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# Supplemental Information

# FcRγ Activation Regulates Inflammation-Associated Squamous Carcinogenesis

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**SUPPLEMENTAL DATA**

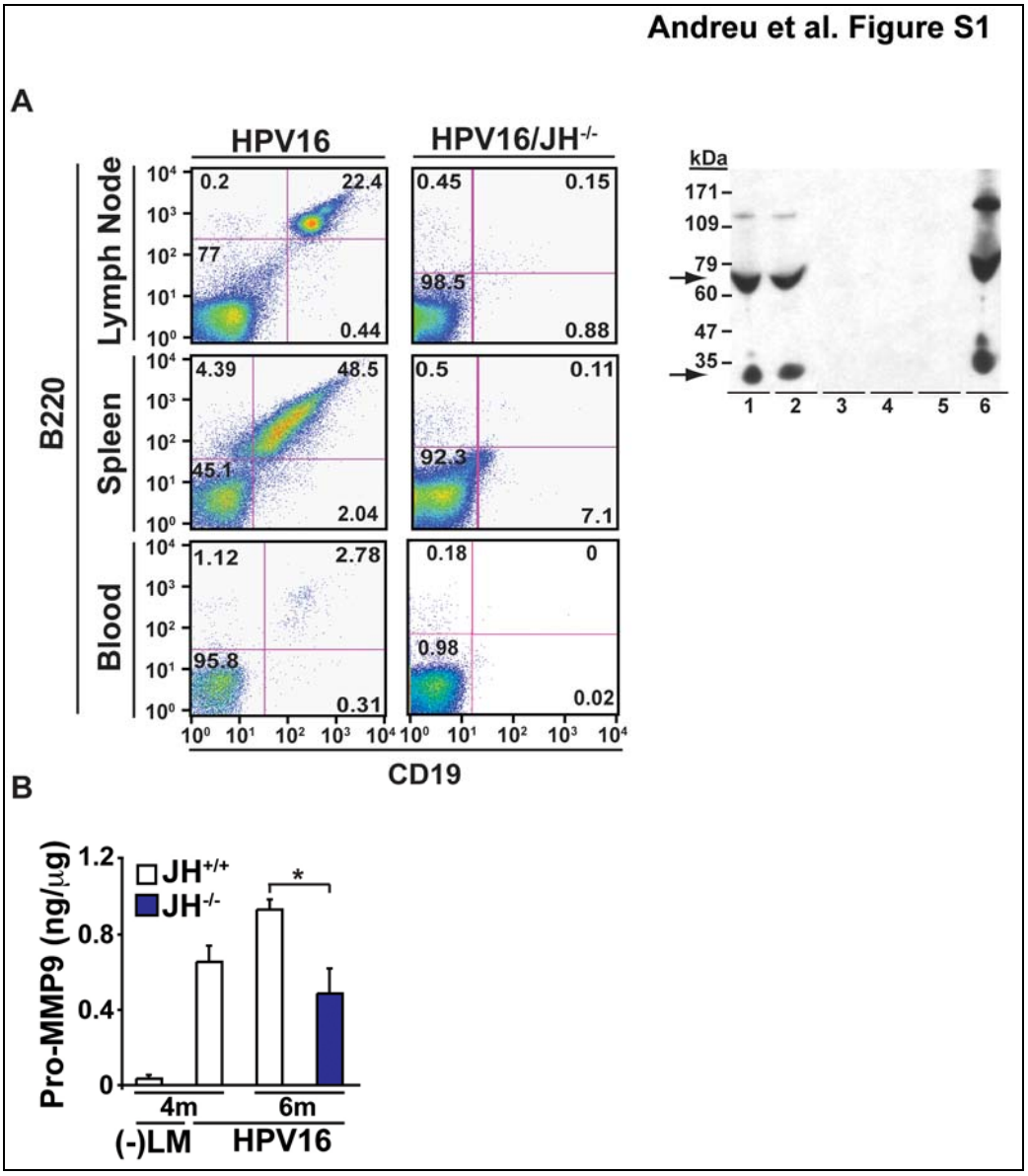


***Figure S1. Generation of B cell-deficient HPV16 mice (accompanies Figure 1)***

**A)** Analysis of $B220^{+}CD19^{+}$ B cells by flow cytometry in lymph node, spleen and peripheral blood from HPV16 and $HPV16/JH^{-/-}$ mice (4-mo). Right panel: Western blot for IgG, IgA and IgM in serum from negative litterate (lane 1), HPV16 (lane 2 and 6) and $HPV16/JH^{-/-}$ (lane 3-5) mice. Ig heavy and light chains are indicated (arrows).

**B)** Protein levels of pro-MMP9 in skin extracts from age-matched (-)LM, $HPV16/JH^{+/-}$ and $HPV16/JH^{-/-}$ mice (4-mo) as assessed by ELISA. Error bars represent SEM, asterisk (*)

indicates statistically significant differences between HPV16/JH$^{+/-}$ and HPV16/JH$^{-/-}$ mice ($p < 0.05$, Mann-Whitney).

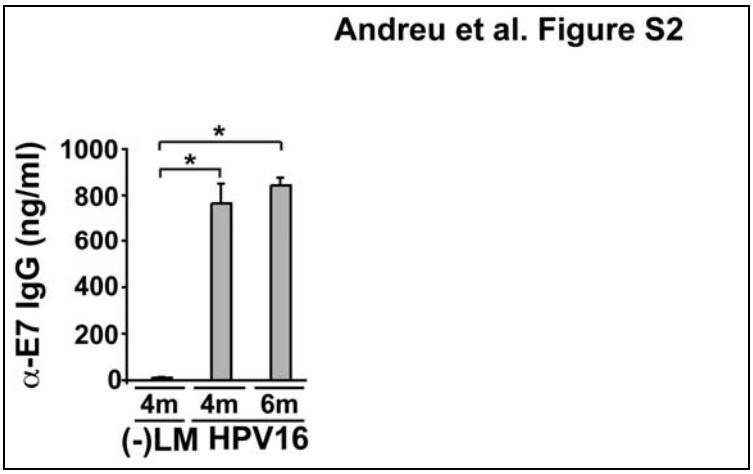


***Figure S2. Anti-E7 IgG quantification (accompanies Figure 2)***

Serum titers of anti-E7 IgG evaluated by indirect ELISA in serum from (-)LM and HPV16 (4- and 6-mo). Error bars represent SEM; asterisk (*) indicates statistically significant differences with (-)LM ($p < 0.05$, Mann-Whitney).

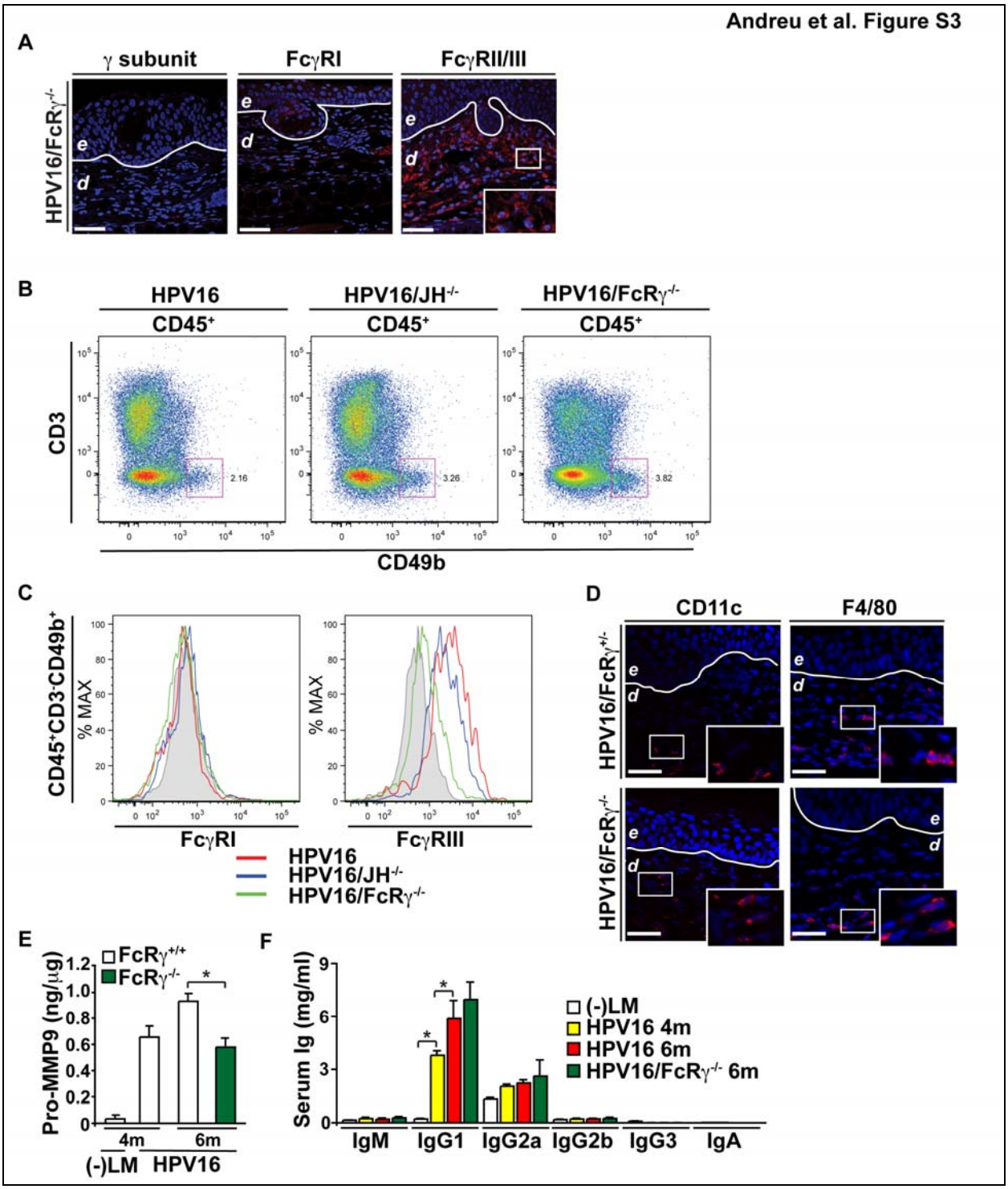


***Figure S3. Deficient carcinogenesis in HPV16/FcRγ-deficient mice (accompanies Figure 4)***

**A**) Immunolocalization of FcRγ (left panel), FcγRI (middle panel) and FcγRII/III (right panel) expression in HPV16/FcγR$^{-/-}$ mice at 4-mo of age. Solid line, epidermal-dermal interface; epidermis, e; dermis, d; scale bar, 50 μm.

**B**) Representative dot-plot graphs showing $CD45^{+}CD49b^{+}CD3^{-}$ NK cells in premalignant skin of HPV16, HPV16/$JH^{-/-}$, and HPV16/$FcR\gamma^{-/-}$ mice (4-mo).

**C**) Differential expression of FcγRI and FcγRIII by NK cells in premalignant skin of HPV16 (red), HPV16/$JH^{-/-}$ (blue) and HPV16/$FcR\gamma^{-/-}$ (green) mice at 4-mo of age. NK cells were gated as live $CD45^{+}CD49b^{+}CD3^{-}$ leukocytes. Grey line: Ig control.

**D**) Immunolocalization of CD11c and F4/80-expressing cells in premalignant skin of HPV16/$Fc\gamma R^{+/-}$ and HPV16/$Fc\gamma R^{-/-}$ mice at 4-mo of age (red staining). White line depicts epidermal-dermal interface; e: epidermis; d: dermis; scale bar, 50 μm.

**E)** Protein levels of pro-MMP9 in skin extracts from (-)LM, HPV16/$FcR\gamma^{+/-}$ and HPV16/$FcR\gamma^{-/-}$ mice assessed by ELISA. Error bars represent SEM, asterisk (*) indicates statistically significant differences between age-matched mice ($p < 0.05$, Mann-Whitney).

**F)** Circulating Ig levels were evaluated by ELISA in serum from (-)LM, HPV16 (4- and 6-mo) and HPV16/$FcR\gamma^{-/-}$ (6-mo). Error bars represent SEM, asterisk (*) indicates statistically significant differences with (-)LM ($p < 0.05$, Mann-Whitney).

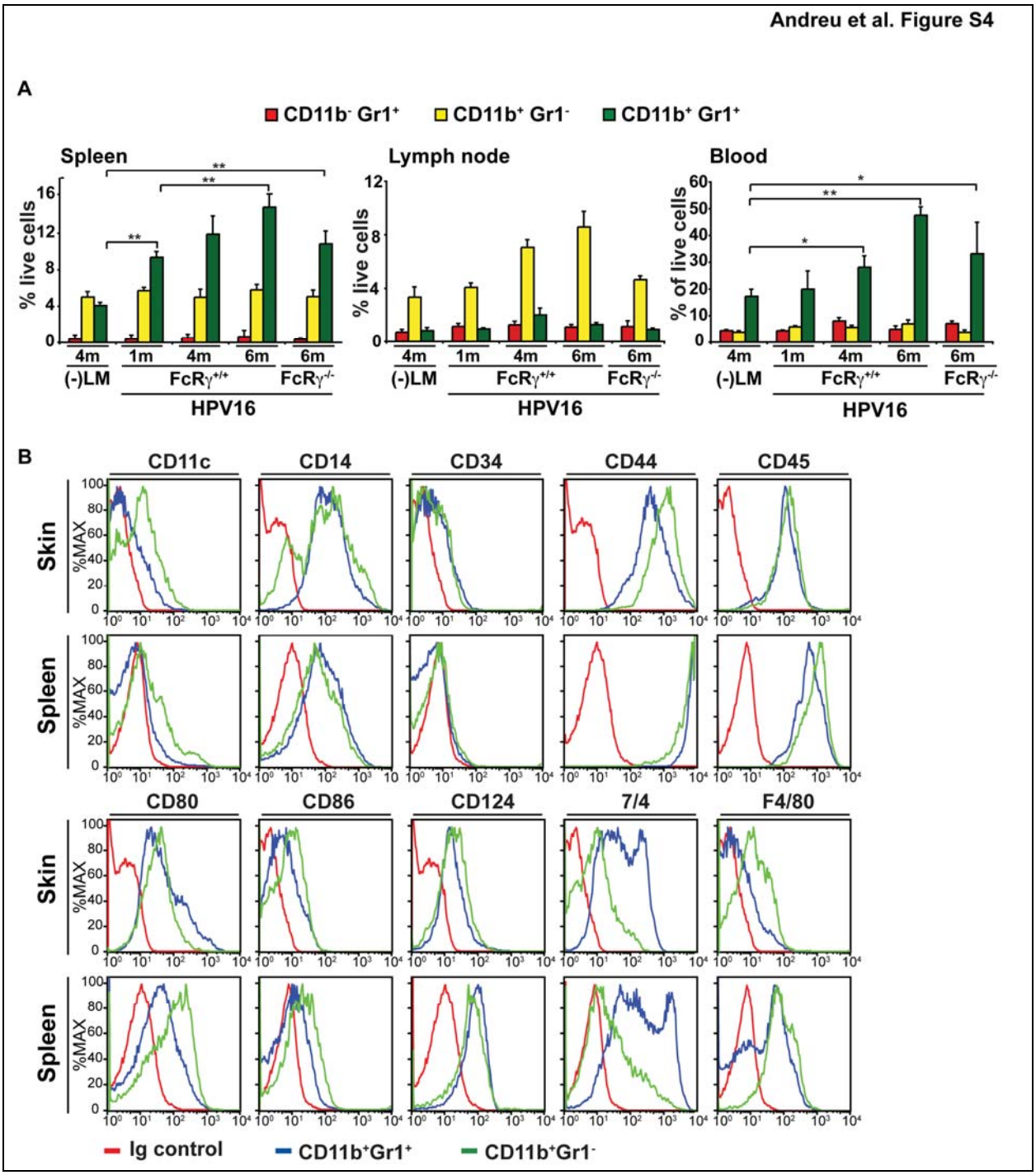


***Figure S4. Lineage characteristics of CD11b$^{+}$Gr1$^{+/-}$ myeloid cell subpopulations in premalignant skin, secondary lymphoid organs and blood of HPV16 mice (accompanies Figure 6)***

**A)** Percentages of CD11b$^{+}$Gr1$^{+/-}$ myeloid cells in spleen, cervical lymph nodes (cLN), and peripheral blood assessed by flow cytometry of single-cell suspensions derived from negative

age-matched negative littermates (-LM), HPV16/FcRγ$^{+/+}$ and HPV16/FcRγ$^{-/-}$ mice. Results shown represent mean percentages (n=5-8 mice) ± SEM. (*) and (**) indicate statistically significant differences; (*) $p < 0.05$ and (**) $p < 0.01$ (Mann-Whitney).

**B)** Phenotype of CD11b$^{+/-}$Gr1$^{+/-}$ myeloid cells evaluated by flow cytometry to assess leukocyte lineage and maturation markers. Single-cell suspensions of neoplastic skin or spleen from mice (4-mo) were gated as live CD11b$^{+}$Gr1$^{+}$ (blue line) and CD11b$^{+}$Gr1$^{-}$ (green line), respectively, and each population was evaluated for expression of the indicated cell surface markers in comparison with isotypic Ig fluorescent levels (red line). At least 3 independent analyses were performed and one representative overlay plot is shown.

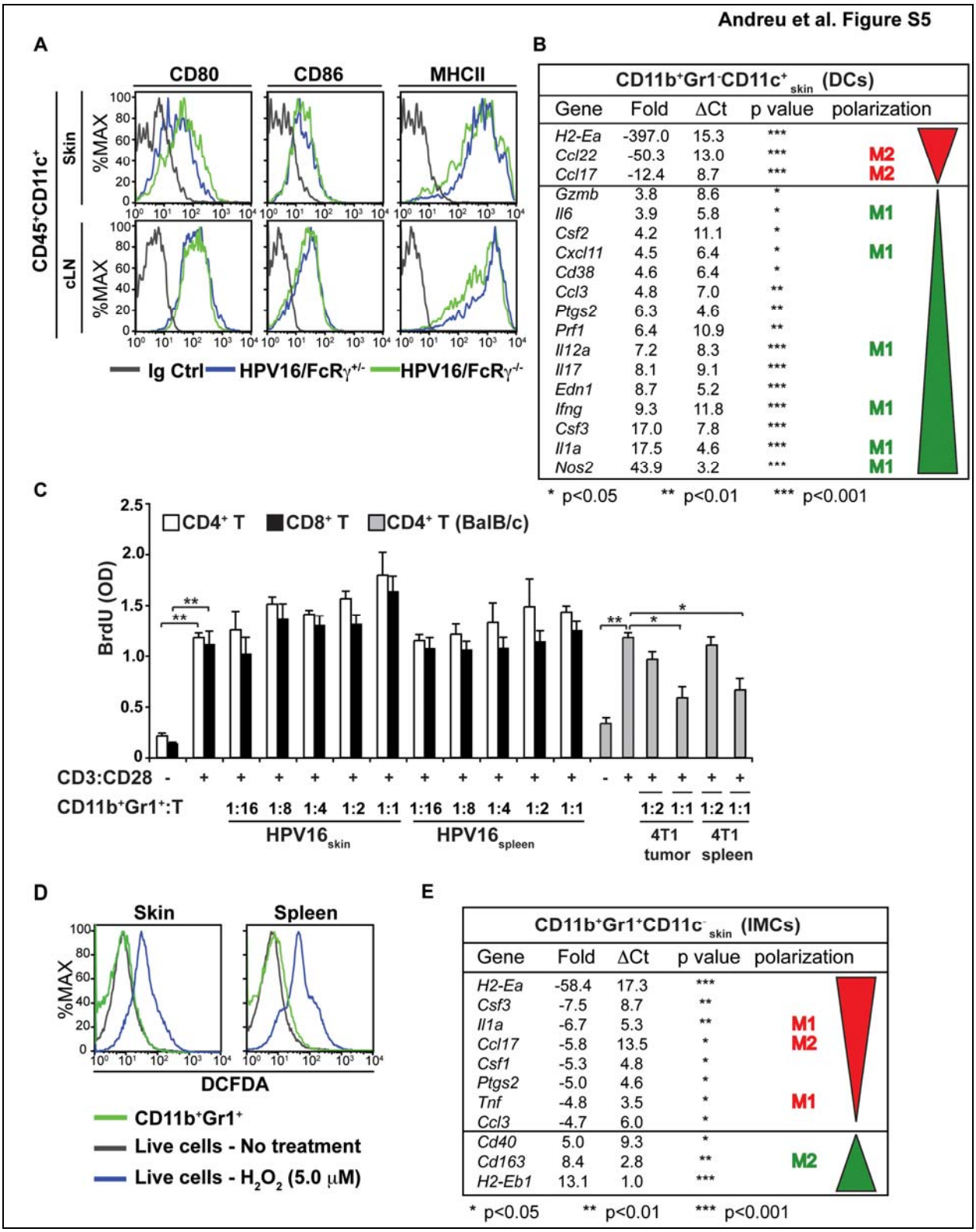


***Figure S5. Functional characterization of myeloid lineage cells infiltrating premalignant skin of HPV16 mice (accompanies Figure 7)***

**A)** FcRγ-deficiency does not modify expression of co-stimulatory molecules on DCs. Single-cell suspensions derived from skin and cLN of HPV16/FcRγ$^{+/-}$ and HPV16/FcRγ$^{-/-}$ mice (4-mo) were gated as CD45$^{+}$CD11c$^{+}$ dendritic cells and evaluated for MHCII, CD80 and CD86 expression levels, as compared to background fluorescent levels (Isotype Ig control, dark grey line). For each antigen, at least 3 independent analyses were performed and overlay plots for one representative experiment are shown.

**B**) Genes differentially expressed in dendritic cells from HPV16/FcRγ$^{+/-}$ and HPV16/FcRγ$^{-/-}$ mice. mRNA expression levels in DCs from HPV16/FcRγ$^{+/-}$ neoplastic skin (4-mo) are indicated as fold-change as compared to HPV16/FcRγ$^{+/-}$ DCs with $\Delta C_T$ of each gene calculated using β*2-microglobulin* as an endogenous control. Genes are considered as statistically significantly de-regulated when p (t test, FcRγ$^{+/-}$ versus FcRγ $^{-/-}$ group) <0.05.

**C)** CD11b$^{+}$Gr1$^{+}$ IMCs purified from either skin or spleen of HPV16 mice do not inhibit T cell polyclonal activation. IMCs were purified by flow cell sorting from premalignant tissue or spleen of HPV16 mice (4-mo) or 4T1 tumor-bearing mice. CD4$^{+}$ and CD8$^{+}$ T cells, respectively, were magnetically purified from (-)LM mouse spleen and co-incubated with isolated IMCs at various ratio (1:16 to 1:1, CD11b$^{+}$Gr1$^{+}$:T cell) for 72-hr in plates coated with anti-CD3/CD28 agonist antibodies. BrdU was added to culture medium for the last 18-hr of co-culture. Incorporation of BrdU was evaluated by ELISA.

**D**) IMCs from skin and spleen of HPV16 mice do not produce reactive-oxygen species. Single-cell suspensions derived from skin and spleen of HPV16 mice (4-mo) were incubated with an oxidation-sensitive probe (DCFDA) in the absence (green line) or presence of $H_2O_2$ (5.0 μM, blue line). Live cells were gated as CD11b$^{+}$Gr1$^{+}$and evaluated for DCFDA fluorescent levels as compared to background fluorescent levels (No treatment, dark grey line). 3 independent analyses were performed and overlay plot for one representative experiment is shown.

**E**) Genes differentially expressed in IMCs from HPV16/FcRγ$^{+/-}$ and HPV16/FcRγ$^{-/-}$ mouse skin. Gene expression levels in IMCs from HPV16/FcRγ$^{-/-}$ neoplastic skin (4-mo) are indicated as fold-change as compared to HPV16/FcRγ$^{+/-}$ IMCs with $\Delta C_T$ of each gene calculated using β*2-microglobulin* as an endogenous control. Gene are considered as statistically significantly de-regulated when p (t test, FcRγ$^{+/-}$ versus FcRγ $^{-/-}$ group) <0.05.

## SUPPLEMENTAL EXPERIMENTAL PROCEDURES

### *Serum collection, immunoglobulin purification and biotinylation*

Blood was collected by cardiac puncture and left at room temperature for 2 hr for coagulation, then centrifuged at 2000 rpm for 15 min. Serum was separated and stored at -80°C. Ig purification was performed as described by the manufacturer's recommendations using the Pierce Thiophilic Adsorption Kit (Pierce Biotechnology, Rockford, IL). Biotinylation of purified IgGs was performed as described by the manufacturer's recommendations using the EZ-Link NHS-PEO Solid Phase Biotinylation Kit (Pierce).

### *Intradermal injection of concentrated immunoglobulins*

Purified IgGs were loaded into the filter unit of Amicon Ultra-4 centrifugal devices (Millipore, Bedford, MA). Devices were spun in a centrifuge with swinging bucket rotor carriers set at 3000 x g for 45 min. Concentrated IgGs were collected immediately after centrifugation, diluted to a final concentration of 12 μg/μl and injected intradermally using 28-gauge insulin syringes to wt mice anesthetized with 2.0% isoflurane/98% $O_2$ mixture.

### *IF-detection of antibody deposition*

Tissue samples were frozen directly in glycerol-based freezing medium (Tissue-Tek® OCT, Sakura Finetek, CA). Tissue sections (10-μm) were cut using a Leica CM1900 cryostat (Leica Microsystem, Bannockburn, IL), air-dried, and fixed in cold acetone. To prevent non-specific binding, tissue sections were incubated for 30 min with rat anti-mouse CD16/CD32 mAb (1:50; 2.4G2, BD Biosciences, San Jose, CA) in blocking buffer (5% goat serum and 2.5% bovine serum albumin (BSA) in PBS). Sections were then incubated with FITC-conjugated goat anti-mouse IgG (γ-chain specific; 1:100; Sigma-Aldrich, St. Louis, MO), anti-mouse IgG1 (1:100; Invitrogen, Carlsbad, CA), anti-mouse IgG2a (1:50; Invitrogen), and IgM (1:50; μ-chain specific, Sigma) in 0.5X blocking buffer for 1 hr at room temperature. Sections were mounted using Vectashield containing DAPI (Vector Laboratories, Burlingame, CA). All IF experiments included negative controls to assess for background staining, which was negligible

### *Immunohistochemistry*

Age-matched tissue samples from transgenic and control animals were immersion-fixed in 10%

neutral-buffered formalin followed by dehydration through graded series of alcohols and xylene and embedded in paraffin. 5-μm thick paraffin sections were cut using a Leica 2135 microtome (Leica Microsystem) and mounted onto SuperFrost/Plus slides (Thermo Fisher Scientific, Waltham, MA). Tissue sections were deparaffinized using xylene and rehydrated through a graded series of alcohol, and subjected to enzyme- and immuno-histochemical (IHC) detection as previously described (Coussens et al., 1996).

To visualize serine esterase activity in mast cells, chloroacetate esterase (CAE) histochemistry was performed as previously described (Coussens et al., 1999). Briefly, 1.0 mg of naphthol AS-D chloroacetate (Sigma) was dissolved in 20 μl of N, N-dimethyl formamide (DMF) and 1.0 ml buffer (8% DMF, 20% ethylene glycol monoethylether in 80mM Tris-maleate pH 7.5). The resulting solution was subsequently added to 1.0 mg of Fast Blue BB salt (Sigma) and applied to tissue sections. Sections were then dehydrated and mounted in glycerol.

To detect endothelial cells and neutrophils, endothelium specific marker PECAM1/CD31 and Gr1 were visualized on paraffin-embedded tissue sections. For antigen retrieval, proteinase K was used (Dako, Glostrup, Denmark). Non-specific binding was blocked using PBS containing 5% goat serum (Thermo Fisher) and 2.5% BSA (Blocking buffer). Sections were incubated with rat anti-mouse CD31 (1:50, BD Biosciences) and rat anti-mouse 7/4, (1:500, Cedarlane Labs, Burlington, NC) in 0.5X blocking buffer for 2 hr at room temperature. Sections were then incubated with biotinylated rabbit anti-rat IgG secondary antibodies (1:200, Vector Laboratories) for 45 min at room temperature. Slides were subsequently incubated with horseradish peroxidase conjugated avidin complex (ABC Elite, Vector Laboratories) for 30 min, followed by incubation with Fast 3,3 diaminobenzidine (DAB, Vector Laboratories). Sections were counterstained with methyl green, dehydrated, and mounted with Cytoseal 60 (Thermo Fisher). To detect FcγR-expressing immune cells, tissue sections were incubated with rat anti-mouse CD45 (1:500, BD Pharmingen) and FcγRI-III (1:500, R&D Systems, Minneapolis, MN) and processed as described above. Hamster anti-mouse CD11c (1:50, BD Pharmingen) and rat anti-mouse F4/80 (1:50, R&D Systems) were used to detect dendritic cells and macrophages respectively. All immunolocalization experiments were repeated on multiple tissue sections and included negative controls for determination of background staining, which was negligible. Photographs were captured at high-magnification (40X) on a Leica DM-RXA microscope attached to a Leica digital camera (Leica Microsystem) operated by OpenLab software™

(Improvision, Waltham, MA). Quantitative analysis of innate immune cells was performed by counting cells in five high-power fields (40X) per age-matched tissue section from five mice per group. Data presented reflect the mean total cell count per field from the ventral ear leaflet.

***Determination of keratinocyte proliferation index***

Mice received intraperitoneal injections of bromodeoxyuridine (BrdU; Roche Diagnostics, Indianapolis, IN) dissolved in PBS (50 μg per g of mouse body weight) 90 min prior to sacrifice. 5-μm thick paraffin sections were deparaffinized in xylene, rehydrated in graded ethanol, and subjected to antigen retrieval by steam heating in Citra™ antigen retrieval solution (BioGenex, San Ramon, CA). BrdU-positive cells were detected according to manufacturers recommendations using the BrdU Labeling Kit II (Roche). Briefly, BrdU staining was revealed using the chromogen Vector Red alkaline phosphatase substrate (Vector Laboratories). Slides were then counterstained with methyl green. Photographs were captured at high-magnification (40X) on a Leica DM-RXA microscope as described above. The proliferative index (percentage of BrdU-positive nuclei over the total number of keratinocytes) was quantified in five high-power fields per tissue section and included five mice per group.

***Flow cytometry***

Ear skin from PBS-perfused mice was manually minced using scissors, followed by a 30 min enzymatic digestion with 2.0 mg/ml collagenase A (Roche) and 1.0 mg/ml Hyaluronidase (Worthington, Lakewood, NJ) in serum-free Dulbecco's modified eagles medium (DMEM) (Invitrogen) at 37$^{o}$C using continuous stirring conditions. The digest was quenched by adding DMEM containing 10% FBS (Invitrogen) and was subsequently filtered through a 70-μm nylon filter (Falcon). To prevent non-specific binding, cells were incubated for 10 min at 4$^{o}$C with rat anti-mouse CD16/CD32 mAb (1:200, BD Bioscience) in PBS containing 1.0 % BSA. Subsequently, cells were washed and incubated for 20 min with 50 μl of fluorophore-conjugated anti-mouse antibodies; B220 (RA3-6B2), CD3e (145-2C11), CD4 (6K1.5), CD8a (53-6.7), CD11b (M1/70), CD11c (N418), CD14 (Sa2-8), CD19 (MB19-1), CD31 (MEC 13.3), CD44 (IM7), CD45 (30-F11), CD80 (16-10A1), CD86 (GL1), CD115 (AFS98), CD117 (2B8), F4/80 (BM8), FcεRI alpha (MAR-1), FcγRII/III (93), FcgammaR III (275003), Gr-1 (RB6-8C5), MHCII (M5/114.15.2) (all from eBioscience, San Diego, CA) CD64 a and b (FcγRI; PE; X54-

5/7.1), CD124 (M1), MHCI (KH114) (from BD-Pharmingen), and polyclonal anti-FcγRIII/CD16 (PE; R&D Systems). Antibodies were used at 1:200 dilution in PBS containing 1% BSA. To delineate between viable and dead cells, 7-amino-actinomycin D (7-AAD; 1:10; BD Biosciences) was used. Data acquisition was performed on a FACSCalibur using CellQuestPro software (BD Biosciences) and analysis was performed using FlowJo software program (Tree Star Inc, Ashland, OR).

***Leukocyte isolation***

Immune cells were isolated from neoplastic skin, lymph node, and spleen by either flow sorting or magnetic sorting. Preparation of single cell suspensions from skin has been described above. Single cell suspensions from lymph nodes or spleens were prepared by passing tissue through 70-μm nylon strainers (Falcon). Cells were then incubated for 10 min at 4°C with 2.4G2 (1:200) in PBS containing 1% of BSA to prevent nonspecific antibody binding. Subsequently, cells were incubated for 20 min with appropriate fluorescent primary antibodies that included anti-Gr-1 (RB6-8G5), -CD11b (93), -CD11c (N418) and/or -F4/80 (BM8, eBioscience) at 1:200 dilution, depending on the population to be isolated. To exclude non-viable cells, single-cell suspensions were labeled with 7-AAD. Selected cells were then flow sorted using a FACSAria using Vantage DiVa software (BD Biosciences). $CD4^+$ and $CD8^+$ splenocytes were isolated using magnetic bead selection according to manufactures specifications (Miltenyi Biotec, Auburn, CA).

***Indirect ELISA***

ELISA plates were coated with recombinant protein (1.0 μg/well) and diluted in sodium carbonate buffer (pH 9.3). The following proteins were reconstituted according to manufacturer recommendations: purified HPV16 E7 (Zymed, South San Francisco, CA), laminin-I, laminin-IV, collagen-I (Sigma-Aldrich), collagen-II (Axxora, San Diego, CA), and collagen-IV (BD Biosciences). After overnight incubation at 4°C, plates were washed and then blocked using 1.0 % BSA for 1 hr. Serial 10 fold successive dilutions of sera in PBS-Tween/1.0 % BSA were added and incubated at room temperature for 2 hr. Plates were incubated with biotinylated goat anti-mouse IgG for 1 hr, followed by streptavidin-HRP (Amersham Biosciences, Buckinghamshire, England) for 30 min, and developed with Sigma FAST OPD kit (Sigma) for

10-min. Enzymatic activity was stopped using sulfuric acid (3M). Optical density (OD) was measured at 450 nm with wavelength correction set to 540 nm on a SpectraMax 340 spectrophotomoter (Molecular Devices, Sunnyvale, CA). Antibody concentrations were calculated using SoftMax Pro 4.1 (Molecular Devices).

***Immunoassays for MMP9, cytokines, CIC and Ig ELISA***

Conditioned medium and/or tissue lysates (50 μg of protein) were analyzed for levels of pro-MMP9, MMP9 and VEGF-A (R&D systems) using immunoassay commercially available antibody-pairs, as described by the manufacturer. OD was measured and analyzed as described above.

To analyze CIC, ELISA was performed on purified serum using the CIC Mouse ELISA Kit according to manufacturer's instructions (Alpha Diagnostic, San Antonio, TX). The method is based on the specific binding of C1q to immune complexes, followed by a secondary step where application of anti-IgG antibodies confirms the presence of CIC. To verify presence of CIC in serum, a high salt "confirmation solution" was added to dissociate C1q-CIC binding. Serum samples displaying 30% decrease in absorbance following addition of "confirmation solution" were considered positive for presence of CIC. CIC levels were also evaluated using C3-IgG ELISA where plates were coated with goat anti-mouse C3 (MP Biomedicals, Solon, OH) and analyzed as described above.

To analyze Ig levels, serum ELISA was performed as described by the manufacturer's recommendations using the clonotyping system-HRP kit (SouthernBiotech, Birmingham AL).

***Preparation of bone marrow-derived mast cells (BMMC) and bone marrow-derived macrophages (BMM)***

Bone marrow cells were flushed out of femurs of 4-week old mice using a 23-gauge needle. Cells were then cultured in RPMI 1640 supplemented with 10% FBS, 2.0 mM L-glutamine, 100U/ml penicillin, 100 μg/ml streptomycin, non-essential amino acids, 14.2 mM 2-mercaptoethanol (cRPMI), to which was added either murine recombinant IL3 (for BMMC, 30 ng/ml; PeproTech, Rocky Hill, NJ) or murine recombinant M-CSF (for BMM, 20 ng/ml; PeproTech, Rocky Hill, NJ). Differentiating mast cells were cultured once per week by transferring non-adherent cells and replenishing half of the medium with a fresh one in the

presence of IL3 (10 ng/ml). To verify differentiation of BMMC, flow cytometric analysis was performed 4 weeks later to assess expression of mast cell markers CD117/c-kit and FcεR-1 (eBiosciences). BMMC differentiation was further assessed with toluidine blue for metachromatic staining. Differentiating macrophages were cultured by transferring non-adherent cells 24 hr after bone-marrow cells isolation and replenishing the medium with a fresh one every 48h. BMM differentiation was further assessed by flow cytometry evaluation of F4/80 and CD45 expression. Purity was usually >97%. The resulting populations were used between weeks 4 and 12. All cell cultures were maintained at 37°C in a humidified atmosphere with 5% $CO_2$.

***Stimulation of leukocytes with IgG***

BMMC were starved for 4 hr in cRPMI medium in the absence of IL3, followed by culturing at high density ($5x10^6$ cells/ ml) in cRPMI medium containing IL3 (5.0 ng/ml) in combination with IL4 (20 ng/ml; PeproTech) for 4 days. BMMC or FACS-sorted myeloid population were then washed, resuspended at 5 x $10^5$ cells/ml in DMEM supplemented with 1.0 % BSA, and incubated with 2.4G2 (10 μg/ml) for 10 min at 4°C. To induce cross-linking, cells were then washed and incubated with 25 μg/ml goat F(ab′2) anti-rat IgG (Jackson Immunoresearch, West Grove, PA) in the presence or absence of the mast cell stabilizer cromolyn (10 μM; Sigma). Cells were plated at $10^5$ cells/200 μl in 96-well flat-bottom plates for 24 hr at 37°C. Conditioned medium was collected, centrifuged to remove cellular debris, and stored at -70°C for subsequent analysis.

***T cell activation assay***

Magnetic bead (Miltenyi Biotech) sorted $CD4^+$ or $CD8^+$ T lymphocytes were added to CD3 coated plates (BD Biosciences) at a concentration of 500,000 cells/ml in presence of CD28 antibody (5.0 mg/ml, eBioscience) in RPMI 1640 supplemented with 10% FBS, 2.0 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, non-essential amino acids, and 14.2 mM 2-mercaptoethanol. T cells were co-cultured for 72 hr with increasing ratio of flow-sorted $CD11b^+Gr1^+$ and 18 hr before termination of the assay BrdU (1.0 μM) was added to the culture medium. BrdU incorporation was evaluated using BrdU Cell Proliferation Assay according to manufactures specifications (Millipore).

***Endothelial cell chemotaxis assays***

Confluent human umbilical vein endothelial cells (HUVEC, ATCC) monolayers were harvested and re-suspended in F-12K medium (ATCC) supplemented with 1.0 % BSA. HUVECs were then seeded at $10^5$ cells (100 μl) onto the top chamber of transwell filters (8-μm, Corning, Corning, NY). The filters were then placed in a 24-well plate that contains conditioned medium collected from cell sorted $CD11b^{+}Gr1^{+/-}$ subpopulations stimulated with LPS (10 ng/ml; Sigma), IFNγ/IL13 (10 ng/ml), or IgG (25 μg/ml), as well as control and IgG-stimulated BMMC (600 μl). Addition of chemotactic factors, including $rVEGF_{165}$ (100 ng/ml, R&D Systems) or 10% FBS to lower chambers served as positive controls. To verify that HUVEC-induced migration was VEGF-dependent, a rat monoclonal function-blocking antibody against VEGFR2 (DC101; 100 μg/ml; UCSF Hybridoma Core) was added to lower chambers when indicated. Chambers were incubated for 8 hr at 37°C in a $CO_2$ incubator. Non-migrating cells were gently removed from the filter surface using cotton swabs. Inserts were fixed in cold methanol, followed by incubation with Diff-Quick stain (IMEB Inc, San Marcos, CA). Inserts were then mounted with Cytoseal 60 (Thermo Fisher). HUVEC migration to the underside of the transwell membrane was quantitated by enumerating the number of migrated cells in four random fields (125x total magnification) per insert.

To evaluate CXCL10 and 11-dependent anti-angiogenic activities, HUVEC were cultured for 48 hr in complete F-12K medium supplemented with CXCL10 or CXCL11 (100 nM; Peprotech). HUVEC pre-treated with CXCL10 and 11 were then seeded at $10^5$ cells onto the top chamber of transwell filters (8-μm) in serum free DMEM medium containing 0.1% BSA. Addition of $VEGF_{165}$ (100 ng/ml) or 10% FBS to the lower chamber compartment served as chemoattractants for HUVEC migration. The chambers were then incubated for 4 hr at 37°C in 5% $CO_2$.

***Leukocyte chemotaxis assay***

For cell migration, PBLs were collected from peripheral blood of HPV16 mice following cardiac puncture, and seeded ($10^5$ cells/ 100 μl DMEM containing 0.1% BSA) onto the top chamber of transwell filters (3-μm; Corning). Filters were placed in a 24-well plate that contains conditioned medium isolated from control or IgG-stimulated BMMC. 8 hr following incubation, the lower chamber compartments were fixed in cold methanol, followed by incubation with Diff-

Quick stain (IMEB Inc.). Leukocyte migration to the lower chamber was quantitated by enumeration of the number of the number of cells in 5-8 random fields of view per well using a Lumar microscope (100x total magnification). Samples were run in triplicates for each experimental group.

***Angiogenesis matrigel plug assay***

Matrigel matrix with reduced growth factor composition (BD Biosciences) was diluted 1:1 in cold PBS. PDSC5 cells (Clone 6) were re-suspended at a density of $1.5x10^6$/100 µl and pre-mixed with diluted Matrigel in a total volume of 300 µl. Matrigel was injected s.c. in the ventral side of 7 weeks old mice in the groin area. At day 26 post-injection, Matrigel plugs appeared as lumps on the ventral side of mice. Mice bearing plugs (n=5 per group) were sacrificed, plugs were recovered and fixed in 10% neutral-buffered formalin and paraffin embedded. The extent of neovascularization was evaluated by staining for rat anti-mouse CD31 (BD biosciences).

***In vivo tumorgenicity assays***

PDSC5 cells (Clone 6) suspended in 100 µl of diluted cold Matrigel in PBS (1:1) ($0.5x10^6$ cells) were inoculated s.c. in the flanks of 7 week-old mice. Tumor dimensions were measured at 2-day interval using a digital caliper, and tumor volume was calculated using the equation: V ($mm^3$) = a x $b^2/2$, where a is the largest diameter and b is the smallest diameter. In some experiments, PDSC5 cells were co-transplanted with $CD11b^+Gr1^{+/-}$ sorted from ears and spleens of HPV16/Fc$\gamma R^{-/-}$ versus HPV16/Fc$\gamma R^{+/-}$ mice or IgG-stimulated BMMC at a ratio of 1:1, 3:1 or 10:1 (PDSC5:cells).

***Individual qPCR assays***

*Whole skin*. Skin pieces from PBS-perfused mice were snap-frozen and ground to a powder in liquid nitrogen. Total mRNA was purified following RNeasy Micro/Mini kit guidelines (Qiagen). RNA was quantified with a NanoDrop ND-1000 **(**Thermo Fisher Scientific**)** and retro-transcribed with SuperScript III. Primers specific for *Cxcl10*, *Cxcr3*, *β-actin* (Superarray) were used and relative gene expression determined using RT2 Real-Timetm SYBR Green/ROX PCR master mix (Superarray) on an ABI 7900HT quantitative PCR machine (ABI biosystems). The comparative threshold cycle method was used to calculate fold change in gene expression, which

was normalized to *β-actin* as reference gene.

*Flow-sorted leukocytes from ear tissue*. Single TaqMan gene expression assays (ABI biosystems) were used to quantify transcripts for the following genes: *Arg1*, *Ccl1*, *Cd163*, *Mrc1* and *Ym1*. SYBER Green chemistry (ABI biosystems) was used to quantify transcripts for *Ccl17* and *Ccl22*. The comparative threshold cycle method was used to calculate fold change in gene expression, which was normalized to *β-2m* as reference gene.

***Low-density qPCR arrays***

For each cell sorting session, ear tissue pooled from 4-6 PBS-perfused mice was excised and minced into single-cell suspensions and selected lineages were flow-sorted to obtain 50-200,000 cells per cell population. Total mRNA was purified following RNeasy Micro/Mini kit guidelines (Qiagen). RNA was quantified with a NanoDrop ND-1000 **(**Thermo Fisher Scientific**)** and retro-transcribed with SuperScript III. qPCR analyses were performed with TaqMan assays using Immune Panel TaqMan array (Applied Biosystems, for flow-sorted leukocytes populations), measuring the expression of 96 genes in 2-3 technical replicates (Pucci et al., 2009). 100 ng-1.0 μg of cDNA was loaded on each array. qPCR was run for 40 cycles (low-density arrays) in standard mode using an ABI7900HT apparatus (Applied Biosystems).

***Collection of raw data and determination of gene expression***

The SDS 2.2.1 software was used to extract raw data ($C_T$ and raw fluorescence). The difference ($\Delta C_T$) between the threshold cycle ($C_T$) of each gene and that of the reference genes (*B2m* or *β-actin*) was used to determine gene expression. A threshold of 0.1 was used. The lower the $\Delta C_T$, the higher the gene expression level.

***Statistical analysis of gene expression data***

To calculate the fold-change of gene expression between HPV16/FcRγ$^{-/-}$ and HPV16/ FcRγ$^{+/-}$ cell populations, we used an implemented covariance model (ANCOVA), as previously describe (Pucci et al., 2009). This multiple regression approach is aimed at modelling jointly the impact of different explicative covariates on the outcome of interest. In this case, the outcome variable is $C_T$, and the covariates are the Experiment (i.e. each biological replicate; $X_{Exp}$), the Gene ($X_{Gene}$), and the genetic background (i.e. HPV16/FcRγ$^{+/-}$, HPV16/FcRγ$^{-/-}$; $X_{BG}$). The multiple regression

formula reads as follows:

$$C_T = \beta_0 + \beta_1 \cdot X_{Exp} + \beta_2 \cdot X_{BG} + \beta_3 \cdot X_{BG \bullet Gene} + \varepsilon$$

where $C_T$ is the threshold cycle, $\beta_i$ are the coefficients calculated by the model that represents the impact of the respective qualitative variable $X_i$, $\varepsilon$ is the residual error. $X_i$ is set to zero when Exp = "first replicate", Gene = "*B2m"*, and BG = "HPV16".

The implemented model leads to a procedure equivalent to test the $\Delta\Delta C_T$ (equal to the interaction BG•Gene) (Yuan et al., 2006). The advantage of this procedure with respect to two-by-two t-test comparisons lies on the joint nature of the modeling of all covariates included. This allows avoiding the high frequency of type I errors (false positive results) when multiple comparisons are performed. Estimation technique is based on Likelihood Ratio Test. The model is implemented in R-statistical software (version 2.6.1; see http://www.R-project.org). Significance level is chosen at $\alpha = 0.05$.

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