FcRγ Activation Regulates Inflammation-Associated Squamous Carcinogenesis

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SUMMARY

Chronically activated leukocytes recruited to premalignant tissues functionally contribute to cancer development; however, mechanisms underlying pro- versus anti-tumor programming of neoplastic tissues by immune cells remain obscure. Using the K14-HPV16 mouse model of squamous carcinogenesis, we report that B cells and humoral immunity foster cancer development by activating $Fc\gamma$ receptors ($Fc\gamma Rs$) on resident and recruited myeloid cells. Stromal accumulation of autoantibodies in premalignant skin, through their interaction with activating $Fc\gamma Rs$, regulate recruitment, composition, and bioeffector functions of leukocytes in neoplastic tissue, which in turn promote neoplastic progression and subsequent carcinoma development. These findings support a model in which B cells, humoral immunity, and activating $Fc\gamma Rs$ are required for establishing chronic inflammatory programs that promote de novo carcinogenesis.

INTRODUCTION

Clinical, epidemiological, and experimental studies have established that chronic inflammation contributes to various aspects of solid tumor development (de Visser et al., 2006; Mantovani et al., 2008). In particular, chronic inflammatory diseases, including several autoimmune disorders, are associated with increased risk of cancer development (Brandtzaeg et al., 2006; Dalgleish and O'Byrne, 2002), revealing that B cell hyperactivity combined with altered cellular immunity cooperate to initiate and/or sustain persistent inflammation that enhances overall cancer risk in afflicted tissues.

Deposition of B lymphocyte-derived immunoglobulins (Igs) is a common occurrence in premalignant and malignant stroma of human cancers (de Visser et al., 2006; Tan and Coussens, 2007). In addition, high levels of circulating immune complexes (CIC) are associated with increased tumor burden and poor prognosis in patients with breast, genitourinary, and head and neck malignancies (Tan and Coussens, 2007). While little is known about the function of CICs in tumor development, the role of CICs in inflammatory and autoimmune diseases is undisputed. CIC deposition in stroma has been implicated as an initiator of inflammatory cascades by mechanisms that include activation of complement pathways and engagement of the receptors for the crystallizable region (Fc) of IgG (Fc γ Rs) on the surface of leukocytes (Takai, 2005). As such, Fc γ Rs represent a functional link between adaptive and innate immunity by coupling interactions between circulating (auto)antibodies and innate immune cells (Nimmerjahn and Ravetch, 2008).

Four classes of IgG receptor Fc γ Rs have been identified, Fc γ RI/CD64, Fc γ RII/CD32, Fc γ RIII/CD16, and Fc γ RIV, differing by their distinct affinity for IgG isotypes, cellular distributions, and effector functions (Nimmerjahn and Ravetch, 2008). Activating types of Fc γ Rs form multimeric complexes including the

Significance

Andreu and colleagues demonstrate that peripheral activation of humoral immunity and subsequent activation of $Fc\gamma Rs$ on myeloid cells regulate critical features of carcinogenesis that foster solid tumor development. This work reveals previously unrecognized targets for therapeutic intervention in solid tumors, namely, B cells and $FcR\gamma$ -signaling pathways that work in concert to differentially regulate not only the composition of leukocytes recruited to premalignant tissues but also their bioeffector functions once present.



Figure 1. B Cells Are Critical Regulators of Premalignant Progression in HPV16 Mice

(A) Percentage of CD45⁺ cells in skin single cell suspensions isolated from negative littermates (-LM), HPV16/JH^{+/-}, and HPV16/JH^{-/-} mice at 1, 4, and 6 months of age assessed by flow cytometry.

(B and C) Mast cells (B, blue staining) and Gr1⁺ myeloid cells (C, brown staining) in skin of HPV16/JH^{+/-} and HPV16/JH^{-/-} mice at 1, 4, and 6 months of age assessed quantitatively after chloroacetate esterase histochemistry or Gr1 immunohistochemistry (IHC), respectively.

(D) Flow cytometric analysis of immune cell lineages expressed as percentages of total CD45⁺ leukocyte infiltrates in ear tissue of negative littermates (–LM), HPV16, HPV16/JH^{+/-}, HPV16/JH^{-/-}, and HPV16/RAG1^{-/-} mice at 1, 4, and 6 months of age.

(E) Dendritic (CD11c⁺) and macrophage (F4/80⁺) lineage cell composition of CD11b⁺Gr1⁺ (left) and CD11b⁺Gr1⁻ (right) myeloid populations evaluated by flow cytometry in skin of negative littermates (-LM), HPV16, and HPV16/JH^{-/-} mice at 4 months of age.

(F) Angiogenic vasculature in skin tissue sections from negative littermates (-LM), HPV16/JH^{+/-}, and HPV16/JH^{-/-} mice at 1, 4, and 6 months of evaluation by CD31/PECAM-1 IHC revealing endothelial cells (brown staining).

(G) Reduced VEGF-A and active MMP-9 protein levels in skin extracts from HPV16/JH^{-/-} versus HPV16/JH^{+/-} mice (4 and 6 months) as assessed by ELISA.

Fc receptor common γ chain (FcR γ) that contains an intracellular tyrosine-based activating motif (ITAM), whose activation triggers oxidative bursts, cytokine release, phagocytosis, antibodydependent cell-mediated cytotoxicity, and degranulation (Takai, 2005). In contrast, engagement of $Fc\gamma RIIB$ (or $Fc\gamma RII$ in mice), which contains an immune tyrosine-based inhibitory motif, abrogates ITAM-mediated inflammatory responses and instead regulates alternative signaling cascades (Takai, 2005). FcRy expression is necessary for assembly and cell-surface localization of Fc γ RI, Fc γ RIII, and Fc γ RIV; as such, FcR $\gamma^{-/-}$ mice (Takai et al., 1994) are deficient for all activating Fc_YRs, whereas Fc_YRII expression is unaltered. Given that developing solid tumors display similar characteristics to tissues damaged by autoimmune dysfunction, e.g., chronic immune cell infiltration, tissue remodeling, angiogenesis, and altered cell survival pathways, we speculated that similar humoral immune-mediated regulatory pathways may be involved in solid tumor development.

Using a transgenic mouse model of multistage epithelial carcinogenesis, i.e., K14-HPV16 mice (Coussens et al., 1996), we previously revealed that adaptive immunity is an important requlator of inflammation-associated cancer development (de Visser et al., 2005). Combined B and T lymphocyte deficiency in HPV16 mice, e.g., HPV16/RAG1^{-/-} mice, resulted in a failure to initiate and/or sustain leukocyte infiltration during premalignancy (de Visser et al., 2005). As a consequence, tissue remodeling, angiogenesis, and epithelial hyperproliferation were significantly reduced, culminating in attenuated premalignant progression and a 43% reduction in carcinoma incidence (de Visser et al., 2005). Importantly, adoptive transfer of B lymphocytes or serum from HPV16 mice into HPV16/RAG1^{-/-} mice reinstated chronic inflammation in premalignant tissues, indicating that B cellderived soluble mediators were necessary to potentiate malignant progression. In the present study, we investigated whether B cell-derived IgGs regulate neoplastic progression and subsequent carcinoma development by engagement of FcyRs expressed on resident and recruited immune cells.

RESULTS

Humoral Immunity-Mediated Promotion of Squamous Carcinogenesis in HPV16 Mice

HPV16 mice express the early region genes of human papillomavirus type 16 (HPV16) under control of the human keratin 14 promotor/enhancer (Arbeit et al., 1994). By 1 month of age, HPV16 mice develop epidermal hyperplasias with 100% penetrance characterized by a terminally differentiated hyperproliferative epidermis. Between 3 and 6 months of age, hyperplastic lesions advance focally into angiogenic dysplasias with prominent hyperproliferative epidermis that fails to undergo terminal differentiation and a dermis containing significant CD45⁺ leukocyte infiltration encompassing CD117⁺ mast cells and CD11b⁺Gr1⁺ immature myeloid cells (IMCs; Coussens et al., 1999; de Visser et al., 2005; Junankar et al., 2006) (Figures 1A–1H). By 1 year of age, 60% of HPV16 mice (FVB/n, N25) develop malignant skin carcinomas, 50% of which are squamous cell carcinomas (SCCs) that metastasize to regional lymph nodes with a ~30% frequency (Coussens et al., 1996). HPV16 mice lacking mast cells (Coussens et al., 1999), leukocyte-derived matrix metalloproteinase (MMP)-9 (Coussens et al., 2000), or B and T lymphocytes, e.g., HPV16/RAG1^{-/-} mice (de Visser et al., 2005), exhibit attenuated parameters of premalignant progression culminating in reduced SCC development.

Given that adoptive transfer of B cells or serum (isolated from HPV16 mice) into HPV16/RAG1-/- mice restored hallmarks of premalignant progression (de Visser et al., 2005), we hypothesized that humoral immunity represented the critical feature of premalignant progression regulating chronic inflammation. To investigate this, we generated HPV16 mice deficient for B220⁺CD19⁺ mature B cells (Chen et al., 1993), e.g., HPV16/ JH^{-/-} mice (Figure S1A, available online). Similar to HPV16/ RAG1^{-/-} mice, HPV16/JH^{-/-} mice exhibited reduced infiltration of premalignant skin by CD45⁺ leukocytes, including mast cells and Gr1⁺ myeloid cells (Figures 1A-1C). Whereas CD11b⁺ Gr1+F4/80-CD11c- IMCs constitute the most abundant CD45+ leukocyte subtype in premalignant HPV16 skin (Figures 1D and 1E), immune cell infiltrates in HPV16/JH^{-/-} mice instead revealed an increased relative proportion of CD11b⁺Gr1⁻ cells (Figure 1D) that contained F4/80⁺ and CD11c⁺ cells (Figure 1E), as well as expanded populations of CD3⁺CD4⁺ and CD3⁺CD8⁺ T cells (Figure 1D). In addition, HPV16/JH $^{-\!/-}$ mice exhibited reduced presence of CD31⁺ blood vessels (Figure 1F), vascular endothelial growth factor (VEGF), and MMP-9 protein levels (Figure 1G and Figure S1B); reduced keratinocyte hyperproliferation (Figure 1H); and diminished presence of focal dysplastic lesions (Figure 1I). Together, these data indicate that B cells are critical components of adaptive immunity regulating premalignant progression and characteristics of early squamous carcinogenesis in HPV16 mice.

HPV16-Induced Autoantibody Complexes Induce Acute Inflammation

Because parameters of premalignant progression were reinstated in HPV16/RAG1^{-/-} mice by adoptive transfer of serum from HPV16 animals, and because dysplastic skin of HPV16 mice is characterized by stromal depositions of IgG and IgM (de Visser et al., 2005), we hypothesized that Igs were the mediators by which B cells promote premalignant progression. To assess this, we first evaluated CIC presence in serum of HPV16 mice and found increased concentrations paralleling premalignant progression (Figure 2A). Using direct immunofluorescence with biotinylated IgGs isolated from HPV16 serum (IgG_{HPV16}) as detector antibodies, we revealed that IgG_{HPV16}

⁽H) Keratinocyte proliferation in skin of negative littermates (–LM), HPV16/JH^{+/-}, and HPV16/JH^{-/-} mice at 1, 4, and 6 months of age, as evaluated by quantitation of bromodeoxyuridine (BrdU)-positive keratinocytes (red staining).

⁽I) Percentage of ear skin in HPV16/JH^{-/-} and HPV16/JH^{+/-} mice developing hyperplastic lesions by 1 month of age (Hyp) or dysplasia by 4 and 6 months of age (Dys).

⁽A–I) Results shown represent mean \pm SEM (n = 5–8 mice) and asterisks (*) indicate statistically significant differences (p < 0.05, Mann-Whitney). Representative images of HPV16/JH^{+/-} and HPV16/JH^{-/-} mouse skin at 4 months of age are shown. Values represent average of five high-power fields of view per mouse and five mice per category. FOV, field of view; solid red line, epidermal-dermal interface; e, epidermis; d, dermis. Scale bars represent 50 μ m. See also Figure S1.



Figure 2. CICs from HPV16 Mice Induce Acute Inflammation

(A) CICs titers in serum collected from negative littermate (–LM), HPV16/JH^{+/+}, and HPV16/JH^{-/-} mice assessed by ELISA with anti-mouse C3 Ig (left) and C1q (right). The specificity of C1q/IgG binding to ICs was assessed by disrupting C1q-CIC binding using a high-salt solution (C1q/IC/d; gray bars).

(B) Antibody titers for laminin (Lm)-111, Lm-332, collagen (Col)-I, Col-II, and Col-IV in serum from littermate (–LM), HPV16, and HPV16/JH^{-/-} (4 months) were evaluated by indirect ELISA. Antigens recognized by biotinylated IgG_{HPV16} were localized by immunofluorescence on skin sections from 4-month-old HPV16 mice (inset; IgGs: red staining).

(C) Immunofluorescence (green staining) staining on syngeneic skin tissue sections reveals presence of intradermally injected purified IgGs isolated from negative littermate (IgG_{wt}) or HPV16 (IgG_{HPV16}) 24 hr after intradermal injections.

(D) Number of infiltrating CD45⁺ leukocytes and Gr1⁺ cells in skin tissue sections 24 hr after intradermal injections of PBS, IgG_{wt}, or IgG_{HPV16} into ears of syngeneic wild-type FVB/n mice. Values reflect averages from five high-power fields per mouse.

(E) Normalized CIC titers of IgG purified from negative littermates (IgG_{wt}) or HPV16 (IgG_{HPV16}) serum assessed by ELISA with anti-mouse C3 Ig (left) and C1q (right).

(B and C) Blue staining, DAPI; white line, epidermal-dermal interface; e, epidermis; d, dermis. Scale bars represent 50 µm.

(B–D) Results shown are mean percentages ± SEM (n = 5–8 mice) and asterisks (*) indicate statistically significant differences (p < 0.05, Mann-Whitney). See also Figure S2.

cognate antigens were localized to both epithelial and dermal compartments of neoplastic skin (Figure 2B, inset). In addition to HPV16 E7 oncoprotein-specific autoantibodies (Figure S2) (Daniel et al., 2005), we identified high titer IgGs specific for type I, II, and IV collagens, but not laminins 111 and 332 (Figure 2B).

To determine whether stromal deposition of autoantibodies was sufficient to induce an acute inflammatory response in vivo, we injected IgG_{HPV16} versus IgG isolated from negative littermate mice (IgG_{wt}) intradermally into syngeneic FVB/n mice. While IgG_{HPV16} and IgG_{wt} antibodies were similarly detected in dermis (Figure 2C), only mice injected with IgG_{HPV16} exhibited an acute inflammatory response characterized by a significant

increase in CD45⁺ and Gr1⁺ cell recruitment (Figure 2D). Since similar concentrations of IgG_{HPV16} versus IgG_{wt} differentially induced leukocyte recruitment in vivo, we hypothesized that higher proportions of IgG_{HPV16} as opposed to IgG_{wt} were present in their active form, e.g., in immune complexes (ICs), and indeed, we found that IgG_{HPV16} contains significantly higher levels of both IgG/C3 and IgG/C1q ICs as compared to IgG_{wt} (Figure 2E).

Differential Expression of $Fc\gamma R$ Ig Receptors on Leukocytes in HPV16 Neoplastic Skin

Premalignant progression in HPV16 mice is independent of complement cascade activation via complement factor C3 (de Visser et al., 2004); thus, we hypothesized that CICs

accumulating in young HPV16 mice promoted neoplastic progression through activation of FcR γ signaling. Fc γ Rs are broadly expressed on immune cells and encompass both activating, e.g., $Fc\gamma RI$, III, and IV (including the $FcR\gamma$ subunit), and inhibitory, e.g., FcyRII, subtype complexes (Nimmerjahn and Ravetch, 2008). Immunodetection of FcRy, FcyRI, and FcyRII/ III revealed increased presence of CD45⁺Fc γ R⁺ cells in dermal regions of premalignant HPV16 skin (Figure 3A). Using flow cytometry, we revealed that while CD11b+Gr1+ IMCs and CD45⁺CD117⁺ mast cells expressed Fc_YRIII, CD11b⁺Gr1⁻ cells, including F4/80⁺ macrophages and CD11c⁺ dendritic cells (DCs), expressed FcyRI and FcyRIII, and no expression of any FcγR was detected on CD3⁺ T cells or CD45⁻ nonimmune cells in neoplastic skin (Figure 3B). B cells were not evaluated given their undetectable levels in HPV16 skin (Figure 1D) (de Visser et al., 2005).

Leukocyte FcR γ Is Necessary for Tumor Development and Squamous Carcinogenesis

Because IgG_{HPV16} induced acute inflammatory responses in syngeneic nontransgenic mice, and proinflammatory-type Fc γ Rs were expressed on infiltrating leukocytes in premalignant HPV16 skin, we evaluated whether carcinoma growth or de novo squamous carcinogenesis were Fc γ R dependent. First we assessed transplantable tumor growth with syngeneic FcR $\gamma^{-/-}$ (Takai et al., 1994) versus FcR $\gamma^{+/-}$ mice injected subcutaneously with PDSC5 cells, a carcinoma cell line derived from a poorly differentiated SCC (Arbeit et al., 1996). Mice lacking FcR γ failed to mount a robust angiogenic response (Figure 3C) as well as to support transplantable tumor growth (Figure 3D), which was independent of humoral immune responsiveness as serum Ig titers increased in PDSC5-injected mice irrespective of FcR γ expression (Figure 3D).

To evaluate whether de novo tumorigenesis was similarly FcR γ dependent, we generated a cohort of HPV16/FcR γ^{-1} mice that retained expression of inhibitory FcvRII (Figure S3A). Quantitative evaluation of CD45⁺ immune cell infiltrates in neoplastic skin by flow cytometry revealed reduced leukocyte infiltration in HPV16/FcR $\gamma^{-/-}$ mice as compared to age-matched HPV16/FcR $\gamma^{+/-}$ tissue (Figure 4A). Similar to HPV16/JH^{-/-} mice, mast cells and IMCs were significantly reduced in HPV16/ $FcR\gamma^{-/-}$ mice, concomitant with an increased relative influx of CD11b⁺Gr1⁻ macrophages and DCs, as well as CD3⁺CD4⁺ and CD3⁺CD8⁺ lymphocytes (Figures 4B-4D). F4/80 and CD11c lineage marker expression on both CD11b⁺Gr1⁺ and CD11b⁺Gr1⁻ cells were unperturbed by absence of FcR γ (Figure 4E and Figure S3D). Fc_YRIII-expressing CD45⁺CD49b⁺CD3⁻ NK cells represented a minor population in control HPV16 skin, recruitment of which was not modified by FcRy deficiency (Figures S3B and S3C).

To determine whether the activating types of Fc γ Rs also mediated other parameters of de novo squamous carcinogenesis, we analyzed age-matched HPV16/FcR $\gamma^{-/-}$ mice at canonical time points (1, 4, and 6 months of age) and found reduced development of angiogenic vasculature (Figure 4F), VEGF, and MMP-9 protein levels (Figure 4G and Figure S3E); reduced keratinocyte hyperproliferation and appearance of focal dysplastic regions (Figures 4H and 4I); and significantly reduced incidence of SCC development (Figure 4I). Diminished de novo carcinogenesis in HPV16/FcR $\gamma^{-/-}$ mice was independent of B cell responses and humoral immunity as shown by Ig isotype switching and accumulation of IgG₁ and IgG_{2a} in HPV16/FcR $\gamma^{-/-}$ mice (Figure S3F). Our interpretation of these findings was that peripheral activation of humoral immunity in young HPV16 mice promoted squamous carcinogenesis by locally activating FcR γ -mediated signaling on resident and recruited immune cells in neoplastic skin. These in turn activate angiogenic programs in vascular cells, thus enabling tissue expansion via keratinocyte hyperproliferation, culminating in increased SCC development.

Mast Cell FcRγ Activation Regulates Parameters of Premalignant Progression and Enhances Tumor Development

Given that we found that combinations of activating Fc γ Rs were expressed on infiltrating leukocytes in HPV16 skin, and given that absence of either humoral immunity or FcR γ altered leukocyte composition during premalignant composition, we sought to delineate which distinct FcR γ^+ leukocyte population exhibited protumorigenic properties.

 $Fc\gamma RIII^+$ mast cells regulate tissue remodeling, angiogenesis, and keratinocyte hyperproliferation in HPV16 mice (Coussens et al., 1999; Coussens et al., 2000), thus we evaluated whether FcR_γ-dependent activation of mast cells was in part necessary for neoplastic progression. Using conditioned medium generated from FcR_Y-deficient versus FcR_Y-proficient bone marrowderived mast cells (BMMCs) previously stimulated with either rat IgG, IgG_{HPV16}, or IgG_{wt}, we found that Ig-activated mast cells induced human umbilical vein endothelial cell (HUVEC) migration (Figure 5A), VEGF expression (Figure 5B), and CD45⁺ peripheral blood leukocyte (PBL) recruitment (Figure 5C), which were dependent on FcRy expression. The diminished capabilities of FcR_Y-deficient mast cells were conserved in vivo as PDSC5 tumor growth was significantly diminished in mast cell-deficient (kit^{sh/sh}) mice, whereas presence of FcRγ-proficient BMMCs significantly enhanced tumor growth, development of angiogenic vasculature, and infiltration by Gr1⁺ leukocytes (Figures 5D and 5E). These features were not significantly altered in PDSC5 tumors grown in the presence of FcR $\gamma^{-\prime-}$ BMMCs as compared to PDSC5 cells alone (Figure 5E). Thus, these findings support a model in which mast cells respond to CIC deposition in early neoplastic stroma by activating FcyR-mediated pathways, leading to PBL recruitment and angiogenesis, which together establish a microenvironment permissive for tumor development.

FcRγ-Independent and -Dependent Properties of CD11b⁺ Myeloid Cells in HPV16 Mice

As described both in cancer patients and mice harboring some transplantable tumors (Ostrand-Rosenberg, 2008), CD11b⁺Gr1⁺ cells accumulate in spleen and peripheral blood of HPV16 mice (Figure S4A). Morphological analysis of CD11b⁺Gr1⁺ cells isolated from neoplastic skin and spleen of HPV16 mice revealed characteristics of immature granulocytes, including presence of elongated band-shaped, nonfragmented nuclei (Figure 6A) in cells encompassing a mixed population expressing CD45, 7/4, CD14, CD44, IL4R α , CD80, and CD86, but not CD34 (Figure S4B). In contrast, CD11b⁺Gr1⁻ cells (from skin) accumulate only in draining lymph nodes of HPV16 mice (Figure S4A),



Figure 3. Infiltration of $Fc\gamma R^+$ Leukocytes during Neoplastic Progression in HPV16 Mice

(A) Infiltration of leukocytes expressing FcR γ (red staining, top), Fc γ RI (red staining, middle), and Fc γ RII/III (brown staining, bottom) in premalignant skin of HPV16 mice evaluated in tissue sections by immunofluorescence. Shown in the right panels is double immunofluorescence staining revealing expression of FcR γ and Fc γ RII/III on CD45⁺ leukocytes. Solid line, epidermal-dermal interface; e, epidermis; d, dermis; blue staining, DAPI. Scale bars represent 50 μ m. (B) Differential expression of Fc γ RII by individual leukocyte populations in premalignant skin of HPV16 (red) and HPV16/JH^{-/-} (blue) mice at 4 months of age. Live cells were gated as CD45⁺ leukocytes, CD45⁺CD3⁺ T lymphocytes, CD11b⁺Gr1⁺ IMCs, CD11b⁺Gr1⁻ mixed macrophages/DCs, and CD45⁺CD117⁺

of age. Live cells were gated as CD45⁺ leukocytes, CD45⁺CD3⁺ T lymphocytes, CD11b⁺Gr1⁺ IMCs, CD11b⁺Gr1⁻ mixed macrophages/DCs, and CD45⁺CD117⁺ mast cells. Grey line, Ig control. (C) PDSC5 tumor cells were injected as matrigel plugs into $FcR\gamma^{+/-}$ and $FcR\gamma^{-/-}$ mice (FVB/n). Neovascularization was evaluated by CD31 IHC (brown staining).

(C) PDSC5 tumor cells were injected as matrigel plugs into FCH γ and FCH γ mice (FVB/n). Neovascularization was evaluated by CD31 IHC (brown staining Values reflect number of CD31⁺ vessels averaged from five high-power fields per mouse (n = 5–8 mice). Scale bars represent 25 μ m.

(D) Deficient tumor growth in mice lacking FcR γ . PDSC5 tumor cells were injected s.c. into FcR $\gamma^{+/-}$ and FcR $\gamma^{-/-}$ syngeneic FVB/n mice. Titers of IgG in serum of wild-type (WT) FVB/n versus transplanted FVB/n mice evaluated by ELISA.

(C and D) Results shown are mean percentages ± SEM. Asterisks (*) indicate statistically significant differences (p < 0.05, unpaired t test).

exhibited a more mature phenotype with larger cellular diameters, dense granules, vacuole-rich cytoplasm, and round nuclei (Figure 6A), and reflected subpopulations orientated toward dendritic (CD11c⁺) and macrophage lineages (F4/80⁺) (Figure S4B).

To delineate which distinct $FcR\gamma^+$ myeloid population present in HPV16 neoplastic skin exerted FcR_Y-dependent protumor properties, and considering the fact that differential activation of FcyRs regulates DC maturation (Takai, 2005), we evaluated expression of CD86, CD80, and MHC-II by flow cytometry in CD11b+Gr1-CD11c+F4/80- DCs from cervical lymph nodes and premalignant skin of HPV16/FcR $\gamma^{+/-}$ and HPV16/FcR $\gamma^{-/-}$ mice and found no significant differences (Figure S5A). Similarly, when we audited gene expression of CD11b+Gr1-CD11c+F4/ 80^{-} skin DCs isolated from age-matched HPV16/FcR $\gamma^{+/-}$ and HPV16/FcR $\gamma^{-/-}$ mice by low-density qPCR arrays, we found no change in expression of genes reflecting DC maturation. However, HPV16/FcR $\gamma^{-/-}$ DCs reflected myeloid population polarized toward a T_H1 state as shown by enhanced expression of Nos2, IL1a, IFN_Y, IL12a, Ptgs2, and IL6 (Figure S5B and Table S1).

CD11b⁺Gr1⁺ myeloid-derived suppressor cells (MDSCs) have been reported to promote tumor development by enhancing angiogenesis and by inhibiting T lymphocyte-mediated antitumor immunity (Ostrand-Rosenberg, 2008). Since CD11b+Gr1+ accumulate in HPV16 neoplastic tissue and peripheral sites and exhibit an immature morphology (Figure 6A and Figure S4B), we assessed their immune-suppressive capabilities as compared to CD11b⁺Gr1⁺ cells isolated from tumors and spleens of 4T1 mammary tumor-bearing mice, a model where immune-suppressive properties of MDSCs have been previously described (Figure S5C) (Ostrand-Rosenberg, 2008). Neither CD11b⁺Gr1⁺ cells isolated from premalignant skin nor from spleen of HPV16 mice demonstrated in vitro inhibition of polyclonal activation of either CD4⁺ or CD8⁺ T cells (Figure S5C). In addition, CD11b⁺ Gr1⁺ cells failed to produce reactive oxygen species, major mediators implicated in myeloid-mediated immune suppression (Figure S5D) (Ostrand-Rosenberg, 2008). Moreover, using low-density qPCR arrays, gene expression analysis of HPV16/ FcR $\gamma^{-/-}$ versus HPV16/FcR $\gamma^{+/-}$ skin CD11b⁺Gr1⁺ cells revealed downregulation of *IL1a*, *Ptgs2*, and *TNF* α , indicative of a diminished proinflammatory state (Figure S5E and Table S1). Moreover, when co-injected with PDSC5, CD11b⁺Gr1⁺ cells isolated from skin of HPV16 mice failed to alter either tumor growth or development of angiogenic vessels (Figures 6B and 6C) and did not exhibit proangiogenic activity in vitro (Figure 6D). Thus, although present in significant numbers, CD11b⁺Gr1⁺ cells infiltrating neoplastic skin are likely to represent a population of bona fide immature cells.

FcRγ Activation Mediates the Protumor and Angiogenic Bioactivities of CD11b⁺Gr1⁻F4/80⁺ Macrophages

In contrast to IMCs, both spleenic and skin CD11b⁺Gr1⁻CD11c⁻ F4/80⁺ macrophages induced HUVEC migration in vitro by a FcR_Y-dependent mechanism (Figure 6D) and significantly enhanced PDSC5 tumor growth in vivo (Figures 6B and 6C). Moreover, macrophage-enhanced tumorgenicity of PDSC5 cells was also FcR_Y dependent (Figures 7A and 7B). Interestingly, bone marrow-derived FcR_Y^{-/-} macrophages, when admixed

with PDSC5 cells, not only failed to promote transplantable tumor development but also impeded tumor growth (Figure 7B).

Given that DC gene expression analyses revealed altered programming toward a T_H 1-type state, we reasoned that perhaps, similarly, in the absence of FcR γ signaling, macrophages were also reprogrammed. Indeed, using low-density qPCR arrays and RT-PCR, gene expression analyses of macrophages isolated from HPV16/FcR $\gamma^{+/-}$ versus HPV16/FcR $\gamma^{-/-}$ skin revealed significant upregulation of genes reflecting classical "M1" activation, including *II1b*, *II12a*, *CxcI10*, *Nos2*, *CxcI11*, and *IL1a*, whereas genes reflecting alternative "M2" (*II13*, *Cd163*, *CcI17*, *II4*, and *Ym1*) or "M2-like" (*CcI1*) activation were significantly downregulated in HPV16/FcR $\gamma^{-/-}$ as compared to HPV16/FcR $\gamma^{+/-}$ macrophages (Figure 7C).

Among the differentially expressed genes, the angiostatic chemokines *Cxcl10 and Cxcl11* were significantly elevated in HPV16/FcR $\gamma^{-/-}$ macrophages (Figure 7C). We confirmed that mRNA expression of *Cxcl10*, as well as its receptor *Cxcr3*, were significantly upregulated in whole neoplastic skin of 4-month-old HPV16/FcR $\gamma^{-/-}$ and HPV16/JH^{-/-}, as compared to age-matched HPV16/FcR $\gamma^{+/-}$ skin by qRT-PCR (Figure 7D). Given that VEGF-induced HUVEC migration was significantly inhibited by CXCL10 in a CXCR3-dependent manner (Figure 7E), we evaluated FcR γ -deficient versus FcR γ -proficient macrophages isolated from the respective HPV16 cohorts and revealed CXCR3-dependent angiostatic activity of FcR $\gamma^{-/-}$ macrophages (Figure 7F).

DISCUSSION

We revealed a provocative and functional role for B cells and activating type FcyRs as potentiators of squamous carcinogenesis. Using a transgenic mouse model of epithelial carcinogenesis and mice lacking either B cells or activating Fc_γRs, we found that IC stimulation of leukocyte FcR_Y is critical for establishing a protumor microenvironment in premalignant tissue that directs not only recruitment of leukocytes from peripheral blood but also leukocyte composition, phenotype, and bioeffector functions once within neoplastic tissue (Figure 8). As such, proangiogenic and protumorigenic functions of mast cells and macrophages are differentially regulated by humoral immunity and functionally contribute to squamous carcinogenesis (Figure 8). These findings have broad clinical implications as they reveal critical signaling pathways regulated by humoral immunity and $FcR\gamma$ to target therapeutically in patients at risk for cancer development, e.g., patients suffering from chronic inflammatory diseases, as well as individuals harboring premalignant lesions where chronic inflammation compromises tissue integrity and enhances risk of malignancy.

Regulation of Protumor Immunity by B Cells, Humoral Immunity, and Activating $Fc\gamma Rs$

While early and persistent inflammatory-type reactions in or around developing neoplasms are thought to regulate tumor development (de Visser et al., 2005; Mantovani et al., 2008), tumor-promoting properties of adaptive leukocytes have not been fully elucidated. As the central component of humoral immunity, B lymphocytes function in antibody production, antigen presentation, and secretion of proinflammatory cytokines. In the



* % ear epidermis evidencing hyperplastic or dysplastic histopathology at age indicated

Figure 4. FcR γ Expression Is a Critical Determinant of Squamous Carcinogenesis in HPV16 Mice

(A) Percentage of CD45⁺ cells in skin of HPV16/FcR $\gamma^{+/-}$ and HPV16/FcR $\gamma^{-/-}$ mice at 1, 4, and 6 months assessed by flow cytometry.

(B and C) Mast cells (B, blue staining) and Gr1⁺ myeloid cells (C, brown staining) in skin of HPV16/FcRY^{+/-} and HPV16/FcRY^{-/-} mice at 1, 4, and 6 months of age assessed quantitatively after chloroacetate esterase histochemistry or Gr1 IHC, respectively.

(D) Flow cytometric analysis of immune cell lineages as percentages of total CD45⁺ leukocytes in single-cell suspensions from nontransgenic, HPV16/FcR_Y^{+/-}, and HPV16/FcR_Y^{-/-} transgenic skin at 1, 4, and 6 months of age.

(E) Dendritic (CD11c⁺) and macrophage (F4/80⁺) cell composition in CD11b⁺Gr1⁺ and CD11b⁺Gr1⁻ myeloid populations evaluated by flow cytometry of single cell suspensions derived from skin of negative littermate (–LM), HPV16, and HPV16/FcR $\gamma^{-/-}$ mice at 4 months of age.

(F) Attenuated angiogenesis in premalignant skin of HPV16/FcR $\gamma^{-/-}$ mice. Density of angiogenic vasculature evaluated quantitatively by CD31 IHC (brown staining) on age-matched tissue sections.

(G) Reduction in total VEGF-A and active MMP-9 protein levels in skin tissue extracts from age-matched HPV16/FcRγ^{-/-} and HPV16/FcRγ^{+/-} mice as determined by ELISA.

(H) Keratinocyte proliferation evaluated as percentage of bromodeoxyuridine (BrdU)-positive keratinocytes (red staining) in ear skin tissue sections representing age-matched negative littermate (-LM), HPV16/FcR $\gamma^{+/-}$, and HPV16/FcR $\gamma^{-/-}$ premalignant skin.

(I) Percentages of ear skin (area) exhibiting hyperplasia by 1 month of age (Hyp) or dysplasia by 4 or 6 months of age (Dys). Values represent percentages of mice with specific neoplastic phenotypes. Statistical significance was determined using the Mann-Whitney test. Lifetime incidence of SCC was determined



Figure 5. Activating FcyRs Regulate Protumor Functions of Mast Cells

(A) $Fc\gamma R$ -dependent chemotaxis of HUVECs in response to $Fc\gamma R$ -stimulated mast cells isolated from $FcR\gamma^{+/-}$ or $FcR\gamma^{-/-}$ mice. BMMCs were stimulated with 2.4G2 and anti-rat IgG (25 µg/ml; $Fc\gamma R$ -stim.), IgG_{HPV16}, or IgG_{wt} (30 µg/ml). HUVEC migration in response to conditioned medium was assessed using a Boyden chamber assay. A specific VEGF-R2 inhibitor (DC101; 100 µg/ml) was used. HUVEC migration was quantitated by enumerating the number of migrating cells in four random fields per membrane (125× magnification). Samples were assayed in quadruplet for each assessed condition.

(B) BMMCs from $FcR\gamma^{+\prime-}$ or $FcR\gamma^{-\prime-}$ mice were $Fc\gamma R$ stimulated or activated with IgG_{HPV16} or IgG_{wt} in the presence or absence of the mast cell stabilizer cromolyn (10 μ M). Levels of VEGF-A in conditioned medium were assessed by ELISA. Representative analysis from three independent experiments is shown. (C) PBL migration in response to conditioned medium from $FcR\gamma^{+\prime-}$ versus $FcR\gamma^{-\prime-}$ BMMCs after $Fc\gamma R$ stimulation was evaluated with a Boyden chamber assay. PBLs migrating to the lower chamber were visualized by H&E staining in five to eight random fields per well. Samples were assayed in triplicate for each tested condition.

(D) FcR γ -proficient mast cells enhance tumorigenicity. PDSC5 tumor cells (blue) alone or admixed with FcR $\gamma^{+/-}$ (red) or FcR $\gamma^{-/-}$ (green) BMMCs were injected s.c. into FVB/n (WT) or kit^{sh/sh} (gray) mice at a 1:1 ratio. The asterisk (*) indicates statistically significant differences between PDSC5 cells in combination with FcR $\gamma^{+/-}$ versus FcR $\gamma^{-/-}$ BMMCs. The number sign (#) indicates statistically significant differences between tumor growth in syngeneic FVB/n or kit^{sh/sh} mice. (E) FcR γ -proficient mast cells induce angiogenesis and leukocyte infiltration of transplantable tumors. Blood vessels and leukocyte infiltration were evaluated by CD31 and Gr1 IHC. Values represent average of five high-power fields of view per tumor and six to ten tumors per category. (A–E) Data are represented as means ± SEM; *p < 0.05; **p < 0.01 (unpaired t test).

context of cancer, in addition to altering local and circulating levels of cytokines, B cells also inhibit T_H1 -mediated anti-tumor immunity. In a transplantable model of colorectal cancer, partial B cell depletion resulted in significantly reduced tumor burden (Barbera-Guillem et al., 2000), while B cell-deficient mice showed resistance to syngeneic tumors (Shah et al., 2005). In addition, overexpression of tumor necrosis factor receptor-associated factor 3 in lymphocytes activates humoral immune responses that result in chronic inflammation and enhanced incidence of cancer, particularly SCCs (Zapata et al., 2009).

Using HPV16/B cell-deficient mice, we found that premalignant progression was significantly attenuated and essentially stalled at an early hyperplastic stage. In the absence of B cells, leukocyte recruitment from peripheral blood was reduced, vasculature failed to mount an angiogenic response, and hyperproliferation of keratinocytes failed to support tissue expansion to a carcinoma in situ state. Thus, peripheral activation of B cells represents an early event in premalignant progression that promotes subsequent neoplastic programming of tissue.

in HPV16/FcR $\gamma^{+/-}$ and HPV16/FcR $\gamma^{-/-}$ mice (97 and 132 mice/group, respectively). Tumor incidence was analyzed with the generalized Wilcoxon test. Hazard ratio was determined by Kaplan Meyer analysis of tumor incidence. All mice reflect similarly backcrossed groups at FVB/n, N5.

⁽A–H) Results shown represent mean \pm SEM (n = 5–8 mice) and asterisks (*) indicate statistically significant differences (p < 0.05, Mann-Whitney). Values represent average of five high-power fields of view per mouse. Representative images of HPV16/FcR $\gamma^{+/-}$ and HPV16/FcR $\gamma^{-/-}$ skin tissue sections at 4 months of age are shown. Red line, epidermal-dermal interface; e, epidermis; d, dermis. Scale bars represent 50 μ m. See also Figure S3.

FcR_Y Activation Potentiates Squamous Carcinogenesis



How do B cells "promote" solid tumor formation? It is well known that cancer patients develop antibodies to tumor-associated antigens-evidence exist for c-myc, HER-2/neu, and p53 (Lu et al., 2008). In addition, high circulating levels of ICs are associated with increased tumor burden and poor prognosis in patients with breast, genitourinary, and head and neck malignancies (Tan and Coussens, 2007). In a chemical carcinogenesis mouse model of papilloma growth, immunization against an inherited oncoprotein failed to eradicate tumor cells but rather induced tumor growth (Siegel et al., 2000). Increased levels of Ig in neoplastic microenvironments result in accumulation of ICs that favor tumor-promoting inflammatory responses including recruitment and activation of several myeloid cell types (Barbera-Guillem et al., 1999). When injected into syngeneic nontransgenic mice, CICs (from HPV16 mice) alone were sufficient to trigger an acute inflammatory response. While largely not described in the context of cancer, the significance of inflammatory responses to autoantibodies has been studied in mouse models of autoimmune disease (Nimmerjahn and Ravetch, 2008) where FcyRs have been recognized as effectors that induce recruitment of CD11b-expressing myeloid cells into tissue (Bergtold et al., 2006). Thus, mice deficient in activating-type $Fc\gamma Rs$ are resistant to IC-mediated hypersensitive reactions, such as alveolitis, glomerulonephritis, and skin Arthus reaction, while mice deficient in FcyRII exhibit enhanced IC-mediated inflammatory responses (Takai, 2005). Herein, we found differential presence of both activating and inhibitory types of FcyRs on infiltrating myeloid cells, and using FcRy-deficient mice, we

Figure 6. Tumor-Promoting Activities of CD11b⁺Gr1^{+/-} Myeloid Cells in HPV16 Mice

(A) Representative micrographs depicting morphology of CD11b⁺Gr1⁺ and CD11b⁺Gr1⁻ myeloid cells purified by FACS sorting from skin or spleen of HPV16 mice (4 month) and visualized via May-Grünwald-Giemsa staining (scale bars represent 50 μm).

(B) Macrophages and spleen IMCs enhance tumorgenicity. Tumor growth of PDSC5 cells alone (red line) or admixed with either IMCs (blue line) or macrophages (MØ, green line; PDSC5/leukocyte = 10:1) FACS sorted from neoplastic skin (top graph) or spleen (bottom graph) of HPV16 mice at 4 months of age. Asterisks (*) and number signs (#) indicate statistically significant differences between PDSC5 cells alone and PDSC5 in combination with macrophages and IMCs, respectively. (C) Macrophages and spleen IMCs enhance tumor angiogenesis. Blood vessels in PDSC5 subcutaneous tumors were evaluated by CD31 and Gr1 IHC. Values represent average of five high-power fields of view per tumor and six to ten tumors per category.

(D) FcyR-stimulated macrophages are chemotactic toward endothelial cells. Macrophages and IMCs were purified by FACS sorting from neoplastic skin and spleens of 4-month-old HPV16 mice and treated for IgG-dependent FcyR stimulation and HUVEC chemotaxis was evaluated in a Boyden chamber assay.

(B-D) Data are represented as means ± SEM (*, *p < 0.01; **p < 0.001, unpaired t test). See also Figure S4 and Table S1.

demonstrated a functional role for the Fc activating receptors in cancer promotion. Therefore IgG-mediated activation of FcRy on resident and recruited leukocytes, including mast cells and macrophages, regulated not only PBL recruitment but also leukocyte activation and bioeffector function within the neoplastic microenvironment.

Activation of complement pathways is an alternative mechanism by which IgG could induce leukocyte activation and recruitment, further leading to chronic inflammation. Markiewski et al. (2008) reported that transplanted renal tumor growth was regulated by complement activation and that C5a deposition was associated with MDSC recruitment and subsequent CTL suppression. Complement factor C3 does not alter parameters of neoplastic progression in HPV16 mice (de Visser et al., 2005); however, C3-independent mechanisms do exist (Baumann et al., 2001). As such, we cannot exclude a role for complement factors as sensors of IC deposition in HPV16 mice, potentially regulating C3-independent FcyR activation.

It is intriguing to speculate that targeting B lymphocyte/lg/ $Fc\gamma R$ pathway by inactivating either B lymphocytes or $FcR\gamma$ signaling may be tractable for anti-cancer therapy. In support of this approach, patients with rheumatoid arthritis, systemic lupus erythematosus, and others, have benefited following B cell-depletion therapy using a chimeric monoclonal antibody specific to human CD20, e.g., Rituximab (Gurcan et al., 2009). Rituximab has also shown clinical efficacy in adult acute lymphoblastic leukemia (Gokbuget and Hoelzer, 2006). Support for extending Rituximab into solid tumor therapy comes from





Figure 7. $FcR\gamma$ Expression Regulates Proangiogenic and Protumorigenic Properties of Macrophages

(A and B) FcR γ expression regulated macrophage protumor activity. PDSC5 tumor cells were injected alone (blue) or admixed with macrophages derived from neoplastic skin (MØ_{skin}) of either HPV16/FcR $\gamma^{+/-}$ (red) or HPV16/FcR $\gamma^{-/-}$ (green) mice (A) or instead from bone marrow-derived macrophages (MØ_{BIN}) isolated from FcR $\gamma^{+/-}$ (red) or FcR $\gamma^{-/-}$ (green) mice (B). The asterisk (°) indicates statistically significant differences between PDSC5 cells admixed with FcR $\gamma^{+/-}$ versus FcR $\gamma^{-/-}$ macrophages. The number sign (#) indicates statistically significant differences between PDSC5 alone and PDSC5 admixed with FcR $\gamma^{-/-}$ macrophages. Data are represented as means \pm SEM; p < 0.05, unpaired t test.

(C) Genes differentially expressed in macrophages isolated from neoplastic skin of HPV16/ FcR $\gamma^{-/-}$ versus HPV16/FcR $\gamma^{+/-}$ mice. mRNA expression levels in macrophages from HPV16/ FcR $\gamma^{-/-}$ neoplastic skin (4 month old) are indicated as fold change as compared to HPV16/ FcR $\gamma^{+/-}$ macrophages with Δ CT of each gene calculated with β 2-microglobulin as endogenous control. Expression was considered as statistically significantly deregulated when p < 0.05 (t test, FcR $\gamma^{-/-}$ versus FcR $\gamma^{+/-}$).

(D) Quantitative real-time PCR analysis of *Cxc110* and *Cxcr3* mRNA expression in ear tissue from 4-month-old negative littermate (–LM), HPV16, HPV16/JH^{-/-}, and HPV16/FcR $\gamma^{-/-}$ mice (n = 3–5 mice/cohort). *p < 0.05; **p < 0.01, unpaired t test.

(E) VEGF₁₆₅-induced (100 ng/ml) HUVEC chemotaxis was evaluated in a Boyden chamber assay after pretreatment with recombinant CXCL10 (100 ng/ml) in the presence or absence of CXCR3 blocking antibody (10 μ g/ml). *p < 0.05; **p < 0.01, unpaired t test.

(F) Macrophages isolated from HPV16/FcR $\gamma^{-/-}$ mice exhibit angiostatic activity. HUVEC chemotaxis in a Boyden chamber assay was evaluated after pretreatment with conditioned medium iso-

lated from macrophages (purified from skin of HPV16/FcR $\gamma^{+/-}$ or HPV16/FcR $\gamma^{-/-}$ mice) following activation with LPS (10 ng/ml) and α CXCR3 blocking Ig (10 µg/ml). *, p < 0.05; **, p < 0.01, unpaired t test.

(E and F) Pretreated HUVECs were evaluated for chemotactic migration in response to VEGF (100 ng/ml) using a Boyden chamber assay. Quantitative values reflect the number of migrating HUVECs averaged from four to five high-power fields per insert and four inserts per treatment ± SEM. At least three independent analyses were performed and one representative experiment is shown. See also Figure S5 and Table S1.

a clinical study with colon cancer patients in which numbers of CD21-hyperpositive lymphocytes were reduced in parallel with reduction in tumor burden (Barbera-Guillem et al., 2000). Although Rituximab effectively deletes circulating B cells, no increased susceptibility to infection has been observed in patients with rheumatoid arthritis or non-Hodgkin's lymphoma (Gokbuget and Hoelzer, 2006), thus supporting the approach and manipulation of humoral immunity and/or its downstream effector pathways as a therapeutic possibility.

Programming Recruited Leukocytes and Inhibiting Protumor Immunity

Clinical and experimental data indicate that chronic presence and activation of immune cells, e.g., mast cells, macrophages, Tie2-expressing monocytes, neutrophils, DCs, IMCs, and CD4⁺ T cells, promote tumor development by activating angiogenic programs, suppressing antitumor immunity (Mantovani et al., 2008), and enhancing tumor cell migration and metastasis (DeNardo et al., 2009; Pollard, 2008). When chronically activated in tumor microenvironments, some myeloid cells are programmed such that they deliver a diversity of bioactive mediators to neoplastic tissues, including chemokines, cytokines, matrix remodeling enzymes, and cytotoxic proteins (Mantovani et al., 2008). Identification of the critical programs that induce these protumor pathways would reveal important mediators to potentially target with anticancer therapeutics.

Mast cells exert duality as cancer mediators with some clinical studies indicating their presence in human cancers correlates



Figure 8. Activation of the FcR γ Pathway in Mast Cells and Macrophages by Humoral Immunity Regulates Squamous Carcinogenesis of HPV16 Mice

HPV16 oncogene expression initiates keratinocytes and triggers early neoplastic progression accompanied by neo- and self-antigen presentation, peripheral B cell activation/maturation, and secretion of Igs. Autoantibodies subsequently accumulate in dermal stroma of neoplastic tissue as vasculature becomes initially angiogenic and "leaky." IgGs interact with Fc γ receptors on resident and recruited myeloid cells where they induce differential recruitment of leukocytes from peripheral blood and regulate mast cell and macrophage bioeffector functions once present in neoplastic tissue. FcR γ deficiency not only impairs the protumorigenic properties of mast cells and macrophages but also reprogram macrophages leading to enhanced angiostatic and M1 bioactivity.

with a favorable clinical outcome, whereas others clearly implicate them as protumor mediators (Theoharides and Conti, 2004). In HPV16 mice (Coussens et al., 1999), and in other models of cancer development (Nakayama et al., 2004; Soucek et al., 2007), mast cell-derived factors foster tumor cell survival and angiogenesis. With IgG-mediated stimulation of mast cells enhancing tumor development and angiogenesis in HPV16 mice, these data indicate a functional requirement for FcR γ engagement for activating protumor cascades, leading to PBL recruitment into neoplastic skin, angiogenesis, and tumor development.

Pharmacologic inhibition of macrophages minimizes cervical carcinogenesis in HPV16 mice (Giraudo et al., 2004), whereas elimination of macrophages during mammary carcinogenesis limits cancer progression and metastasis (Pollard, 2008) by reducing angiogenesis in premalignant tissues. Interestingly, pro- versus anti-metastatic properties of infiltrating macrophages in MMTV-PyMT mice are programmed by CD4⁺ T lymphocytes via an IL-4-dependent mechanism (DeNardo et al., 2009). In contrast, in ovarian cancer, macrophage phenotype is regulated by IL-1R and MyD88, which together maintain a macrophage immunosuppressive M2 phenotype (Hagemann et al., 2008). In the present study, we report that B cells and FcR γ are key parameters regulating pro- versus antitumor programming of macrophages during squamous carcinogenesis

through a mechanism that not only involves modified M1 versus M2 or M2-like polarization but also the differential expression of an angiostatic chemokine *Cxcl10* and its receptor *Cxcr3*. As such, activation of angiogenic vasculature in developing tumors is regulated by multiple subsets of myeloid cells, each exhibiting a distinct signature of pro- versus antiangiogenic molecules, as well as likely being programmed by distinct effector pathways dependent on the tissue microenvironment.

These experimental findings imply that reprogramming myeloid cell phenotypes and/or altering the immune microenvironment to foster antitumor versus protumor activity could improve survival of patients with cancer by limiting cancer development and perhaps stabilizing premalignant or premetastatic disease. Proof-of-concept studies supporting this notion were recently reported by De Palma et al. (2008) demonstrating that myeloid cells could be used as vehicles to deliver immune mediators to tumor microenvironments and essentially reprogram them.

Conclusions

Results from this study demonstrate a key role for B lymphocytes, humoral immunity, and activation of FcR γ signaling pathways in myeloid cells as promoting forces for squamous carcinogenesis. With regards to therapy, our data indicate that anti-cancer strategies targeting B cells, Ig, or FcR γ may harbor therapeutic efficacy in limiting risk of malignant conversion in patients suffering from chronic inflammatory diseases or in patients harboring premalignant lesions whose molecular and/or immunologic characteristics favor tumor development. The efficacy and safety of Rituximab for various autoimmune disorders and some hematological cancers could be extended to potentially combat squamous neoplasms in which IC deposition is prominent and/or activation of FcR γ -mediated signaling is evident.

EXPERIMENTAL PROCEDURES

Animal Husbandry

Generation and characterization of HPV16, JH^{-/-}, FcR $\gamma^{-/-}$, and c-kit^{sh/sh} mice have previously been described (Arbeit et al., 1994; Coussens et al., 1996; Lyon and Glenister, 1982; Takai et al., 1994). To generate HPV16 mice in the JH^{-/-} and FcR $\gamma^{-/-}$ backgrounds, JH^{+/-} and FcR $\gamma^{+/-}$ mice were backcrossed into the FVB/n strain to N5 and intercrossed with HPV16 mice. c-kit^{sh/+} mice were backcrossed five generations into FVB/n. All mouse experiments complied with National Institutes of Health guidelines and were approved by the University of California San Francisco Institutional Animal Care and Use Committee. Characterization of neoplastic stages has been reported previously (Coussens et al., 1996).

In Vivo Assays

For evaluation of proinflammatory properties of IgGs, serum-purified IgGs (20 μ l; 12 μ g/ μ l) were injected intradermally into ears of (–)littermates. For performing matrigel plug assays, PDSC5 cells (Arbeit et al., 1996) (1.5 × 10⁶/100 μ l) were suspended in 300 μ l of Matrigel and injected s.c. in the groin area of 7-week-old mice. For tumorgenicity assays, PDSC5 cells (0.5 × 10⁶ cells) were suspended in 100 μ l of Matrigel in PBS (1:1) and inoculated s.c. into flanks of 7-week-old mice. Tumors were measured at 2 day intervals with a digital caliper, and tumor volume was calculated with the equation V (mm³) = a × b²/2 (a is the largest diameter and b the smallest diameter).

Low-Density qPCR Arrays

Immune Panel TaqMan arrays (Applied Biosystems) were used to measure the expression of 96 genes in three biological replicates. Analysis of raw data

obtained with Immune Panel TaqMan arrays have been performed using an implemented covariance model, as previously described (Pucci et al., 2009).

Statistical Analyses

Statistical analyses were performed using GraphPad Prism version 4 and/or InStat version 3.0a for Macintosh (GraphPad Software). Specific tests included Mann-Whitney (unpaired, nonparametric, two-tailed), unpaired t test, Fisher's exact test, chi-square test, and log rank analysis. p values < 0.05 were considered statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures, Supplemental Experimental Procedures, and one table and can be found with this article online at doi:10.1016/j.ccr.2009.12.019.

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Supplemental Information

FcRy 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 (4and 6-mo). Error bars represent SEM; asterisk (*) indicates statistically significant differences with (-)LM (p < 0.05, Mann-Whitney).



Figure S3. Deficient carcinogenesis in HPV16/FcR₇-deficient mice (accompanies Figure 4)

A) Immunolocalization of FcR γ (left panel), Fc γ RI (middle panel) and Fc γ RII/III (right panel) expression in HPV16/Fc γ R^{-/-} mice at 4-mo of age. Solid line, epidermal-dermal interface; epidermis, e; dermis, d; scale bar, 50 μ m.

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 Fc γ RI and Fc γ RIII 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 γ R^{+/-} and HPV16/Fc γ 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/FcR $\gamma^{+/+}$ and HPV16/FcR $\gamma^{-/-}$ 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) FcR γ -deficiency does not modify expression of co-stimulatory molecules on DCs. Single-cell suspensions derived from skin and cLN of HPV16/FcR $\gamma^{+/-}$ and HPV16/FcR $\gamma^{-/-}$ 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/FcR $\gamma^{+/-}$ and HPV16/FcR $\gamma^{-/-}$ mice. mRNA expression levels in DCs from HPV16/FcR $\gamma^{+/-}$ neoplastic skin (4-mo) are indicated as fold-change as compared to HPV16/FcR $\gamma^{+/-}$ DCs with Δ CT of each gene calculated using β 2-*microglobulin* as an endogenous control. Genes are considered as statistically significantly deregulated when p (t test, FcR $\gamma^{+/-}$ versus FcR $\gamma^{-/-}$ 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. Singlecell 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 μ M, 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/FcR $\gamma^{+/-}$ and HPV16/FcR $\gamma^{-/-}$ mouse skin. Gene expression levels in IMCs from HPV16/FcR $\gamma^{-/-}$ neoplastic skin (4-mo) are indicated as fold-change as compared to HPV16/FcR $\gamma^{+/-}$ IMCs with Δ CT of each gene calculated using β 2-*microglobulin* as an endogenous control. Gene are considered as statistically significantly deregulated when p (t test, FcR $\gamma^{+/-}$ versus FcR $\gamma^{-/-}$ 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 μ g/ μ l and injected intradermally using 28-gauge insulin syringes to wt mice anesthetized with 2.0% isoflurane/98% O₂ 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- μ m) 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 μ l 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 FcyR-expressing immune cells, tissue sections were incubated with rat antimouse CD45 (1:500, BD Pharmingen) and FcyRI-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 softwareTM

(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 CitraTM 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 highpower 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°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°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), FccRI 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 μ g/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 μ g/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 FccR-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% CO₂.

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 $(5x10^{6} \text{ cells/ ml})$ 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 10⁵ 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 10⁵ 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 10⁵ 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), IFNy/IL13 (10 ng/ml), or IgG (25 µg/ml), as well as control and IgG-stimulated BMMC (600 µl). Addition of chemotactic factors, including rVEGF₁₆₅ (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 CO₂ 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 VEGF₁₆₅ (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% CO₂.

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 $1.5 \times 10^6/100 \,\mu$ l and premixed with diluted Matrigel in a total volume of 300 μ l. 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 μ l of diluted cold Matrigel in PBS (1:1) (0.5x10⁶ 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 (mm³) = a x b²/2, 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 γ R^{-/-} versus HPV16/Fc γ 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 (ΔC_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 ΔC_T , the higher the gene expression level.

Statistical analysis of gene expression data

To calculate the fold-change of gene expression between HPV16/FcR $\gamma^{-/-}$ and HPV16/ FcR $\gamma^{+/-}$ 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/FcR $\gamma^{+/-}$, HPV16/FcR $\gamma^{-/-}$; 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, β_i are the coefficients calculated by the model that represents the impact of the respective qualitative variable X_i , ε 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 twoby-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 <u>http://www.R-project.org</u>). Significance level is chosen at $\alpha = 0.05$.

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