

Celecoxib promotes degranulation of CD8⁺ T cells in HPV-induced lesions of K14-HPV16 transgenic mice

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Abstract

Aims: Human papillomavirus (HPV) is a known biologic carcinogen which is commonly transmitted through sexual intercourse. CD8⁺ T cells are known effectors against tumour cells and an important prognostic marker in HPV-induced cancers. COX-2 inhibitors enhance CD8⁺ T cell activity against some cancers. In this work, we sought to study the presence and activation of CD8⁺ T lymphocytes in lesions from K14-HPV16 transgenic mice and the immunomodulatory effect of celecoxib (CXB) over these cells.

Main methods: Skin samples of CXB-treated and untreated HPV16^{-/-} and HPV16^{+/-} mice were enzymatically digested and analysed by flow cytometry to assess CD8⁺ and CD8⁺CD107a⁺ T cell infiltrates. Matched skin samples were classified histologically.

Key findings: HPV16^{+/-} mice presented higher CD8⁺ T cell infiltration than HPV16^{-/-} animals ($P < 0.001$). Older HPV16^{+/-} animals showed epidermal dysplasia and increased percentages of CD8⁺CD107a⁺ T cells compared with younger animals with hyperplasia ($P < 0.001$), validating this model for testing the effects of celecoxib on CD8⁺ T cells. CXB-treated HPV16^{+/-} mice showed higher percentages of CD8⁺CD107a⁺ T cells compared with untreated HPV16^{+/-} animals ($P < 0.01$), but no differences were observed concerning the progression of epidermal lesions.

Significance: These findings indicate that celecoxib enhances the degranulation of CD8⁺ T cells on HPV16-induced lesions, suggesting the potential clinical use of COX-2 inhibitors. Additionally, this study demonstrates the usefulness of the K14-HPV16 mouse model for testing therapeutic immunomodulatory approaches.

Keywords: CD8⁺ T lymphocytes; Celecoxib; COX-2 inhibition; Human Papillomavirus; K14-HPV16 Transgenic Mice.

Introduction

Human papillomavirus (HPV) has been described as an etiologic factor of several diseases, such as cervical and other anogenital cancers, and a subset of HPV-positive head and neck cancers [1-4]. Oropharyngeal cancers have lately drawn considerable attention, given their growing incidence [5]. In order for a HPV-associated neoplasm to develop, a persistent infection by an oncogenic HPV type (mostly types 16 and 18) is mandatory [6]. Only in a minority of cases, in which the immune system fails to eradicate the virus, will the infection progress to cancer [7].

Cytotoxic T lymphocytes (CTL), differentiated from CD8⁺ T cells, play an important role in the complex immune response which accompanies HPV-induced carcinogenesis [8,9]. CD8⁺ T cells are recruited to the lesions microenvironment by chemokines released by stromal and/or innate immune cells on site [10]. In the presence of activator stimuli, differentiated CTL can specifically target virus-infected and tumour cells, which are eliminated either through the release of lytic granules, engagement of death receptors (e.g. FAS-FASL or TRAIL) or following interferon- γ -dependent mechanisms [11-13]. In particular, CTL are critical to control cervical lesions and were recently demonstrated to drive the immune response in patients following the administration of an experimental therapeutic vaccine [14]. Recent studies have also shown the number of CD8⁺ T cells to be an important independent prognostic marker in HPV-positive head and neck cancer patients, while high CD8⁺ T cell levels correlate with a better prognosis, enhancing overall survival as well as progression-free survival [15-17]. Understanding and enhancing CTL function in patients with HPV-induced malignancies seems, therefore, a priority for cancer therapy.

It has recently been suggested that cyclooxygenase-2 (COX-2) and its product prostaglandin E2 down-regulate the function of activated CD8⁺ T cells and induce their senescence [18]. This effect may explain how the abrogation of COX-2 signalling reduces tumour growth in mouse models of glioma [19] and mammary cancer [20]. In light of these findings, it is appealing to study whether CD8⁺ T cells present in HPV-induced lesions are affected by COX-2 inhibition.

K14-HPV16 transgenic mice, created nearly two decades ago [21], are an useful *in vivo* animal model for the study of HPV-induced carcinogenesis. This model shares a number of morphologic and molecular similarities to HPV-related human disease [22], thus functioning as an excellent replica of the multi-stage process of carcinogenesis. Targeting of HPV16 oncogenes to keratinocytes by the keratin-14 (K14) promoter/enhancer is the key characteristic of this model [21].

In this study, we aimed to examine the effect of a selective COX-2 inhibitor, celecoxib, on the infiltration and activation of CD8⁺ T lymphocytes in HPV-induced lesions. To accomplish our primary objective, we first sought to validate the K14-HPV16 transgenic mouse model employing untreated wild-type (WT) and transgenic animals of different ages, comparing lesions and cell infiltration percentages between younger and older mice.

Material & Methods

Animals

Generation of K14/HPV16 mice on a FVB/n background has been previously reported [21]. K14-HPV16 transgenic mice were kindly donated by Drs. Jeffrey Arbeit and Douglas Hanahan (University of California) through the USA National Cancer Institute Mouse Repository. The animal experiments were approved by the Universidade de Trás-os-Montes e Alto Douro Ethics Committee (10/2013) and the Portuguese General Veterinary Directorate (approval no. 0421/000/000/2014). Animals were maintained and bred according to Portuguese (Decreto-Lei 113, August 7th) and European (EU Directive 2010/63/EU) legislation, under controlled conditions of temperature (23 ± 2 °C), light-dark cycle (12h light/12h dark) and relative humidity (50 ± 10 %), using corncob bedding. Food and water were provided *ad libitum*.

Mice genotyping

Animals were genotyped at weaning, using tail tip samples as described previously [23,24]. Briefly, nucleic acids were extracted and DNA quality and purity were assessed. HPV16-E6 and -E2 genes were amplified to confirm the presence of HPV DNA and a fragment of mouse β -globin was also amplified as endogenous control. Lengths of the amplicons were confirmed by agarose gel electrophoresis. Only hemizygous females were used for the transgenic mouse groups in the experiment.

Experimental design

Experiment 1: To validate the usefulness of the K14-HPV16 transgenic mouse model to examine CD8⁺ T cell infiltration and activation, twenty-four 18 to 20 weeks-old mice were divided into four groups according to their genotype: group 1 (HPV16^{-/-} untreated animals, n = 6), group 2 (HPV16^{+/-} untreated animals, n = 6), group 3 (HPV16^{-/-} untreated animals, n = 6), group 4 (HPV16^{+/-} untreated animals, n = 6). Mice from groups 1 and 2 were humanely euthanized at 24-26 weeks of age while animals from groups 3 and 4 were euthanized at 28-30 weeks-old. All sacrifices were performed by intraperitoneal pentobarbital overdose, followed by exsanguination by cardiac puncture. Chest skin samples (approximately 4 cm²) were collected for flow cytometry analysis and matched adjacent skin samples were collected for histological analysis.

Experiment 2: To assess the effect of celecoxib over CD8⁺ T cell infiltration and activation, twenty-four 18 to 20 weeks-old female mice were divided into four experimental groups according to their genotype: group A (HPV16^{-/-} untreated animals, n = 6), group B (HPV16^{+/-} untreated animals, n = 6), group C (HPV16^{-/-} CXB-treated animals, n = 6), group D (HPV16^{+/-} CXB-treated animals, n = 6). For ethical reasons, in order to reduce the number of sacrificed animals, the control groups used in this experiment (groups A and B) were the same as groups 1 and 2 of experiment 1, respectively. All surviving mice were humanely euthanized at 24-26 weeks of age by intraperitoneal pentobarbital overdose, followed by exsanguination by cardiac puncture. Chest skin samples (approximately 4 cm²) were collected for flow cytometry analysis and matched adjacent skin samples were collected for histological analysis.

Celecoxib administration

Celecoxib (Pfizer, New York, NY) was dissolved in drinking water at a concentration of 0.5 mg/ml, estimating an average daily water intake of 5.0ml per mouse, and a dose of 46.7 mg/kg/day and 2.5 mg/animal/day in an average mouse weighting 30 g. This is a well-tolerated moderate dose, as shown in previous assays [25]. However, K14-HPV16 animals dramatically increased their water intake when CXB was added (possibly due to the highly palatable lactose present in the vehicle) reaching up to 15 ml per animal. The CXB concentration was accordingly reduced from the start of the third week onwards down to 0.2 mg/ml, resulting in a decrease in consumption and in an effective dose of 93 mg/kg/day and 2.8 mg/animal/day. The average dose during the overall experimental period was 124 mg/kg/day and 3.72 mg/animal/day, which is still a moderate dose [25].

Histological analysis

Skin samples were fixated in 10% neutral buffered formalin for 48 hours. Samples were dehydrated through graded alcohols and xylene and paraffin embedded in an automatic STP 120 processor (Micron, Boise, ID). 2 µm-thick sections were stained with haematoxylin-eosin (H&E) for histological evaluation on light microscopy. Skin samples were classified as normal skin, epidermal hyperplasia and epidermal dysplasia.

Preparation of single-cell suspensions

Chest skin samples were cut into small pieces after excessive blood vessels and fat tissue removal. Skin fragments were incubated for 2 hours with 125 U/ml type I collagenase (Gibco, Life Technologies, Paisley, UK) in RPMI-1640 medium complemented with 1% glutamine, 1% penicillin-streptomycin-amphotericin B, 1% HEPES buffer (all from Sigma, St. Louis, MO) and 10% foetal bovine serum (BioWest, Nuaillé, France) at 37 °C and 150 rpm in a 3031 orbital incubator (GFL, Burgwedel, Germany). Subsequently, the resulting cell suspension was filtered and centrifuged at 300 *g* for 10 min at 4 °C. Cells were resuspended in phosphate buffered saline containing 1% bovine serum albumin and 20 mM sodium azide followed by flow cytometry analysis.

Immunophenotyping

Following cell isolation, the surface phenotype of the collected cells was assessed by flow cytometry using specific monoclonal antibodies (mAb). Cells were incubated with anti-mouse CD16/CD32 mAb for FcγR blocking, to prevent non-specific antibody binding. Next, cells were incubated with anti-CD8 mAb phycoerythrin-cychrome 5-conjugate (clone 53-6.7, BD Biosciences, San Diego, CA) and anti-CD107a (LAMP1) mAb phycoerythrin-conjugate (clone eBio1d4b, eBioscience, San Diego, CA). Following extracellular staining, the cells were washed, fixed in 2 % formaldehyde and washed with phosphate buffered saline containing 1% bovine serum albumin and 20 mM sodium azide. Antibody-labelled cells were analysed in an EPICS XL flow cytometer using the EXPO32ADC software

(Beckman Coulter, Miami, FL). The assembled data files were analysed using the FlowJo software v10.0.7 (FLOWJO, LLC, Ashland, OR).

Statistical analysis

Statistical analyses were performed using the GraphPad software (version 6.0, GraphPad Software, Inc. La Jolla, CA). In column and dot graphs each point represents individual mice while bars represent the mean for the respective group. Statistical analysis was performed using one-way analysis of variance with the Tukey post-hoc test.

Results

General findings

All K14-HPV16 mice showed typical cutaneous changes, including diffuse hyperkeratosis and erythema. One celecoxib-treated HPV^{+/+} mice (group D) succumbed before the end of the study. All mice in groups 1/A, 2/B, 3, 4 and C survived until the end of the study.

Transgenic mice show epidermal hyperplasia and dysplasia

Histological analysis of skin samples (Table 1) showed that the totality of WT mice (groups 1, 3 and C) presented normal skin histology (Fig. 1a) whilst all transgenic mice presented skin lesions. All transgenic mice sacrificed at 24-26 weeks of age, either untreated or CXB-treated (groups 2/B and D), showed simple to papillary, diffuse, variably severe epidermal hyperplasia and papillomatosis with orthokeratotic hyperkeratosis (Fig. 1b). Inflammation was mild, with a few macrophages, lymphocytes and mast cells present in the superficial dermis. Also, in group 2/B, two animals (33.3%) presented small epidermal dysplastic foci. In group 4, three animals (50.0%) presented diffuse epidermal dysplasia (Fig. 1c); and the other three animals (50.0%) showed multifocal epidermal dysplasia in a hyperplastic background. Sub-epidermal angiogenesis and dermal inflammatory infiltrates were prominent, showing numerous mixed mononuclear leukocytes and neutrophils.

Table 1 Histological classification of HPV-induced skin lesions from WT and K14-HPV16 transgenic female mice.

Group	Age (weeks)	Cutaneous lesions		
		Normal skin	Epidermal hyperplasia	Epidermal dysplasia
1/A (HPV ^{-/-} , n = 6)	24-26	6/6 (100%)	0/0 (0%)	0/0 (0%)
2/B (HPV ^{+/+} , n = 6)	24-26	0/0 (0%)	6/6 (100%)	2/6 (33.3%)
3 (HPV ^{-/-} , n = 6)	28-30	6/6 (100%)	0/0 (0%)	0/0 (0%)
4 (HPV ^{+/+} , n = 6)	28-30	0/0 (0%)	3/6 (50%)	6/6 (100%)
C (HPV ^{-/-} + CXB, n = 6)*	24-26	6/6 (100%)	0/0 (0%)	0/0 (0%)
D (HPV ^{+/+} + CXB, n = 5)*	24-26	0/0 (0%)	5/5 (100%)	0/0 (0%)

* CXB – Celecoxib

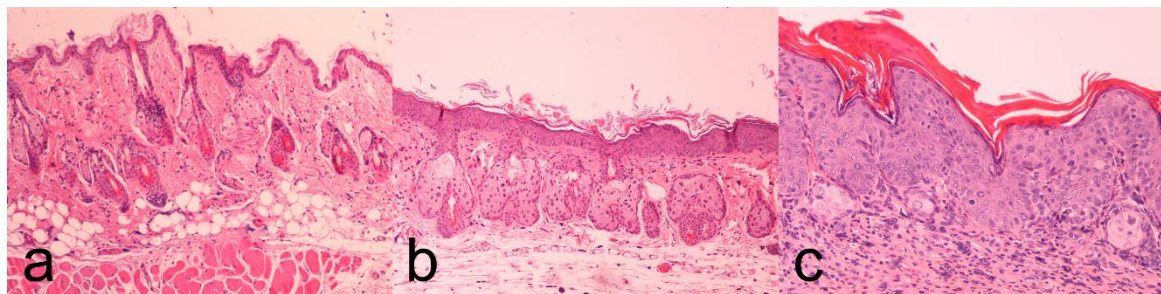


Fig. 1 Histopathological changes induced by HPV16 oncogenes in FVB/n mice, H&E. a –Normal skin histology, 100 ×. b – Epidermal hyperplasia extending to the follicular infundibulum and isthmus, 100 ×. c –Epidermal dysplasia, 200 ×. Note marked parakeratotic hyperkeratosis, loss of cell polarity, enhanced anisokaryosis and mitotic activity. The dermal-epidermal junction is obscured by severe inflammatory cell infiltration.

HPV16^{+/-} mice show increased CD8⁺ T lymphocyte infiltration and activation

In order to assess the presence of CD8⁺ T cells in HPV-associated lesions, we isolated lymphoid cells from chest skin tissue and analysed them by flow cytometry (Fig. 2a). As shown in Fig. 2b, chest skin samples from HPV16^{+/-} mice presented a significantly higher percentage of CD8⁺ T cells when compared with those of WT animals, regardless of their age. Although the percentage of skin CD8⁺ T cells was slightly higher in older transgenic mice comparing to the younger transgenic ones, it did not reach statistical significant difference.

To determine if the CD8⁺ T cells found in the skin of HPV16^{+/-} mice presented evidence of cytotoxic activity we evaluated the surface expression of CD107a (also known as LAMP1). In CD8⁺ T lymphocytes CD107a reaches the cell surface when lytic granules suffer exocytosis, thus exposing its membrane proteins [26]. Therefore, this lysosome-associated membrane protein is a commonly used surrogate marker of CTL degranulation [27]. As shown in Fig. 2c, the percentage of CD8⁺CD107a⁺ T cells in chest skin samples of 28-30 weeks-old transgenic mice is significantly higher when compared to HPV^{-/-} mice of both ages ($P < 0.001$). This indicates that a great number of skin infiltrating CTL from older HPV16^{+/-} mice releases cytotoxic granules. Fig. 2c also shows a significant relation between both WT groups ($P < 0.05$). Such significance is due to the two outliers in the 28-30 weeks-old HPV^{-/-} group and no conclusions should be taken from this relation. Moreover, older transgenic mice, 100% of which show multifocal or diffuse dysplasia, present a higher percentage of CD8⁺ T cells expressing CD107a ($P < 0.001$) than the younger HPV^{+/-} mice, of which only 33.3% show focal dysplastic lesions. This result suggests a positive correlation in the proportions of activated CD8⁺ T cells and lesion severity.

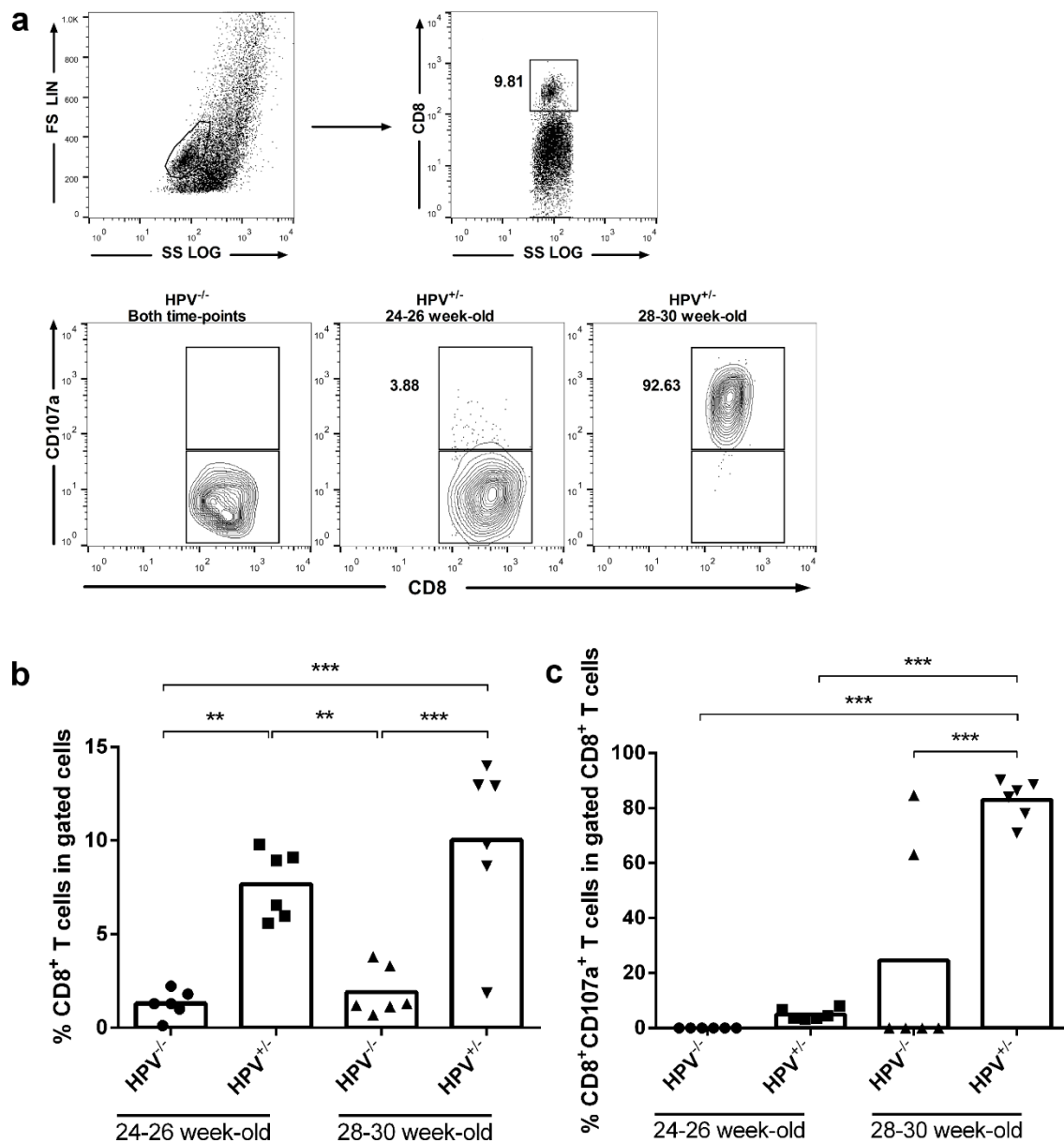


Fig. 2 Percentage of CD8⁺ and CD8⁺CD107a⁺ T cells within total lymphoid-gated cells obtained from chest skin samples from WT and K14-HPV16 transgenic mice. **a** – Representative analysis of the gating strategy employed. Numbers within graphs correspond to the percentage of the gated population. **b** – Percentages of CD8⁺ T cells and **c** – percentages of CD8⁺CD107a⁺ T cells in gated CD8⁺ T cells were determined by flow cytometry after skin cytometry after skin tissue collection and digestion with collagenase. Chest skin samples were collected from 24-26 weeks-old HPV^{-/-} and HPV^{+/-} mice (Groups 1 and 2, respectively) and 28-30 weeks-old HPV^{-/-} and HPV^{+/-} mice (Groups 3 and 4, respectively). Group 1, n = 6; Group 2, n = 6; Group 3, n = 6; Group 4, n = 6. Each dot represents an individual animal. Bars represent the mean value in each group. ** $P < 0.01$; *** $P < 0.001$.

Celecoxib-treated mice show enhanced CTL degranulation

Given the usefulness of the K14-HPV16 mouse model to examine CD8⁺ T cell infiltration and activation as shown above, we studied the effect of the selective COX-2 inhibitor celecoxib over the infiltration and activation of CD8⁺ T lymphocytes. Subsequently, lymphoid cells were isolated from mice chest skin tissue and a flow cytometry analysis of the recovered cells was performed (Fig. 3a). Again, skin samples from untreated HPV^{+/-} mice showed a significantly higher percentage of CD8⁺ T cells than untreated WT animals ($P < 0.001$) (Fig. 3b). Also, HPV^{-/-} + CXB mice show decreased CD8⁺ T cell infiltration when compared to untreated HPV^{+/-} animals (Fig. 3b). Moreover, Fig. 3b shows a decrease in

the percentage of infiltrating CD8⁺ T lymphocytes in HPV^{+/-} + CXB mice when compared with untreated transgenic animals ($P < 0.001$).

Once more we assessed the expression of CD107a to examine if these CD8⁺ T lymphocytes were degranulating. Fig. 3c shows a significantly higher percentage of CD8⁺CD107a⁺ T cells in samples from HPV^{+/-} + CXB mice comparing to the remaining groups ($P < 0.001$), indicating that a higher number of the infiltrating CTL are degranulating in this group.

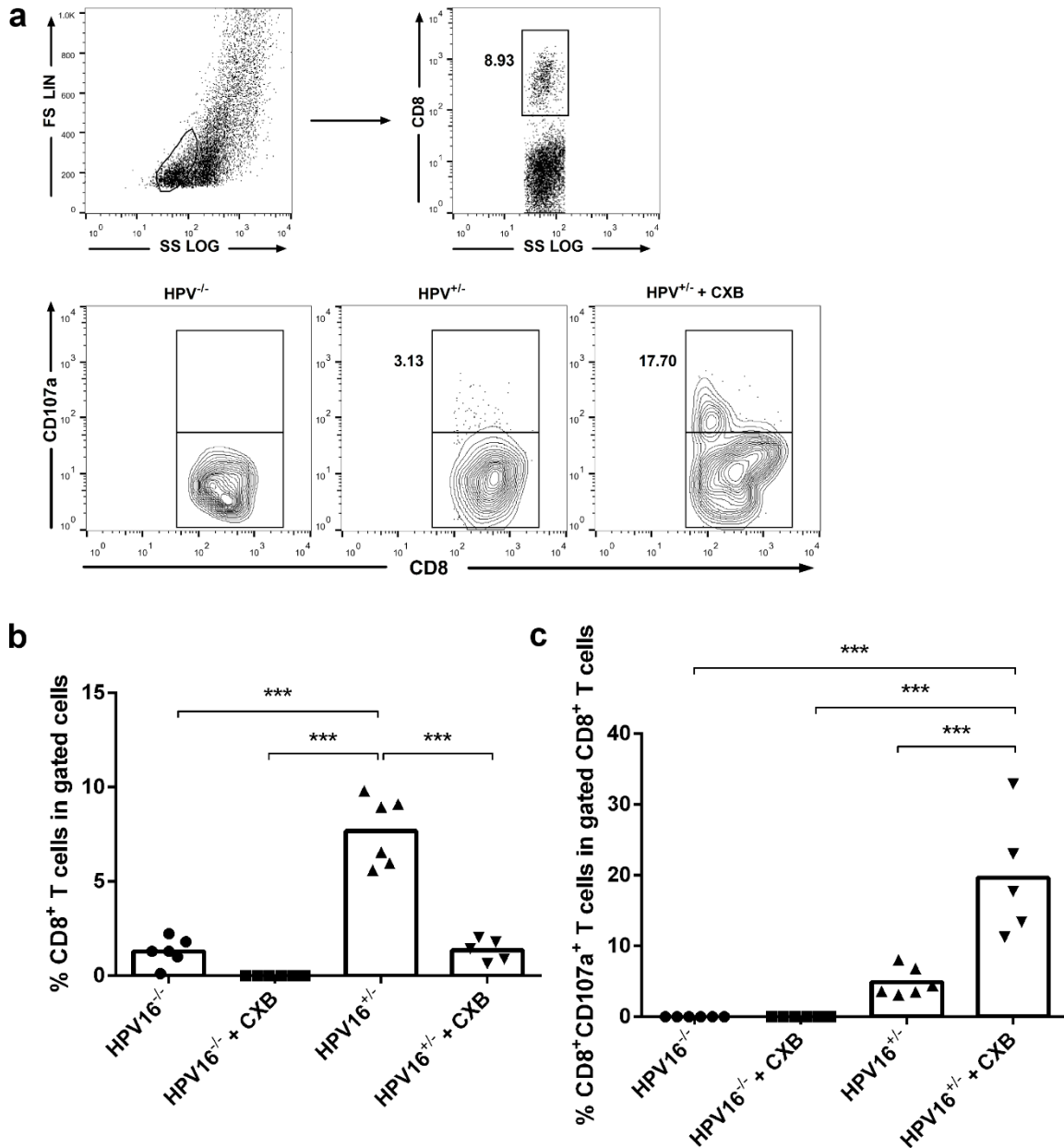


Fig. 3 Percentage of CD8⁺ and CD8⁺CD107a⁺ T cells within total lymphoid-gated cells obtained from chest skin samples from WT and K14-HPV16 transgenic mice. **a** – Representative analysis of the gating strategy employed. Numbers within graphs correspond to the percentage of the gated population. **b** – Percentages of CD8⁺ T cells and **c** – percentages of CD8⁺CD107a⁺ T cells in gated CD8⁺ T cells were determined by flow cytometry after skin tissue collection and digestion with collagenase. Chest skin samples were collected from WT and HPV^{+/-} mice (Groups A and B, respectively) and CXB-treated (CXB) WT and HPV^{+/-} mice (Groups C and D, respectively) at 24-26 weeks of age. Group A, n = 6; Group B, n = 6; Group C, n = 6; Group D, n = 5. Each dot represents an individual animal. Bars represent the mean value in each group. *** $P < 0.001$.

Discussion

The development of HPV-associated malignancies depends on a persistent HPV infection. Ultimately, the ability of the immune system to eliminate the virus is the key element to decide whether a HPV infection is cleared or evolves to cancer [7]. HPV presents well-known mechanisms to evade host immunity and delay its elimination, thus facilitating viral persistence [28]. When the virus is detected, an innate immune response occurs, and leads to the development of an adaptive immune response. A fundamental part of this adaptive response is cell-mediated immunity, characterized by the activity of a vast number of CD8⁺ and CD4⁺ T lymphocytes [29]. In fact, infiltration by these cells in HPV-associated lesions has already been shown to lead to regression [8,30]. CTL infiltration drives the response induced by an experimental therapeutic vaccine in patients with cervical intraepithelial lesions [14] and correlates with a better prognosis in patients with HPV-positive head and neck cancer [15-17].

Chronic inflammation is a key feature associated with carcinogenesis, namely in the case of HPV infection [31]. COX-2 is an enzyme with an imperative role in the metabolism of arachidonic acid, which leads to the production of prostaglandins, which in turn promote inflammation [32]. In fact, COX-2 is over-expressed in several malignancies, including cervical cancer [33-37]. As inflammation is known to contribute for cancer progression [31], COX-2 inhibition is expected to result in tumour growth inhibition whilst reducing inflammation. Some non-steroidal anti-inflammatory drugs, like aspirin and ibuprofen, have already shown promising results as anti-tumour therapy in both patients and pre-clinical animal models [38-41]. However, these drugs have very low specificity. Selective COX-2 inhibitors such as celecoxib and rofecoxib have already been used to prevent the development of colorectal adenomas [42,43]. Furthermore, celecoxib showed promising results in a tumour model of human colon cancer when combined with chemotherapeutic drugs [44].

The K14-HPV16 transgenic mouse model appears as an appropriate *in vivo* animal model to improve the knowledge on HPV-induced carcinogenesis and elicited immune response. Besides cervical cancers, these mice develop aggressive skin lesions with greater incidence in the ear and the chest [45]. As expected, we observed a higher incidence of aggressive lesions (epidermal dysplasia) in older transgenic mice than in younger ones, which presented mostly hyperplastic lesions. The development of dysplastic lesions was accompanied by a dramatic intensification of sub-epidermal inflammation. As this process of carcinogenesis is associated with progressive chronic inflammation, this model will allow the study of the intervening immune effectors. Celecoxib treated animals presented hyperplastic lesions similar to the observed in age-matched untreated animals. However, 16.7 % of the untreated HPV^{+/−} animals showed multifocal dysplastic lesions. These findings suggest that CXB blocked tumour progression at the hyperplastic stage, but the small number of dysplastic lesions observed does not allow for any definitive conclusions.

In the present study, we observed that CD8⁺ T cells heavily infiltrate HPV-induced chest skin lesions in K14-HPV16 transgenic female mice. This data is in agreement with previous studies which described the presence of CD8⁺ T cells in HPV-induced cervical and skin lesions in human patients [46,8], validating the usefulness of this animal model. According to one of the studies, the presence of CD8⁺ T cells in cervical tissue was associated with tumour regression [8]. Our results suggest a trend towards the increase of infiltrating CD8⁺ T cells in chest skin samples of 28-30 weeks-old mice when

compared to 24-26 week-old animals. Although indicating that CD8⁺ T lymphocytes migrate in greater number towards more severe lesions than to less aggressive ones, these results should be further confirmed. Nevertheless, our data clearly show that a significant proportion of the infiltrating CD8⁺ T cells are activated and degranulate. In older transgenic animals, a higher percentage of CD8⁺ T lymphocytes is activated when compared to the correspondent cells isolated from younger mice. This suggests that more CTL become activated in response to a more severe stimulus than when confronted with a less aggressive lesion. However, despite the increase in the percentage of cytotoxic cells and its enhanced activation, the lesions still tend to progress to a poor phenotype with aging. In fact, low tumour infiltration by activated CTL has been associated with the limiting efficacy of the immune response in eliminating established tumours [10]. Therefore, the results reported herein indicate that however important, CTL recruitment and degranulation into lesion areas do not suffice to prevent progressive carcinogenesis in the K14-HPV16 transgenic mice.

Our results show that celecoxib reduces the number of tumour-infiltrating CD8⁺ T cells when compared with untreated mice. Still, despite the decrease in cell numbers, celecoxib-treated mice have a higher percentage of degranulating CD8⁺ T cells compared with untreated animals. These findings suggest that celecoxib reduces the number of tumour-infiltrating CTL while enhancing their effector functions. These data are in agreement with a previous study using glioma-bearing mice where *COX2*^{-/-} mice had increasing percentages of tumour-infiltrating CD8⁺CD107a⁺ lymphocytes [19]. This may be explained by another study reporting that COX-2 activity leads to CTL senescence and this trajectory may be opposed by COX-2 inhibition, as shown by increased levels of CD28 and interleukin-2 in CD8⁺ T cells [18]. In fact, COX-2 inhibition boosted the efficacy of a DNA vaccine expressing the HPV E7 oncogene, by enhancing tumour-infiltrating CD8⁺ T cells and slowing tumour growth [47]. However, this study was performed in mice bearing allografted TC1 lung cells immortalized by the HPV16 E6 and E7 oncogenes and transformed by the c-Ha-*ras* oncogene. Comparisons between this model and K14-HPV16 mice are limited, because allografts do not reproduce HPV-associated multi-step carcinogenesis, being directly implanted in the subcutis with a fully malignant phenotype. Also, keratinocytes and not lung cells are the targets for papillomavirus infection. Further studies are needed to clarify whether the present effects of celecoxib are mediated by COX-2 inhibition or are related to some of its known COX-2-independent effects.

It remains unclear whether CTL are activated at regional lymph nodes or at the lesion location and this would be an interesting point to address in the future. The results presented herein suggest that celecoxib induces augmented CTL degranulation in HPV-induced lesions, possibly contributing to prevent malignant progression in this animal model. Enhancing CD8⁺ T cell activation seems a promising therapeutic strategy considering the role played by CTL in the regression of HPV-associated cervical lesions [8], in HPV-positive head and neck cancer [15-17] and the response to vaccines [14], and COX-2 inhibitors may prove useful for this purpose.

Conclusion

The results presented here clearly show that CD8⁺T cells infiltrate HPV-induced skin lesions in K14-HPV16 mice, reproducing the features observed in human patients. Thus, these findings support the usefulness of this model to study the host immune response associated with HPV-induced lesions. Moreover, these data confirm the potential of celecoxib to enhance degranulation by tumour-infiltrating CTL. These results suggest possible applications of affordable and widely available COX-2 inhibitors for enhancing the immune response against HPV-induced malignancies and, possibly, against other cancers. However, the effect of celecoxib seems to be more complex than previously thought, as it also reduced the overall number of tumour-infiltrating CD8⁺ T cells. Future studies addressing the impact of COX-2 inhibitors on the prognosis of patients bearing HPV-induced lesions should take into account both the number of infiltrating CD8⁺ T cells and their activation status.

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Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

References

1. Zaravinos A (2014) An updated overview of HPV-associated head and neck carcinomas. *Oncotarget* 5 (12):3956-3969. doi:1934 [pii]
2. Hoots BE, Palefsky JM, Pimenta JM, Smith JS (2009) Human papillomavirus type distribution in anal cancer and anal intraepithelial lesions. *Int J Cancer* 124 (10):2375-2383. doi:10.1002/ijc.24215
3. Bzhalava D, Guan P, Franceschi S, Dillner J, Clifford G (2013) A systematic review of the prevalence of mucosal and cutaneous human papillomavirus types. *Virology* 445 (1-2):224-231. doi:10.1016/j.virol.2013.07.015
S0042-6822(13)00435-2 [pii]
4. Bosch FX, de Sanjose S (2007) The epidemiology of human papillomavirus infection and cervical cancer. *Dis Markers* 23 (4):213-227
5. Pytynia KB, Dahlstrom KR, Sturgis EM (2014) Epidemiology of HPV-associated oropharyngeal cancer. *Oral Oncol* 50 (5):380-386. doi:10.1016/j.oraloncology.2013.12.019
S1368-8375(13)00807-5 [pii]
6. Schiffman M, Wentzensen N, Wacholder S, Kinney W, Gage JC, Castle PE (2011) Human papillomavirus testing in the prevention of cervical cancer. *J Natl Cancer Inst* 103 (5):368-383. doi:10.1093/jnci/djq562
djq562 [pii]
7. Zandberg DP, Bhargava R, Badin S, Cullen KJ (2013) The role of human papillomavirus in nongenital cancers. *CA Cancer J Clin* 63 (1):57-81. doi:10.3322/caac.21167
8. Trimble CL, Clark RA, Thoburn C, Hanson NC, Tassello J, Frosina D, Kos F, Teague J, Jiang Y, Barat NC, Jungbluth AA (2010) Human papillomavirus 16-associated cervical intraepithelial neoplasia in humans excludes CD8 T cells from dysplastic epithelium. *J Immunol* 185 (11):7107-7114. doi:10.4049/jimmunol.1002756
jimmunol.1002756 [pii]
9. Fernandes JV, TA DEMF, JC DEA, Cobucci RN, MG DEC, Andrade VS, JM DEA (2015) Link between chronic inflammation and human papillomavirus-induced carcinogenesis (Review). *Oncol Lett* 9 (3):1015-1026. doi:10.3892/ol.2015.2884
ol-09-03-1015 [pii]
10. Sharma RK, Chheda ZS, Jala VR, Haribabu B (2015) Regulation of cytotoxic T-Lymphocyte trafficking to tumors by chemoattractants: implications for immunotherapy. *Expert Rev Vaccines* 14 (4):537-549. doi:10.1586/14760584.2015.982101
11. van Montfoort N, van der Aa E, Woltman AM (2014) Understanding MHC class I presentation of viral antigens by human dendritic cells as a basis for rational design of therapeutic vaccines. *Front Immunol* 5:182. doi:10.3389/fimmu.2014.00182
12. Mescher MF, Curtsinger JM, Agarwal P, Casey KA, Gerner M, Hammerbeck CD, Popescu F, Xiao Z (2006) Signals required for programming effector and memory development by CD8+ T cells. *Immunol Rev* 211:81-92. doi:IMR382 [pii]
10.1111/j.0105-2896.2006.00382.x

13. Dunn GP, Old LJ, Schreiber RD (2004) The three Es of cancer immunoediting. *Annu Rev Immunol* 22:329-360. doi:10.1146/annurev.immunol.22.012703.104803
14. Kim TJ, Jin HT, Hur SY, Yang HG, Seo YB, Hong SR, Lee CW, Kim S, Woo JW, Park KS, Hwang YY, Park J, Lee IH, Lim KT, Lee KH, Jeong MS, Surh CD, Suh YS, Park JS, Sung YC (2014) Clearance of persistent HPV infection and cervical lesion by therapeutic DNA vaccine in CIN3 patients. *Nat Commun* 5:5317. doi:10.1038/ncomms6317
ncomms6317 [pii]
15. Tertipis N, Hammar U, Nasman A, Vlastos A, Nordfors C, Grun N, Ahrlund-Richter A, Sivars L, Haeggbloom L, Marklund L, Hammarstedt-Nordenvall L, Chaturvedi AK, Munck-Wikland E, Ramqvist T, Bottai M, Dalianis T (2015) A model for predicting clinical outcome in patients with human papillomavirus-positive tonsillar and base of tongue cancer. *Eur J Cancer* 51 (12):1580-1587. doi:10.1016/j.ejca.2015.04.024
S0959-8049(15)00386-X [pii]
16. Oguejiofor K, Hall J, Slater C, Betts G, Hall G, Slevin N, Dovedi S, Stern PL, West CM (2015) Stromal infiltration of CD8 T cells is associated with improved clinical outcome in HPV-positive oropharyngeal squamous carcinoma. *Br J Cancer*. doi:10.1038/bjc.2015.277
bjc2015277 [pii]
17. Balermipas P, Rodel F, Rodel C, Krause M, Linge A, Lohaus F, Baumann M, Tinhofer I, Budach V, Gkika E, Stuschke M, Avlar M, Grosu AL, Abdollahi A, Debus J, Bayer C, Stangl S, Belka C, Pigorsch S, Multhoff G, Combs SE, Monnich D, Zips D, Fokas E (2015) CD8+ tumour-infiltrating lymphocytes in relation to HPV status and clinical outcome in patients with head and neck cancer after postoperative chemoradiotherapy: A multicentre study of the German cancer consortium radiation oncology group (DKTK-ROG). *Int J Cancer*. doi:10.1002/ijc.29683
18. Chou JP, Ramirez CM, Ryba DM, Koduri MP, Effros RB (2014) Prostaglandin E2 promotes features of replicative senescence in chronically activated human CD8+ T cells. *PLoS One* 9 (6):e99432. doi:10.1371/journal.pone.0099432
PONE-D-14-10938 [pii]
19. Fujita M, Kohanbash G, Fellows-Mayle W, Hamilton RL, Komohara Y, Decker SA, Ohlfest JR, Okada H (2011) COX-2 blockade suppresses gliomagenesis by inhibiting myeloid-derived suppressor cells. *Cancer Res* 71 (7):2664-2674. doi:10.1158/0008-5472.CAN-10-3055
0008-5472.CAN-10-3055 [pii]
20. Chen EP, Markosyan N, Connolly E, Lawson JA, Li X, Grant GR, Grosser T, Fitzgerald GA, Smyth EM (2014) Myeloid Cell COX-2 deletion reduces mammary tumor growth through enhanced cytotoxic T-lymphocyte function. *Carcinogenesis* 35 (8):1788-1797. doi:10.1093/carcin/bgu053
bgu053 [pii]
21. Arbeit JM, Munger K, Howley PM, Hanahan D (1994) Progressive squamous epithelial neoplasia in K14-human papillomavirus type 16 transgenic mice. *J Virol* 68 (7):4358-4368
22. Smith-McCune K, Zhu YH, Hanahan D, Arbeit J (1997) Cross-species comparison of angiogenesis during the premalignant stages of squamous carcinogenesis in the human cervix and K14-HPV16 transgenic mice. *Cancer Res* 57 (7):1294-1300

23. Paiva I, Gil da Costa RM, Ribeiro J, Sousa H, Bastos MM, Faustino-Rocha A, Lopes C, Oliveira PA, Medeiros R (2015) MicroRNA-21 expression and susceptibility to HPV-induced carcinogenesis - role of microenvironment in K14-HPV16 mice model. *Life Sci* 128:8-14. doi:10.1016/j.lfs.2015.02.020 S0024-3205(15)00137-X [pii]
24. Paiva I, Gil da Costa RM, Ribeiro J, Sousa H, Bastos M, Rocha AF, Oliveira PA, Medeiros R (2015) A Role for MicroRNA-155 Expression in Microenvironment Associated to HPV-Induced Carcinogenesis in K14-HPV16 Transgenic Mice. *PLoS One* 10 (1):e0116868. doi:10.1371/journal.pone.0116868 PONE-D-14-33446 [pii]
25. Paulson K, Maziasz, T (2004) Role of preclinical metabolism and pharmacokinetics in the development of Celecoxib. In: Rajesh K (ed) *Application of Pharmacokinetic Principles in Drug Development*. 1 edn. Springer US, pp 405-425. doi:10.1007/978-1-4419-9216-1
26. Peters PJ, Borst J, Oorschot V, Fukuda M, Krahenbuhl O, Tschopp J, Slot JW, Geuze HJ (1991) Cytotoxic T lymphocyte granules are secretory lysosomes, containing both perforin and granzymes. *J Exp Med* 173 (5):1099-1109
27. Betts MR, Brenchley JM, Price DA, De Rosa SC, Douek DC, Roederer M, Koup RA (2003) Sensitive and viable identification of antigen-specific CD8+ T cells by a flow cytometric assay for degranulation. *J Immunol Methods* 281 (1-2):65-78. doi:S0022175903002655 [pii]
28. Venuti A, Paolini F, Nasir L, Corteggio A, Roperto S, Campo MS, Borzacchiello G (2011) Papillomavirus E5: the smallest oncoprotein with many functions. *Mol Cancer* 10:140. doi:10.1186/1476-4598-10-140 1476-4598-10-140 [pii]
29. Hibma MH (2012) The immune response to papillomavirus during infection persistence and regression. *Open Virol J* 6:241-248. doi:10.2174/1874357901206010241 TOVJ-6-241 [pii]
30. Peng S, Trimble C, Wu L, Pardoll D, Roden R, Hung CF, Wu TC (2007) HLA-DQB1*02-restricted HPV-16 E7 peptide-specific CD4+ T-cell immune responses correlate with regression of HPV-16-associated high-grade squamous intraepithelial lesions. *Clin Cancer Res* 13 (8):2479-2487. doi:13/8/2479 [pii] 10.1158/1078-0432.CCR-06-2916
31. Balkwill F, Mantovani A (2001) Inflammation and cancer: back to Virchow? *Lancet* 357 (9255):539-545. doi:S0140-6736(00)04046-0 [pii] 10.1016/S0140-6736(00)04046-0
32. Adefuye A, Sales K (2012) Regulation of inflammatory pathways in cancer and infectious disease of the cervix. *Scientifica (Cairo)* 2012:548150. doi:10.6064/2012/548150
33. Wolff H, Saukkonen K, Anttila S, Karjalainen A, Vainio H, Ristimäki A (1998) Expression of cyclooxygenase-2 in human lung carcinoma. *Cancer Res* 58 (22):4997-5001
34. Sano H, Kawahito Y, Wilder RL, Hashiramoto A, Mukai S, Asai K, Kimura S, Kato H, Kondo M, Hla T (1995) Expression of cyclooxygenase-1 and -2 in human colorectal cancer. *Cancer Res* 55 (17):3785-3789

35. Ryu HS, Chang KH, Yang HW, Kim MS, Kwon HC, Oh KS (2000) High cyclooxygenase-2 expression in stage IB cervical cancer with lymph node metastasis or parametrial invasion. *Gynecol Oncol* 76 (3):320-325. doi:10.1006/gyno.1999.5690
S0090-8258(99)95690-1 [pii]
36. Ristimäki A, Honkanen N, Jankala H, Sipponen P, Harkonen M (1997) Expression of cyclooxygenase-2 in human gastric carcinoma. *Cancer Res* 57 (7):1276-1280
37. Golijanin D, Tan JY, Kazior A, Cohen EG, Russo P, Dalbagni G, Auburn KJ, Subbaramaiah K, Dannenberg AJ (2004) Cyclooxygenase-2 and microsomal prostaglandin E synthase-1 are overexpressed in squamous cell carcinoma of the penis. *Clin Cancer Res* 10 (3):1024-1031
38. Thun MJ, Namboodiri MM, Heath CW, Jr. (1991) Aspirin use and reduced risk of fatal colon cancer. *N Engl J Med* 325 (23):1593-1596. doi:10.1056/NEJM199112053252301
39. Robertson FM, Parrett ML, Joarder FS, Ross M, Abou-Issa HM, Alshafie G, Harris RE (1998) Ibuprofen-induced inhibition of cyclooxygenase isoform gene expression and regression of rat mammary carcinomas. *Cancer Lett* 122 (1-2):165-175. doi:S0304-3835(97)00387-X [pii]
40. Pathi S, Jutooru I, Chadalapaka G, Nair V, Lee SO, Safe S (2012) Aspirin inhibits colon cancer cell and tumor growth and downregulates specificity protein (Sp) transcription factors. *PLoS One* 7 (10):e48208. doi:10.1371/journal.pone.0048208
PONE-D-12-23952 [pii]
41. Harris RE, Chlebowski RT, Jackson RD, Frid DJ, Ascenseo JL, Anderson G, Loar A, Rodabough RJ, White E, McTiernan A (2003) Breast cancer and nonsteroidal anti-inflammatory drugs: prospective results from the Women's Health Initiative. *Cancer Res* 63 (18):6096-6101
42. Bertagnolli MM, Eagle CJ, Zauber AG, Redston M, Solomon SD, Kim K, Tang J, Rosenstein RB, Wittes J, Corle D, Hess TM, Woloj GM, Boissier F, Anderson WF, Viner JL, Bagheri D, Burn J, Chung DC, Dewar T, Foley TR, Hoffman N, Macrae F, Pruitt RE, Saltzman JR, Salzberg B, Sylwestrowicz T, Gordon GB, Hawk ET (2006) Celecoxib for the prevention of sporadic colorectal adenomas. *N Engl J Med* 355 (9):873-884. doi:355/9/873 [pii]
10.1056/NEJMoa061355
43. Baron JA, Sandler RS, Bresalier RS, Quan H, Riddell R, Lanos A, Bolognese JA, Oxenius B, Horgan K, Loftus S, Morton DG (2006) A randomized trial of rofecoxib for the chemoprevention of colorectal adenomas. *Gastroenterology* 131 (6):1674-1682. doi:S0016-5085(06)01994-9 [pii]
10.1053/j.gastro.2006.08.079
44. Zhang DQ, Guo Q, Zhu JH, Chen WC (2013) Increase of cyclooxygenase-2 inhibition with celecoxib combined with 5-FU enhances tumor cell apoptosis and antitumor efficacy in a subcutaneous implantation tumor model of human colon cancer. *World J Surg Oncol* 11:16. doi:10.1186/1477-7819-11-16
1477-7819-11-16 [pii]
45. Coussens LM, Hanahan D, Arbeit JM (1996) Genetic predisposition and parameters of malignant progression in K14-HPV16 transgenic mice. *Am J Pathol* 149 (6):1899-1917
46. van den Hende M, van Poelgeest MI, van der Hulst JM, de Jong J, Drijfhout JW, Fleuren GJ, Valentijn AR, Wafelman AR, Slappendel GM, Melief CJ, Offringa R, van der Burg SH, Kenter GG

(2008) Skin reactions to human papillomavirus (HPV) 16 specific antigens intradermally injected in healthy subjects and patients with cervical neoplasia. *Int J Cancer* 123 (1):146-152. doi:10.1002/ijc.23502

47. Haas AR, Sun J, Vachani A, Wallace AF, Silverberg M, Kapoor V, Albelda SM (2006) Cyclooxygenase-2 inhibition augments the efficacy of a cancer vaccine. *Clin Cancer Res* 12 (1):214-222. doi:12/1/214 [pii]

10.1158/1078-0432.CCR-05-1178