D14, *t*-test). From day 21 onwards, TC-1 tumors gradually enlarged in IU NS (paired comparison, p=0.026 for D28–D21, *t*-test) as opposed to IU E7 (paired comparison, p=0.301 for D28–D21, *t*-test). At last, four of six IU E7 recipients could achieve tumor clearance on palpation. (B) Bar charts showed mean tumor volumes of IU E7 and IU NS every 7 days. The difference in tumor burden between IU E7 and IU NS reached statistical significance (*t*-test) on days 28, 35 and 42. (C) Within a 2-month follow-up, the HPV E7 recipients compared favorably in survivals with saline controls (p=0.027, log-rank test). Circles represented cases censored. HPV, human papilloma virus.

Lymphocyte polarization in HPV E7 fetal recipients was assessed by IFNγ, IL4 and IL5 secretion in in vitro lymphocyte culture under HPV E7 stimulation at the age of 6–8 weeks. HPV E7 recipient lymphocytes significantly generated higher levels of IFNγ, as opposed to those of saline controls (figure 1E). However, it made no difference in IL4 and IL5 secretions between HPV E7 recipients and saline controls. These findings indicated a Th1-skewed cytokine profile, suggesting that in utero exposure to HPV E7 endowed the recipient mice with Th1-skewed lymphocytes.

TC-1 cell challenge in recipients with in utero exposure to HPV E7

GD_{14} C57BL/6 murine fetuses were subjected to in utero exposure to HPV E7. Postnatally, they were given subcutaneous inoculation of TC-1 cells at the age of 6 weeks to simulate leftover transformed cancer cells egressing during embryogenesis. The tumor cells slowly expanded by day 14. From day 14 onwards, the TC-1 tumor burden significantly decreased in HPV E7 recipients, but rapidly increased in saline controls (figure 2A). Among six HPV E7 recipients, four were able to eliminate TC-1 tumors by day 28, and then afterwards stayed tumor-free locally as well as in the lung, liver and spleen on histopathological examinations at sacrifice. The other two showed slow tumor regrowth from day 21 onwards after initial shrinkage within days 14–21. Overall, HPV E7 recipients were not in a state of lower tumor burdens until day 28, as compared with their counterpart controls (figure 2B). Within 2-month follow-up, two saline control cases succumbed to tumor expansion on days 43 and 48, respectively, and the third was ethically euthanized on day 61 due to restricted mobility from an excessive tumor burden. In contrast, all the HPV E7 recipients survived TC-1 cell challenge, experiencing superior overall survivals to their controls (figure 2C).

Histological examinations of cutaneous TC-1 tumors

In the control group with in utero saline injection, the tumor was composed of TC-1 cells with nuclear pleomorphism and focal areas of tumor necrosis (figure 3A). In HPV E7 recipients that failed to completely eliminate TC-1 tumors, the residual tumor mass developed massive central necrosis with capsular infiltration by tumor cells and mononuclear lymphocytes (figure 3B). It supported the immune-mediated cytotoxicity against TC-1 cells. Additionally, numerous $F4/80^{+}CD11c^{+}$ macrophages were found to reside within capsular TC-1 cell nests of the residual tumor (figure 3C), exhibiting the capacity of tumor cell phagocytosis. These antitumor activities might contribute to less tumor burden in HPV E7 recipients. Remarkably, TC-1 cells also invaded beyond the tumor capsule (figure 3B). This provided the pathological evidence of tumor invasiveness as the barrier to complete tumor eradication.

Surface phenotyping of fetal MPs by flow cytometry

Cells harvested from GD_{14} fetal peritoneum and liver were pulsed with OVA-647 and subjected to immunophenotyping for surface markers of CD45, F4/80, CD11b and Gr1. Fetal MPs, functionally defined by their phagocytosis of OVA-647, were $CD45^{+}$ hematopoietic cells and accounted for about 60% and 75% of cells harvested from fetal peritoneum and liver, respectively (figure 4A). Conventional $F4/80^{+}$ macrophages were estimated as <1% of fetal phagocytes in the peritoneum and <3% in the liver (figure 4A), presenting as $F4/80^{+}CD11b^{+}Gr1^{+}$, $F4/80^{+}CD11b^{+}Gr1^{-}$ and $F4/80^{+}CD11b^{-}Gr1^{-}$ rather than $F4/80^{+}CD11b^{-}Gr1^{+}$ at that time (figure 4B). $F4/80^{-}$ cells comprised over 95% of functionally defined fetal MPs and were rarely present as $F4/80^{-}CD11b^{-}Gr1^{+}$.

Endocytosis of HPV E7 by fetal MPs and their ability to elicit Th1 cytotoxic immunity

After cocultured with HPV E7 overnight, fetal MPs were demonstrated to take up HPV E7 (figure 5A,B). To assess

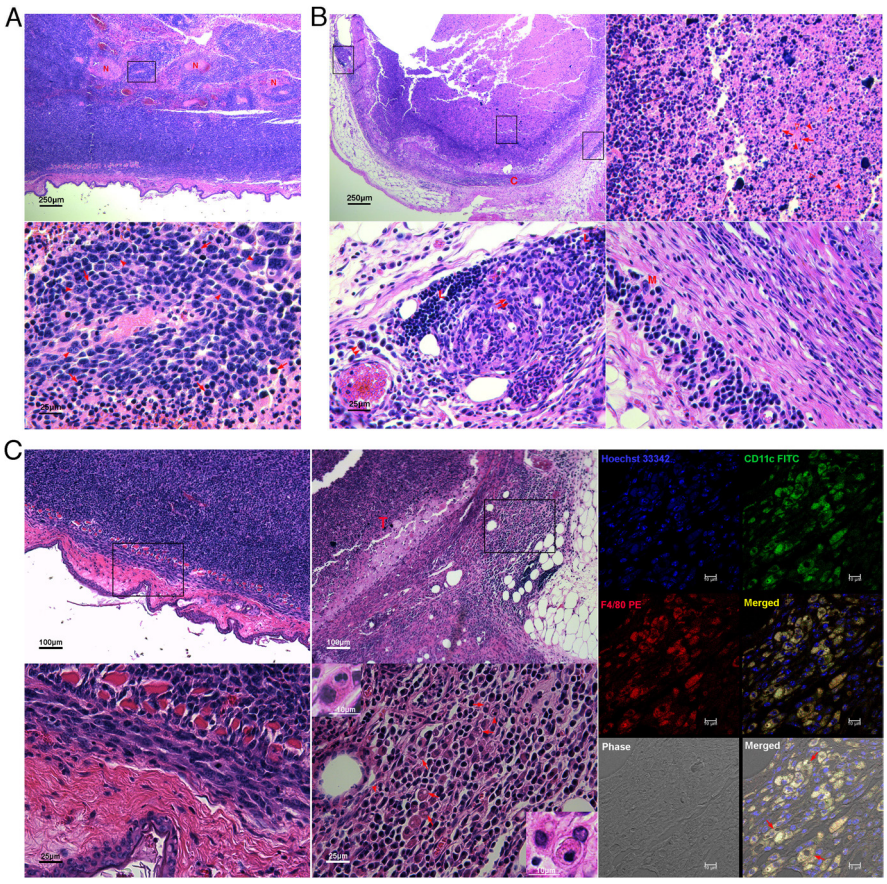

Figure 3 Histopathological examinations of cutaneous TC-1 tumors. The TC-1 tumors harvested from recipients with in utero injection of saline or HPV E7 were processed for H&E staining and microscopic examinations. (A) The tumor from a representative saline control was surrounded by the thin capsule and had focal areas of tumor necrosis (N). A magnified view of the boxed area showed that TC-1 cells exhibited the high nucleus-cytoplasm ratio with nuclear pleomorphism in size, shape and staining. Their nuclei were either stained darkly (hyperchromasia, arrows) or characterized by irregular coarse clumped chromatin and multiple prominent nucleoli (arrowheads). (B) The residual tumor mass was harvested from a representative HPV E7 recipient unable to completely eliminate TC-1 tumors. It was encased by the thickened capsule (C, left upper panel). The boxed areas were magnified and showed in a fashion of 90° clockwise rotation. The tumor was centrally filled with hypereosinophilic TC-1 cells with nuclear condensing (karyopyknosis, arrows), fragmenting (karyorrhexis, arrowheads) or fading (karyolysis, blank arrowheads), indicating tumor cell necrosis that was surrounded by subcapsular leukocyte infiltration (right upper panel). Tumor capsule was invaded by TC-1 cells with nuclear hyperchromasia (double arrowheads) or coarse clumped chromatin and prominent nucleoli (double arrows, left lower panel). There were mononuclear lymphocytes, either gathering by (L) or scattering among tumor cells. Notably, hyperchromatic TC-1 cells spread beyond the capsule and showed mitosis (M, right lower panel). (C) In residual TC-1 tumors of HPV E7 recipients (T, middle panels), numerous macrophages were present among penetrating TC-1 cells within a focus of capsular tumor invasion. They were large in size (10–20 µm), characterized by round or bean-shaped nuclei and very abundant eosinophilic cytoplasm that might contain vacuoles (arrows, magnified boxed area). The macrophages were capable of engulfing hyperchromatic tumor cells to form a cytoplasmic phagosome (arrowheads and insets). Further immunofluorescence staining of a neighboring section disclosed that macrophages simultaneously expressed F4/80 and CD11c (right panels). Cytoplasmic vacuoles were also evident (arrows). As for TC-1 tumors growing in control mice, their tumor capsule was barely infiltrated by immune cells. Hyperchromatic TC-1 cells invaded the muscle layer and capsule as well (magnified boxed area, left panels). HPV, human papilloma virus.

their ability to elicit lymphocytic cytotoxicity, we first subjected C57BL/6 mice to the injection of C57BL/6 fetal MPs loaded with HPV E7. One month later, C57BL/6 recipients were found to generate significant levels of serum anti-E7 IgG_1 (figure 5C). The activation and proliferation of recipient lymphocytes in response to HPV E7 were also examined in in vitro cell culture systems. Recipient lymphocytes exhibited the activity of lymphocyte activation and proliferation specifically in response to HPV E7 stimulation as evidenced by significant IL2 secretion (figure 5D) and tritium incorporation (figure 5E). Further evaluation of lymphocyte polarization in recipients by ELISA disclosed a Th1-skewed phenotype (figure 5F).

Following adoptive transfer of HPV E7-loaded fetal MPs, C57BL/6 recipients were examined for the generation of HPV E7-specific cytotoxic $CD8^+$ T-cells using HPV E7 peptide/MHC class I ($H\text{-}2D^b$) pentamers. Circulating and splenic $CD8^+$ T-cells specific to an HPV E7 epitope could be detected 1 week after intraperitoneal challenge of TC-1 tumor cells (figure 6A). The cell-mediated

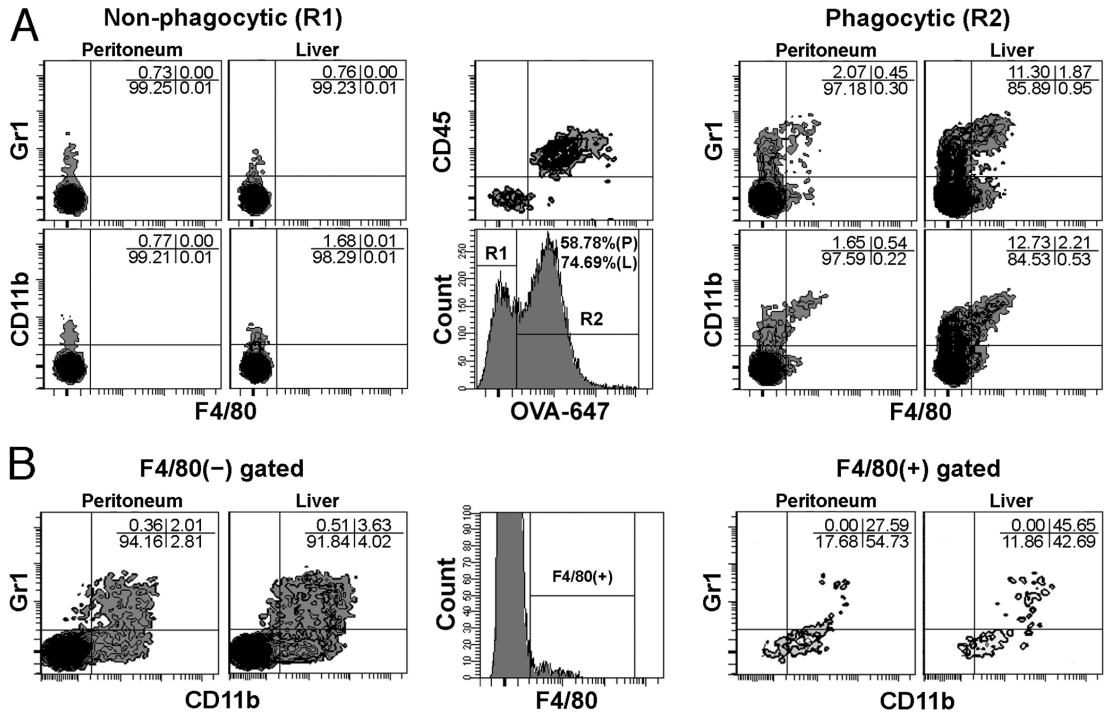

Figure 4 Phenotyping of fetal MPs for myeloid surface markers of F4/80, CD11b and Gr1. GD_{14} fetal MPs were functionally determined by their ability to phagocytose OVA-647. (A) Fetal liver significantly harbored more phagocytes than fetal peritoneum (74.69% (L) vs 58.78% (P), n=5, p<0.001, *t*-test). Fetal MPs expressed leukocyte common antigen of CD45. Over 80%–90% of phagocytes were $F4/80^{-}Gr1^{-}$ or $F4/80^{-}CD11b^{-}$. $F4/80^{+}$ macrophages accounted for <1% and <3% of phagocytes in the peritoneum and liver, respectively. Nonphagocytic cells did not comprise F4/80-expressing cells, and barely expressed myeloid GR1 or CD11b. (B) $F4/80^{+}$ macrophages might be present as $F4/80^{+}CD11b^{+}Gr1^{+}$, $F4/80^{+}CD11b^{+}Gr1^{-}$ or $F4/80^{+}CD11b^{-}Gr1^{-}$ instead of $F4/80^{+}CD11b^{-}Gr1^{+}$. Among $F4/80^{-}$cells, the rarity of $F4/80^{-}CD11b^{-}Gr1^{+}$ phenotype was evident. The percentage of each quadrant was shown as the mean of a data set consisting of five murine samples. Isotype controls used as manufacturers' suggestions revealed background staining of <0.05%. HPV, human papilloma virus.

cytotoxicity of recipient lymphocytes against TC-1 cells was morphologically evaluated by DAPI viability dye, which is impermeant to live cells but penetrates the membrane and binds to nucleic acid in nonviable cells. After incubated with the recipient lymphocytes, TC-1 tumor cells displayed very bright fluorescence under a fluorescence microscope (figure 6B). The lymphocytic cytotoxicity was further quantified by LDH release from dead cells. It showed that TC-1 cytolysis significantly increased in recipients subjected to adoptive transfer of HPV E7-loaded fetal MPs (figure 6C), especially when the recipient lymphocytes were rechallenged in advance with HPV E7 before their incubation with TC-1 tumor cells.

Preventive and therapeutic effects of HPV E7-loaded fetal MPs on TC-1 tumorigenesis

To evaluate the protective effects of HPV E7-loaded fetal MPs on TC-1 tumorigenesis, we treated seven C57BL/6 mice with HPV E7-loaded fetal MPs 4 weeks prior to subcutaneous challenge of TC-1 tumor cells (figure 7A,B). After given a tumorigenic dose of TC-1 cells, five of the seven recipients were tumor-free 3 weeks after tumor cell inoculation and remained so afterwards. The other two exhibited sluggish tumor growth, in sharp contrast to rapid tumor growth in wild types as well as the controls receiving fetal MPs without HPV E7 loading. Thus, this preventive HPV E7-loaded fetal MP vaccination effectively rendered the recipients resistant to TC-1 tumorigenesis, in line with the results observed following in utero exposure to HPV E7.

To further evaluate the therapeutic effects of HPV E7-loaded fetal MPs on pre-existing or growing TC-1 tumor cells, we first subjected C57BL/6 mice to subcutaneous inoculation of TC-1 cells, followed 2 or 4 days later by the injection of HPV E7-loaded fetal MPs. Neither of the strategies could cure mice with the tumors derived from a tumorigenic dose of TC-1 cells, as evidenced by the observation that the recipients either lived with progressive enlargement of cutaneous TC-1 tumors or died prematurely during follow-up (figure 7C). However, both strategies significantly slowed down cutaneous TC-1 tumor growth, achieving less cutaneous tumor burdens in comparison with wild types and the controls receiving fetal MPs only (figure 7D). Thus, the therapeutic HPV E7-loaded fetal MP vaccination could only attenuated cutaneous TC-1 tumor growth but never achieved complete tumor clearance.

The effects of HPV E7-loaded fetal MPs on recipient survivals following tumor challenge

Given HPV E7-loaded fetal MPs as preventive or therapeutic cellular vaccines described above, the recipients

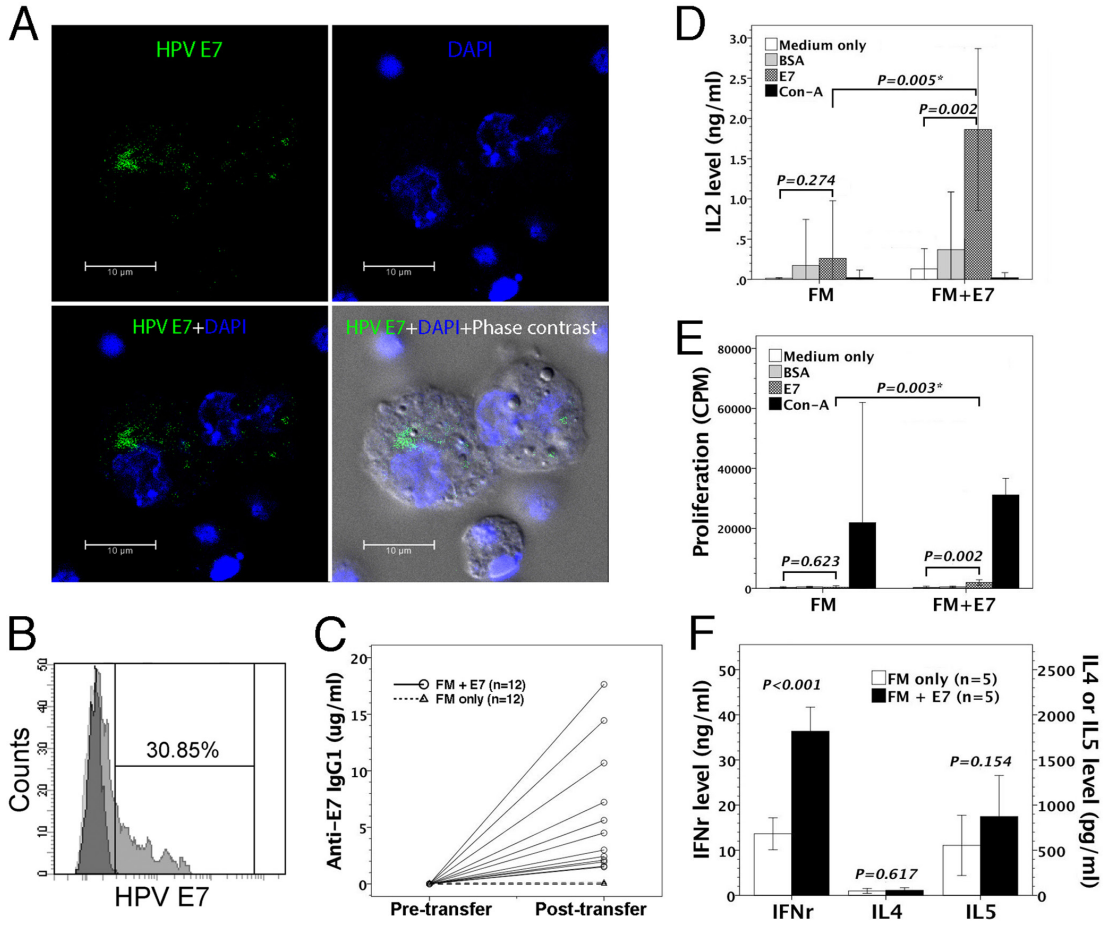

Figure 5 HPV E7 endocytosis by fetal MPs and their ability to elicit Th1 immunity. Cells harvested from GD_{14} C57BL/6 fetal peritoneum or liver were pulsed with HPV E7 overnight and then subjected to immunofluorescence staining by mouse anti-E7 IgG_1 and FITC-conjugated anti-mouse IgG secondary antibody, followed by DAPI nuclear counterstain. Cells were observed under a confocal microscope and analyzed by flow cytometry. (A) HPV E7 was detected within the cytoplasm of fetal MPs that contained several intracellular vacuoles. (B) The histogram obtained from a representative sample showed 30.85% of cells to take up HPV E7 following HPV E7 pulse. Area in black represented the background control. (C) Adoptive transfer of HPV E7-loaded fetal MPs (FM+E7) induced the generation of anti-E7 IgG_1 (paired comparison, p=0.003, *t*-test) in recipients' serum. The controls received fetal MPs without HPV E7 pulse (FM only; paired comparison, p=0.336, *t*-test). Coupled circles or triangles represent paired data of pre and post-cell transfer from an individual mouse. Additionally, recipients were evaluated for their lymphocyte activation, proliferation and polarization in cultures supplemented with BSA, HPV E7 (E7) or Con-A. (D) T-cell activation was measured by IL2 secretion in culture supernatants. FM+E7 (n=3) showed HPV E7-specific IL2 secretion in the culture supernatants, as opposed to the controls with adoptive transfer of fetal MPs only (FM, n=3). Lymphocytes of FM+E7 and FM hardly generated IL2 under Con-A stimulation. All bar data are shown as 95% CIs for the means. (E) The proliferative responses were measured by the readout of incorporated tritium as CPM. The lymphocytes of FM+E7 (n=3) proliferated specifically to HPV E7 stimulation, whereas the control lymphocytes (FM, n=3) proliferated only under Con-A stimulation. All p values were obtained from ANOVA multiple comparisons except for p* that derived from *t*-tests between FM+E7 and FM for IL2 secretion and lymphocyte proliferation under HPV E7 stimulation. (F) As for lymphocyte polarization in recipients under HPV E7 stimulation, FM+E7 compared favorably in IFNγ but not IL4 and IL5 secretions with FM only, indicating a Th1-skewed phenotype of FM+E7 lymphocytes. All p values derived from *t*-tests. CI, confidence interval; ANOVA, analysis of variance; BSA, bovine serum albumin; CPM, counts per minute; HPV, human papilloma virus.

were examined for survivals following TC-1 tumor challenge within a period of 2 months (figure 7E). It was worth noting that preventive HPV E7-loaded fetal MPs conferred an excellent protection against TC-1 tumor challenge, accounting for 71% (5/7) of animals immunized with HPV E7-loaded MPs to live free from cutaneous TC-1 tumors and metastatic lesions at 2-month follow-up, which was superior to wild types or their counterpart controls. However, the recipients receiving the therapeutic MP vaccines either died of tumor expansion or were ethically sacrificed due to excessive tumor burden at follow-up. Overall, the therapeutic MP vaccines provided no survival benefits to the recipients.

DISCUSSION

According to Medawar's theory of 'actively acquired tolerance',[18] self-nonself discrimination by the immune system

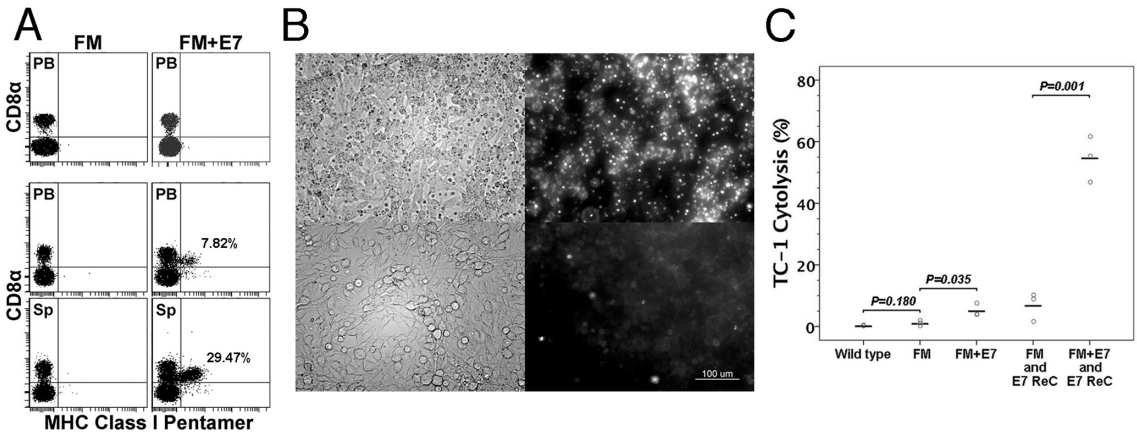

Figure 6 Detection of HPV E7-specific CD8$^+$ T-cells and their cytotoxicity against TC-1 tumor cells. C57BL/6 mice were subjected to adoptive transfer of HPV E7-loaded fetal MPs (FM+E7) or fetal MPs without carrying a load of HPV E7 peptides (FM). (A) Prior to TC-1 cell challenge, HPV E7-specific cytotoxic CD8$^+$ T-cells were barely detected in PB (upper two panels) of FM and FM+E7 recipients by anti-CD8α monoclonal antibodies and Pro5® MHC Class I pentamers of H-2D^b/RAHYNIVTF (HPV16 E7 49–57). One week after intraperitoneal challenge of TC-1 cells, FM+E7 recipients displayed a significant level of HPV E7-specific cytotoxic CD8$^+$ T-cell clones in PB and Sp, as opposed to FM recipients (lower four panels). The percentage of HPV E7-specific CD8$^+$ T-cells among total CD8$^+$ T-cells was shown in the dot plot. Data shown were a representative of three independent experiments. (B) Recipient (FM+E7) splenic lymphocytes rechallenged with HPV E7 in vitro were added to TC-1 cells grown in culture plates and incubated overnight. TC-1 cells were examined under a fluorescence microscope following the treatment of DAPI viability dye. There were numerous small nucleated lymphocytes (phase contrast fields, left upper panel). Areas of TC-1 cells with bright fluorescence-stained large nuclei (right upper panel) were indicative of nonviable or dying TC-1 cells. The controls (FM) showed inconspicuous lymphocyte proliferation and very dim fluorescence (lower panels). (C) The cytotoxicity of recipient lymphocytes against TC-1 cells were further quantified in vitro by LDH release in cell culture supernatants. FM+E7 had a higher TC-1 lysis than FM and wild-type mice. Rechallenged with HPV E7 (E7 ReC), lymphocytes of FM+E7 obtained an markedly increased capacity of TC-1 lysis, as compared with those of FM. All p values were obtained by *t*-test. HPV, human papilloma virus; PB, peripheral blood; Sp, spleen.

is not an inherited property but rather gradually learned in the course of its development. Through a steady contact with self-substances in fetal life, the developing immune system might commit to memory of their patterns by deleting self-reactive lymphocytes.[19] In this way, artificial introduction of a foreign antigen to the fetus early before full T-cell maturation may impress this foreign antigen as self. However, this argument seems to suffer from over-simplification given that complete skin tolerance did not universally develop following in utero exposure to various forms of alloantigens including bone marrow cells,[20 21] MHC exosomes or B-cells,[22] but rather ensued conditionally on marrow inocula capable of achieving a significant level of hematopoietic chimerism.[20 21] The picture of fetal tolerance induction was even more clouded by scattered examples of opposite immunization reported in the literature.[23 24] In this study, fetal exposure to HPV E7 did not cause tolerance but rather elicited T-cell immunity, in keeping with fetal exposure to OVA.[9] It strengthened the notion that the fetuses were not immune-privileged but competent to generate adaptive immunity. Likewise, fetal MPs played an critical role in shepherding the immune responses to HPV E7, endowing a developing fetus with the capability of sensing nonself from self even early before full T-cell development.

During embryonic development, innate phagocytes that can survey their surroundings to eliminate unfit (apoptotic) cells, metabolic waste and foreign antigens are commonly referred to as macrophages.[25 26] In our previous study,[9] GD_{14} antigen-laden fetal MPs lacked the expression of CD11c (dendritic cells) and CD45R (B-cells). However, a minority of them might express F4/80, which has been the most well-known and widely used surface marker for murine macrophage population. These conventional F4/80$^+$ fetal macrophages showed up as F4/80$^+$CD11b$^+$Gr1$^+$, F4/80$^+$CD11b$^+$Gr1$^-$ or F4/80$^+$CD-11b$^-$Gr1$^-$ rather than F4/80$^+$CD11b$^-$Gr1$^+$. As dendritic cell progenitors,[9] fetal MPs might express MHC-I but lacked MHC-II on their surface. However, they might intracellularly express MHC-II and CD80/CD86. Additionally, fetal MPs might be positive for FcγRIII/II(CD16/32) or FcγRI(CD64),[9] endowed with the capability of targeting IgG-antigen complexes to facilitate antigen clearance or downstream immune responses. In this study, over 95% of GD_{14} murine fetal phagocytes were virtually devoid of F4/80 expression. F4/80$^-$ cells mostly presented as the phenotypes of F4/80$^-$CD11b$^-$Gr1$^-$, F4/80$^-$CD11b$^+$Gr1$^-$ or F4/80$^-$CD11b$^+$Gr1$^+$, but rarely F4/80$^-$CD11b$^-$Gr1$^+$. It was reported that F4/80$^+$ macrophages might arise from F4/80$^-$ precursors[10] . Thus, F/80$^-$ phagocytes are likely to be macrophage precursors, reflecting the dispensability of F4/80 expression for macrophages' development and function as demonstrated in transgenic mice with conditional F4/80 inactivation.[27] Taken together, it suggested that phagocytic function of fetal macrophages might show up earlier than surface F4/80 or even CD11b

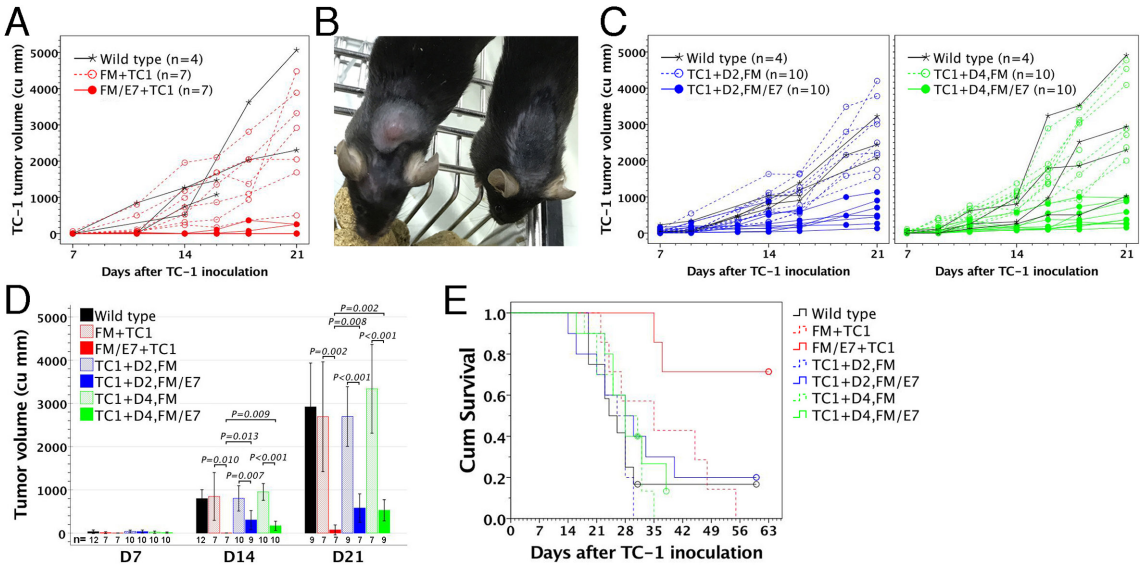

Figure 7 TC-1 tumorigenesis and survival analyses following preventive and therapeutic administration of HPV E7-loaded fetal MPs. (A) The preventive effects of HPV E7-loaded fetal MPs (FM/E7) on TC-1 tumor development were evaluated by subjecting C57BL/6 mice to adoptive transfer of FM/E7, followed 4 weeks later by subcutaneous challenge of TC-1 cells (FM/E7+TC1). Tumor growth was recorded within days 7–21 after TC-1 inoculation. FM/E7+TC1 significantly prevented from TC-1 tumor growth (paired comparison, p=0.356 for D14–D7 and p=0.172 for D21–D14, *t*-test), as compared with the controls receiving fetal MPs and then TC-1 cell challenge (FM+TC1) (paired comparison, p=0.009 for D14–D7 and p=0.010 for D21–D14, *t*-test). (B) A representative FM+TC1 mouse (left) developed a tumor mass at the inoculation site on day 14, whereas a FM/E7+TC1 mouse (right) was absent from TC-1 tumor growth. (C) The therapeutic effects of FM/E7 on pre-existing TC-1 tumor cells were examined. C57BL/6 mice were first given the inoculation of TC-1 cells, followed by FM/E7 transfer 2 days (TC1+D2,FM/E7) or 4 days (TC1+D4,FM/E7) later. There was progressive tumor enlargement in groups TC1+D2,FM/E7 (paired comparison, p=0.021 for D14–D7 & p=0.013 for D21–D14, *t*-test) and TC1+D4,FM/E7 (p=0.004 for D14–D7 & p=0.003 for D21–D14, *t*-test) as well as their respective controls of TC1+D2,FM (paired comparison, p<*0.001* for D14–D7 & p<0.001 for D21–D14, *t*-test) and TC1+D4,FM (p<*0.001* for D14–D7 & p=0.006 for D21–D14, *t*-test). (D) The mean tumor volumes of all groups were shown in bar charts. On days 14 and 21, both TC1+D2,FM/E7 and TC1+D4,FM/E7 had greater TC-1 tumor volumes than FM/E7+TC1, but less tumor burden than TC1+D2,FM and TC1+D4,FM respectively. However, it made no difference in mean tumor volumes among wild types, FM+TC1, TC1+D2,FM and TC1+D4,FM. All p values were obtained by *t*-test. (E) C57BL/6 recipients were subjected to survival analyses by Kaplan-Meier method. FM/E7+TC1 experienced better survivals than other treatment combinations (p<0.001 – p=0.021, log-rank test). There was no significant difference in survivals among wild types, FM+TC1, TC1+D2,FM, TC1+D2,FM/E7, TC1+D4,FM and TC1+D4,FM/E7. Circles represented cases censored. HPV, human papilloma virus.

expression. Thus, the studies of early gestational macrophages selected by their surface expression of F4/80 or CD11b might actually involve only a minority of functionally active phagocytes.[28]

Macrophages can be functionally divided into two major subgroups with opposing activities: M1 for defense 'kill' and M2 for nondefense 'heal' activities,[29] phenotypically linked to Th1/Th2 T-cell responses.[30] However, there was no evidence showing that M1/M2 macrophages were clonally distinguishable to function individually in response to distinct antigens.[31] In contrast, macrophages might exhibit functional plasticity as a continuum of phenotypes that could be shaped by a huge array of environmental factors.[32] In mice, yolk sac progenitors give rise to fetal macrophages until at least $GD_{16.5}$ and then almost all the tissue-resident macrophages in postnatal life.[8] Those yolk sac-derived tissue macrophages exhibit default M2 activities,[29 33] benefitting a variety of constructive M2 processes during embryonic organogenesis. Thus, macrophage differentiation represents an integral part of organogenesis during embryonic development.[26] In Th2-biasing fetal milieu, GD_{14} M2-defaulted macrophages loaded with Th1-promoting HPV E7 elicited Th1 immune deviation, compatible with their functional plasticity conditional on the antigen nature. Thus, Th2 sensitization following fetal exposure to OVA in our previous study[9] could not be attributed to Th2-biasing fetal milieu but rather the innocuous allergen nature of OVA. It further fortified that the nature of antigens rather than the bias of fetal or neonatal immune milieu determined T-cell polarization.[34]

Pediatric cancers were infiltrated monotonously by macrophages with a paucity of dendritic cells, as compared with adult tumors that lodged diverse leukocyte types.[35] This phenomenon reflected the distinct tumor nature and etiology of blastomas in children versus epithelial carcinomas in adults and likely a more crucial role of macrophages in the microenvironment of pediatric blastoma. Antitumor activities by macrophages occur either in a direct way of tumor cell killing through the release

of cytotoxic mediators or phagocytosis, or in an indirect way of bridging toward T-cell cytotoxicity through antigen processing and presentation.[36] The direct way is associated with tumoricidal M1 phenotypes, as opposed to tumor-associated macrophages (TAMs) that have tumorigenic effects and functionally belong to M2 phenotype.[37] However, accumulating evidence indicates that not all TAMs were akin to M2 phenotypes,[38] highlighting the heterogeneity of TAM population.[39] Thus, strategies have been pursued for the reprogramming of TAMs toward M1-like macrophages to facilitate tumor regression.[40] In this study, we found that F4/80$^+$CD11c$^+$ macrophages residing within the capsule of residual tumors in HPV E7 recipients exhibited the capacity of tumor cell phagocytosis with the formation of phagosome-associated vacuoles, as the finding relevant to phagocytosis of antibody-opsonized tumor cells.[41] F4/80$^+$CD11c$^+$ macrophages were proinflammatory as M1-polarized phenotype[42] and positively correlated with overall survivals of patients following curative resection of hepatocellular carcinoma.[43] Thus, F4/80$^+$CD11c$^+$ macrophages might have important implication for the inhibition of TC-1 tumorigenesis, essentially in line with the histological finding of tumor cell phagocytosis by F4/80$^+$CD11c$^+$ macrophages in this study. HPV E6/E7-related vaccines might trigger humoral immunity to protect against TC-1 tumorigenesis.[44 45] It is immunologically relevant to complement-mediated cytolysis and cell-mediated cytotoxicity involving NK cells, macrophages and neutrophils.[46] These effector cells can be bridged toward tumor cells via their Fc receptors following specific antibody binding to tumor cells (antibody opsonization), leading to antibody-dependent cell cytotoxicity (ADCC). It had been reported that anti-HPV E6/E7 antibodies could recognize E6/E7 peptides on the surface of TC-1 tumor cells and thereby trigger ADCC to eliminate tumor cells.[45] However, there was increasing evidence that macrophages were the prominent effector cells to eliminate tumor cells through the mechanism of antibody-dependent cell phagocytosis (ADCP).[46] Given the generation of anti-E7 IgG and the formation of discrete tumor cell-containing phagosome-associated vacuoles in F4/80$^+$CD11c$^+$ macrophages observed in this study, ADCP might play a role in tumor cell elimination by TAMs following fetal oncoantigen exposure.

The indirect way is linked to macrophages' capability of dealing with tumor antigens such as oncofetal proteins. Although macrophages exhibited the comparable capacity for activating anti-tumor cytotoxic T-cell clone to dendritic cells,[47] they were rarely reported as professional antigen presenting cells to initiate antitumor T-cell cytotoxicity in the literature,[48] let alone developing fetal macrophages. In this study, we simulated the influence of fetal exposure to oncoproteins on the cytoablation of transformed cancer cells, showing that in utero exposure to HPV E7 rendered fetal recipients capable of eliminating inoculated TC-1 tumor cells through T cell-mediated cytotoxicity in postnatal life. It could be attributed to fetal MPs that endocytosed HPV E7 oncoprotein and then acted as antigen presenting cells to trigger Th1 cytotoxic immunity with rapid clonal expansion of HPV E7-specific CD8$^+$ T-cells in response to TC-1 cell challenge, similar to the scenario observed following in utero exposure to OVA.[9] Our results might provide further implications for the immunological mechanisms of spontaneous neuroblastoma regression in perinatal life,[49 50] which involved antitumor humoral and cellular immune responses via the recognition of neuroblastoma-specific antigens.[49 50] It cast light on the critical role of fetal MPs in sampling transformed cells or their related tumor antigens egressing during embryogenesis so as to later trigger tumor antigen-specific B-cell humoral and T-cell cytotoxic immunity as the tumor immune surveillance against the developmental tumorigenesis in perinatal life.

The potent immunogenicity of fetal MPs could be attributed to their specialty of limited proteolytic capacity,[9] thereby favoring antigen accumulation, processing and then dissemination throughout the immune system to enhance antigen presentation and immunogenic T-cell responses. The capacity of HPV E7-loaded fetal MPs to prevent TC-1 tumorigenesis was further fortified by their adoptive transfer in adult mice, which achieved a tumor-free survival of around 70% at 2-month follow-up, comparable to that of 67% following fetal exposure to HPV E7. However, the therapeutic transfer of HPV E7-loaded fetal MPs to treat pre-existing TC-1 tumors was less fruitful, only achieving the attenuation of cutaneous tumor growth but providing no substantial survival benefits.

Conclusively, the developing fetuses even prior to T-cell maturation were not in an immune-privileged stage permissive for tolerance induction to foreign peptides. The nature of antigens, either Th1 or Th2-promoting, determined T-cell polarization in preference to Th2-biasing fetal milieu. Our results bear momentous implications for the role of fetal MPs in bridging toward adaptive T-cell and humoral immunity potentially as a tumor immune surveillance system against developmental tumorigenesis once they endocytose tumor antigens egressing during embryogenesis. Additionally, fetal MPs might be manipulated for developing novel approaches to meet the health challenges of pediatric life, such as enhancing vaccine responsiveness, or increasing resistance to pathogenic microorganisms.

Contributors J-CC performed in utero injection, cytometry, cytotoxic assays and immunofluorescence staining, analyzed the data, prepared the figures and wrote the manuscript. L-SO performed cell culture, quantified immunoglobulin/cytokine levels and measured lymphocyte proliferation. M-LK helped with data analyses and manuscript preparation. L-YT and H-LC assisted in experiments and animal surgery and care.

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Competing interests None declared.

Patient consent for publication Not required.

Ethics approval This study was conducted under the approval of the CGMH Committee on Animal Research.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement All data relevant to the study are included in the article.

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