Human Papillomavirus E7 Oncoprotein Transgenic Skin Develops an Enhanced Inflammatory Response to 2,4-Dinitrochlorobenzene by an Arginase-1-Dependent Mechanism

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We have shown that the expression of human papillomavirus type 16 E7 (HPV16.E7) protein within epithelial cells results in local immune suppression and a weak and ineffective immune response to E7 similar to that occuring in HPV-associated premalignancy and cancers. However, a robust acute inflammatory stimulus can overcome this to enable immune elimination of HPV16.E7-transformed epithelial cells. 2,4-Dinitrochlorobenzene (DNCB) can elicit acute inflammation and it has been shown to initiate the regression of HPV-associated genital warts. Although the clinical use of DNCB is discouraged owing to its mutagenic potential, understanding how DNCB-induced acute inflammation alters local HPV16.E7-mediated immune suppression might lead to better treatments. Here, we show that topical DNCB application to skin expressing HPV16.E7 as a transgene induces a hyperinflammatory response, which is not seen in nontransgenic control animals. The E7-associated inflammatory response is characterized by enhanced expression of Th2 cytokines and increased infiltration of $CD11b^+Gr1^{int}F4/80^+Ly6C^{hi}Ly6G^{low}$ myeloid cells, producing arginase-1. Inhibition of arginase with an arginase-specific inhibitor, *N*(omega)-hydroxy-nor-L-arginine, ameliorates the DNCB-induced inflammatory response. Our results demonstrate that HPV16.E7 protein enhances DNCB-associated production of arginase-1 by myeloid cells and consequent inflammatory cellular infiltration of skin.

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INTRODUCTION

Persistent infection with oncogenic human papillomaviruses (HPV), particularly HPV16, is associated with selective expression of two virally encoded proteins (E6 and E7). One action of HPV16.E7 protein is to subvert the innate immune system (Frazer *et al.*, 1998) through the downregulation of IFNγ pathways, modulation of antigen presentation, and suppression of Toll-like receptor 9 protein (Bhat *et al.*, 2011).

K14.E7 transgenic mice, which express HPV16.E7 oncoprotein within basal keratinocytes under the control of the keratin 14 transcriptional promoter, have been extensively used as a model of HPV oncoprotein–induced immune suppression associated with human squamous cancers, in which only the *E6* and *E7* genes of the papillomavirus are expressed (Trimble and Frazer, 2009). We have previously shown that skin grafts expressing HPV16.E7 oncoprotein are not spontaneously rejected when transplanted onto syngeneic animals, but they are rejected when certain components of the innate immune system are unavailable, confirming that the expression of HPV16.E7 in the epithelium results in the establishment of a local suppressive environment and the subversion of antigen-specific T cells (Mattarollo *et al.*, 2010; Mittal *et al.*, 2013). Therefore, successful strategies targeting HPV-associated cancer need to circumvent or disrupt the local suppressive environment.

Topical immunotherapy with immunostimulatory agents has been used clinically to treat cancerous lesions including squamous cell and basal cell carcinoma in immunocompetent and immunosuppressed patients (Hengge *et al.*, 2001). Topical application of 2,4-dinitrochlorobenzene (DNCB) is an effective therapy for condylomata acuminata caused by HPV infection. DNCB induced the complete clearance of HPV-associated warts in 13/15 patients (Georgala *et al.*, 1989). The efficacy of this treatment is attributed to the immunostimulatory role of DNCB that might activate cell-mediated

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Abbreviations: CIN, cervical intraepithelial neoplasia; DNCB, 2,4-dinitrochlorobenzene; HPV, human papillomavirus; Nor-NOHA, N^{ω}-hydroxy-nor-L-arginine; mRNA, messenger RNA

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immunity (Belij *et al.*, 2012). However, the use of DNCB for treatment has been discouraged because of its mutagenic potential (Black *et al.*, 1985). We speculated that understanding how induction of a vigorous acute inflammatory response by DNCB can break the locally immune-suppressive environment and restore the effector function of adaptive immunity might lead to more acceptable treatments for persisting HPV infection.

Arginase, which metabolizes L-arginine to *N*-ornithine and urea, has been identified as a crucial regulator of inflammation. Mammalian cells express two different isoforms, arginase-1 and arginase-2, which are encoded by two distinct genes and are different in their tissue distribution and subcellular localization (Bronte and Zanovello, 2005). Arginase can function as an immunosuppressive factor in the tumor environment (Ochoa *et al.*, 2007), in virus (Tacke *et al.*, 2012), or in parasite infection (Mou *et al.*, 2013). However, this enzyme has also been identified as an important proinflammatory factor in numerous disease models (Kenyon *et al.*, 2008; Zhang *et al.*, 2009; Stoermer *et al.*, 2012). As inflammation decides the fate of HPV infection and arginase is an important regulator of inflammation and immunity, we investigated the interaction between DNCB-induced inflammation and HPV16.E7 protein in induction of arginase in K14.E7 transgenic mice. We show here that K14.E7 transgenic mice exhibit an enhanced local inflammatory response to DNCB treatment, compared with nontransgenic mice, and that myeloid cells express increased arginase-1, which specifically promotes DNCB-induced inflammation.

RESULTS

K14.E7 mice develop a robust inflammation response to DNCB

An inflammatory response was induced in wild-type nontransgenic C57BL/6 and in E7 transgenic K14.E7 mice by applying DNCB topically to the ear skin. The mean increase in ear thickness was monitored as an indicator of inflammatory reaction (Pinto *et al.*, 2010). K14.E7 mice displayed a significantly higher degree of ear swelling than C57BL/6 mice, which peaked at day 3 in response to a single application of DNCB (Figure 1a).

T lymphocytes have been found in several skin inflammation diseases and have an important role in the production of inflammatory cytokines and in the recruitment of innate immune cells (Bromley *et al.*, 2013). They also drive the enhanced inflammatory response seen on repeated exposure to DNCB (Wang *et al.*, 2000). In addition, our previous study showed that lymphocytes are increased in number in K14.E7 skin (Choyce *et al.*, 2013). Therefore, we investigated whether the enhanced inflammatory response to first exposure to DNCB in K14.E7 skin was dependent on local lymphocyte function. K14.E7 mice deficient in lymphocytes ($Rag^{-/-} \times E7$), when exposed to DNCB, exhibited a similar level of ear swelling as K14.E7 mice, and stronger than control $Rag^{-/-}$ mice over 5 days after DNCB treatment (Figure 1a). Thus, DNCB-treated K14.E7 mice exhibit a hyperinflammatory response on first exposure to DNCB, which is independent of an adaptive immune response.

DNCB-treated K14.E7 skin displays an enhanced infiltration of myeloid cells and Th2 cytokine expression

To characterize further the inflammation in DNCB-treated K14.E7 skin, we examined the infiltration of immune cells. Histological examination suggested that the greater ear swelling in K14.E7 mice corresponded to a significant increase in the number of immune cells infiltrating the dermis (Figure 1b). As expected, the thickness of the K14.E7 epidermal layer remained unchanged following DNCB treatment (control: $42.4 \pm 3.7\,\mu m$, DNCB: $40 \pm 3.5\,\mu m$). $Rag^{-/-} \times E7$ mice develop similar ear swelling and inflammatory cell infiltration in the dermis as K14.E7 mice, whereas control $Rag^{-/-}$ mice do not (Figure 1a and b). Thus, the increased ear swelling in K14.E7 in response to DNCB mice was mainly contributed by infiltration of cells of the innate immune system in the dermis, and it was independent of lymphocytes.

Flow cytometry analysis revealed that DNCB-treated K14.E7 ears recruited significantly higher numbers of myeloid cells ($CD45.2^{+}CD11b^{+}$) than similarly treated C57BL/6 ears. We observed the same trend when we compared $Rag^{-/-} \times E7$ mice with $Rag^{-/-}$ mice (Figure 2a). In contrast, the number of non-myeloid bone marrow–derived cells ($CD45.2^{+}CD11b^{-}$) remained unchanged and was comparable in all four groups following DNCB treatment (Figure 2b). Thus, the expression of HPV16.E7 in the skin mediates the enhanced recruitment of myeloid cells, and this effect is independent of adaptive immunity.

IL-1β and IL-6 are major cytokines secreted from the local inflammation site and promote the recruitment of innate immune cells (Zohlnhofer *et al.*, 2000; Fielding *et al.*, 2008). DNCB treatment resulted in a significant increase in IL-1β messenger RNA (mRNA) expression in both C57BL/6 and K14.E7 skin (Table 1). In addition, IL-6 mRNA expression was significantly increased in DNCB-treated K14.E7 skin but not in nontransgenic skin, suggesting that IL-6 might be responsible for the enhanced recruitment of myeloid cells in K14.E7 skin.

Further, DNCB treatment of K14.E7 mice significantly induced mRNA expression of prostaglandin E2 synthase and Th2 cytokines IL-4 and IL-10. In contrast, there was no significant change in the expression of these factors in DNCB-treated C57BL/6 mice. Th1 cytokines displayed decreased (tumor necrosis factor-α) or unaltered (interferon gamma) expression after DNCB treatment (Table 1).

Together, these results suggest that after a single DNCB treatment to previously unexposed animals, K14.E7 mice mounted an enhanced inflammatory response, which is accompanied by $CD45.2^{+}CD11b^{+}$ myeloid cell infiltration and amplified Th2 cytokine expression.

Arginase-1 is specifically induced in DNCB-treated K14.E7 mice

Inflammation and myeloid cell activation can be associated with the induction of arginase (Bronte *et al.*, 2003). We speculated that arginase activity might contribute to enhanced inflammation of K14.E7 skin in response to DNCB. To test this hypothesis, we first investigated arginase activity in the skin of C57BL/6 and K14.E7 mice following DNCB treatment. Arginase activity was comparable in control K14E7 and

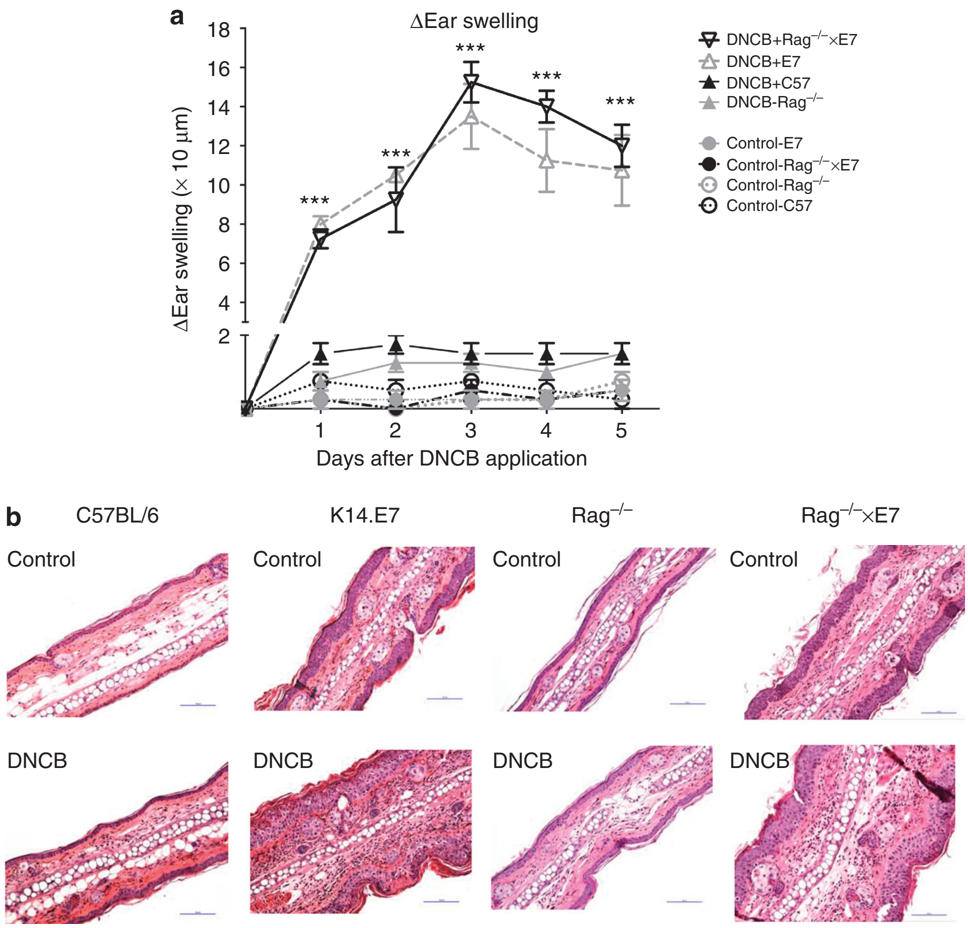

Figure 1. K14.E7 mice exhibit an enhanced inflammatory response to 2,4-dinitrochlorobenzene (DNCB). Ear swelling of C57BL/6, K14.E7, $Rag^{-/-}$, and $Rag^{-/-} \times$ E7 mice in response to DNCB or vehicle over 5 consecutive days (**a**) measured by caliper. Data are means ± SEM, and are representative of two independent experiments with four mice per group. ***$P<0.001$. Histology of representative sections from C57BL/6, K14.E7, $Rag^{-/-} \times$ E7, and $Rag^{-/-}$ mice (**b**) at 1 day post DNCB or vehicle application. Hematoxylin and eosin stain, original magnification ×200, scale bar = 100 μm (representative of four mice per group).

C57BL/6 ear skin (Figure 3a), as was arginase mRNA expression (Figure 3b and RNAseq data not shown). DNCB-treated K14.E7 skin, however, demonstrated a substantially higher amount of arginase activity than DNCB-treated C57BL/6 skin (Figure 3a). Arginase activity is contributed by two arginase isoforms. Increased arginase activity in K14.E7 mice corresponded to markedly increased (fourfold) arginase-1 mRNA expression. Conversely, the expression of arginase-1 mRNA remained unchanged in DNCB-treated C57BL/6 mice (Figure 3b). In contrast to arginase-1, there was no significant induction of arginase-2 mRNA in either C57BL/6 or K14.E7 skin (Figure 3c).

K14.E7 mice lacking lymphocytes ($Rag^{-/-} \times$ E7) not only exhibited a similar level of ear swelling as K14.E7 mice but also of arginase-1 mRNA and arginase activity following DNCB treatment. Notably, Arginase-1 and arginase-2 mRNA expression are not increased in control $Rag^{-/-}$ mice following DNCB treatment (Figure 3b and c). Thus, the induction of arginase in K14.E7 skin is independent of adaptive immunity.

To examine whether the induction of arginase was unique to skin expressing HPV16.E7 oncoprotein as a transgene, K14.hGh and K5.OVA mice expressing human growth hormone or ovalbumin, respectively, under the control of keratin promoters were treated with DNCB. In contrast to K14.E7 mice, there was no change in the level of arginase activity of K14.hGh or K5.OVA mice following DNCB treatment (Figure 3d). Furthermore, the ear swelling of these transgenic mice (K5.OVA: 2.25 ± 0.5 μm; K14.hGh: 1.75 ± 0.5 μm, day 5) was similar to that of C57BL/6 mice (1.75 ± 0.5 μm, day 5) and markedly lower than in K14.E7 mice (19.8 ± 9.9 μm, day 5). Thus, these results suggest that increased arginase activity, likely derived from activated myeloid cells, might be a consequence of exposure of K14.E7-expressing epithelial cells to DNCB.

$CD11b^+Gr1^{int}F4/80^+Ly6C^{hi}Ly6G^{low}$ cells are the major source of arginase-1 in DNCB-treated K14.E7 mice

Arginase-1 can be induced in myeloid cells including macrophages, dendritic cells, and myeloid-derived suppressive cells

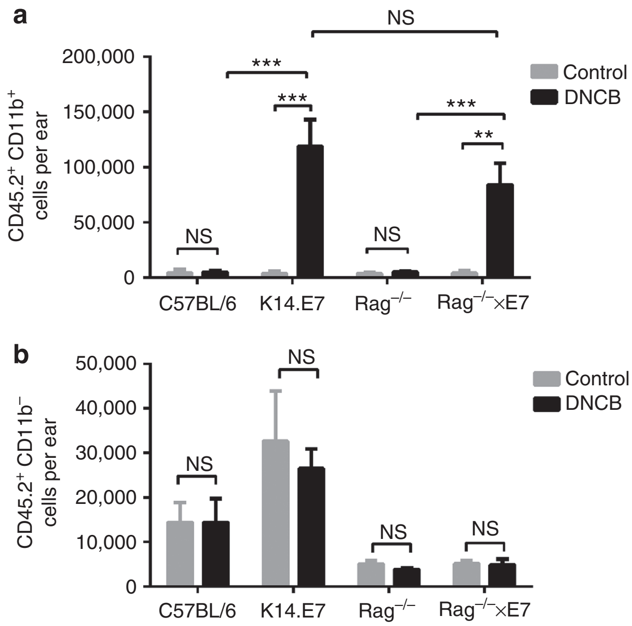

Figure 2. K14.E7 mouse skin has an enhanced myeloid cell infiltration in response to 2,4-dinitrochlorobenzene (DNCB). Absolute counts of leukocyte cells in the skin of C57BL/6 ($n=4$), K14.E7 ($n=6$), $Rag^{-/-}$ ($n=5$), and $Rag^{-/-}$ $\times$ E7 ($n=5$) mice following DNCB treatment. Absolute numbers of $CD45.2^{+}CD11b^{+}$ myeloid cells (**a**) and $CD45.2^{+}CD11b^{-}$ cells (**b**) were determined by flow cytometry. Data are presented as means ± SEM and are representative of two independent experiments. NS, not significant. $^{**}P<0.01$, $^{***}P<0.001$.

Table 1. Expression of cytokine mRNA in response to DNCB or vehicle in K14E7 and C57 mice

	Expression relative to housekeeping gene *RPL32*				
	C57BL/6		K14.E7		
Cytokine	Vehicle	DNCB	Vehicle	DNCB	*P*
IL-1β ($\times 10^4$)	6.8 ± 0.8	51 ± 4	221 ± 132	1490 ± 900	#, +, *, @
IL-6 ($\times 10^4$)	50 ± 8	55 ± 29	142 ± 103	5310 ± 2870	+, @
IL-4 ($\times 10^{11}$)	1.3 ± 2.2	3.0 ± 3.1	15.1 ± 14.2	74.5 ± 53.3	+, @, *
Ptge2s ($\times 10^4$)	75 ± 74	91 ± 127	158 ± 34	480 ± 124	+, @
IL-10 ($\times 10^4$)	0.4 ± 0.3	0.8 ± 0.8	4.0 ± 1.1	7.0 ± 1.6	+, *
IFNγ ($\times 10^4$)	0.2 ± 0.2	0.11 ± 0.12	1.9 ± 1.1	1.1 ± 0.9	*
TNFα ($\times 10^4$)	3.2 ± 2.9	2.7 ± 1.6	41.3 ± 15.2	3.4 ± 2.2	*, @

Abbreviations: DNCB, dinitrochlorobenzene; mRNA, messenger RNA; Ptge2s, prostaglandin E2 synthase; TNFα, tumor necrosis factor-α.
$^{\#}P<0.05$ C57 DNCB versus vehicle.
$^{+}P<0.05$ K14E7 DNCB versus vehicle.
$^{*}P<0.05$ K14E7 vehicle versus C57 vehicle.
$^{@}P<0.05$ change in expression for DNCB-treated K14E7 versus DNCB-treated C57.

in response to a wide range of stimuli (Sindrilaru *et al.*, 2011; Chang *et al.*, 2013). These myeloid cells express surface CD11b (Hammad and Lambrecht, 2008). To confirm whether myeloid cells were the source of DNCB-induced arginase-1 in K14.E7 skin, $CD45.2^{+}CD11b^{-}$ and $CD45.2^{+}CD11b^{+}$ cells (Supplementary Figure S1 online) were isolated from DNCB-treated C57BL/6 and K14.E7 mice and analyzed for arginase activity and arginase-1 mRNA expression. $CD11b^{+}$ myeloid cells from DNCB-treated K14.E7 mice produced significantly higher levels of arginase activity per cell compared with $CD11b^{-}$ cells, and compared with $CD11b^{+}$ myeloid cells from DNCB-treated C57BL/6 mice (Figure 4a). In addition, arginase-1 mRNA expression was substantially detected in $CD45.2^{+}CD11b^{+}$ cells in DNCB-treated K14.E7 mice, but not in $CD11b^{-}$ or $CD11b^{+}$ cells from C57BL/6 mice (Figure 4b). Although arginase-2 mRNA was also detected in $CD45.2^{+}CD11b^{+}$ cells in K14.E7 skin, arginase-2 mRNA expression was 10 times lower than arginase-1 (Figure 4b). Thus, DNCB-activated myeloid cells ($CD45.2^{+}CD11b^{+}$) produce the increased arginase-1 observed in DNCB-treated K14.E7 skin.

CD11b is expressed on different myeloid subsets including macrophages/monocytes and granulocytes. To further define which cell subset expresses arginase-1 in DNCB-treated K14.E7 mice, we examined the expression level of arginase-1 in different populations of $CD11b^{+}$ cells based on the expression of macrophage marker F4/80 and granulocyte marker Gr1. We found that $F4/80^{+}Gr1^{int}$ cells expressed significantly higher levels of arginase-1 than other cell populations (Figure 4c). Furthermore, arginase-1 was abundantly expressed in inflammatory monocytes that express $Ly6C^{hi}Ly6G^{low}$ but not in $Ly6C^{low}Ly6G^{hi}$ granulocytes (Figure 4d).

Suppression of arginase ameliorates the ear swelling of inflamed K14.E7 skins

Arginase can be paradoxically involved in the upregulation or downregulation of inflammatory responses. *N*(omega)-hydroxy-nor-L-arginine (nor-NOHA), which is an intermediate in the L-arginine/NO pathway, is widely used as a specific, reversible inhibitor of arginase both *in vitro* and *in vivo* (Tenu *et al.*, 1999; Bratt *et al.*, 2009). To understand whether arginase promoted or suppressed the inflammatory response of K14.E7 mice to DNCB, we administered nor-NOHA or saline buffer to K14.E7 mice 1 day before DNCB treatment and daily for 4 days.

Ear tissue was harvested after 24 hours of DNCB treatment with or without arginase inhibitor, and the expression and activity of arginase were determined. Nor-NOHA, as expected, did not suppress the transcription of arginase-1 gene (Figure 5a), but reduced arginase activity in DNCB-treated K14.E7 skin (Figure 5b).

Furthermore, swelling of DNCB-treated K14.E7 skin was reduced in animals treated with arginase inhibitor (Figure 5c). Consistent with the ear swelling data, arginase inhibition decreased the number of infiltrating cells in the dermis of DNCB-treated K14.E7 mice (Figure 5d and e). These results demonstrate that arginase-1 induced by DNCB in K14.E7 transgenic ear skin, but not in C57BL/6 ear skin, itself contributes to exacerbated inflammatory response to DNCB in K14.E7 skin by recruitment of further inflammatory cells.

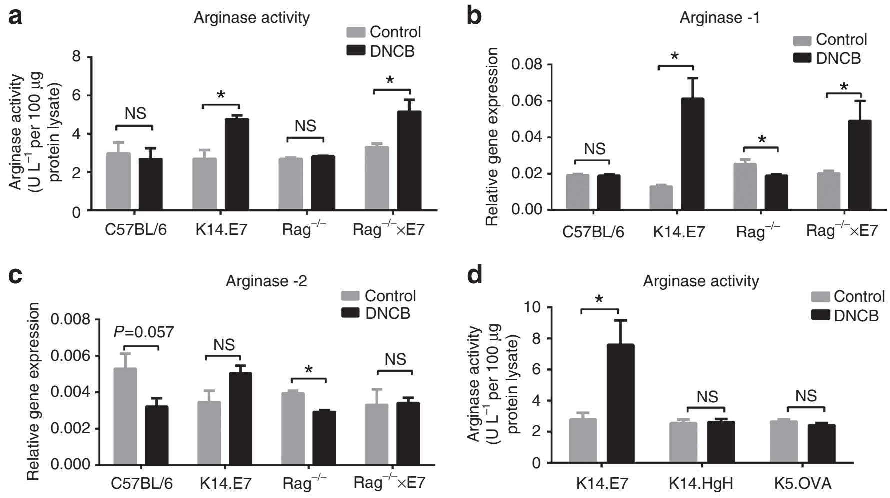

Figure 3. Arginase-1, but not arginase-2, is induced in K14.E7 mice following 2,4-dinitrochlorobenzene (DNCB) treatment, and this regulation is lymphocyte independent. Arginase activity was measured by determining the release of urea product from 100 μg of protein lysate per sample. Protein lysates were prepared from ear tissues of DNCB or vehicle-treated C57BL/6, K14.E7, Rag$^{-/-}$, Rag$^{-/-}$ × E7, K14.HgH, and K5.OVA ear tissues. Relative gene expression levels of (**b**) arginase-1 and (**c**) arginase-2 messenger RNAs were determined by real-time PCR in the skin of C57BL/6, K14.E7, Rag$^{-/-}$, and Rag$^{-/-}$ × E7 mice 1 day following DNCB treatment, normalized against the housekeeping gene *RPL32*. Data are presented as means ± SEM and are representative of two independent experiments ($n=4$ mice per group). NS, not significant. *$P<0.05$.

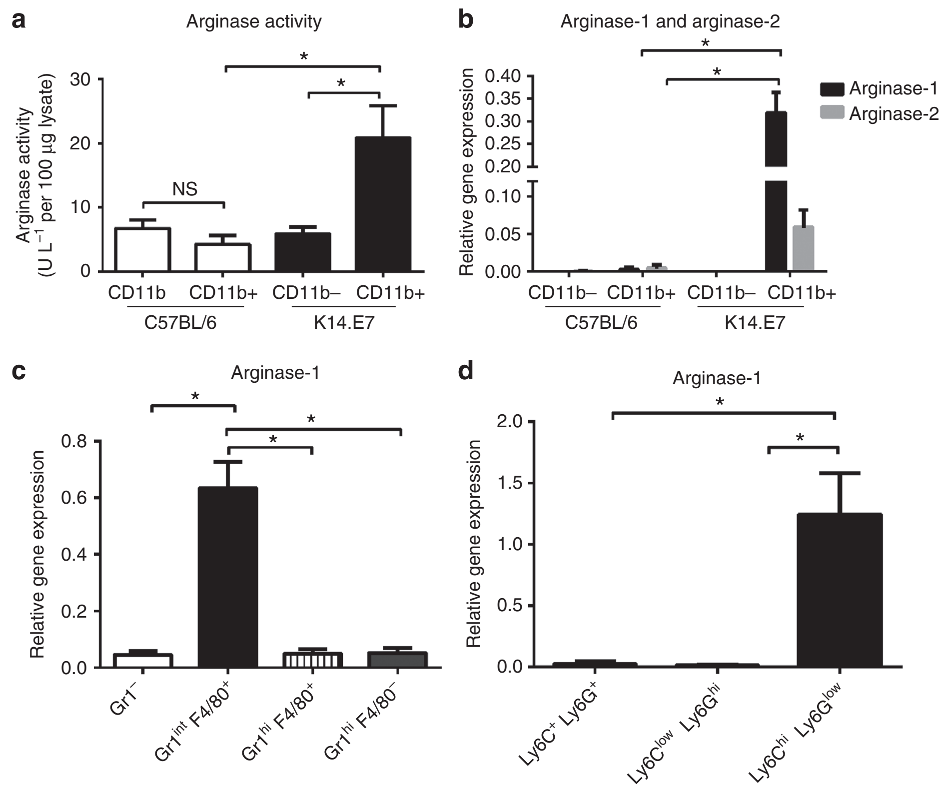

Figure 4. CD11b^{+} Gr1^{int} F4/80^{+} Ly6G^{low}Ly6C^{hi} cells produce arginase in 2,4-dinitrochlorobenzene (DNCB)-treated K14.E7 skin. Arginase activity produced by CD45.2^{+}CD11b^{+} and CD45.2^{+}CD11b^{-} populations from DNCB-treated C57BL/6 and K14.E7 mice (**a**) was determined as described in Materials and Methods. Arginase-1 and arginase-2 mRNA expression (**b**) levels were determined by real-time PCR. Arginase-1 messenger RNA (mRNA) expression levels in Gr1^{-}F4/80^{+}; Gr1^{int}F4/80^{+}; Gr1^{hi}F4/80^{+}; and Gr1^{-}F4/80^{-} subsets from DNCB-treated K14.E7 mice were determined (**c**) by real-time PCR. Real-time PCR analysis of arginase-1 mRNA expression in different cell subsets (**d**) was based on the expression of Ly6C and Ly6G antigens. Data were pooled from four independent experiments. In each experiment, six C57BL/6 and two K14.E7 mice were treated with DNCB or vehicle. Means ± SEM. NS, not significant. *$P<0.05$.

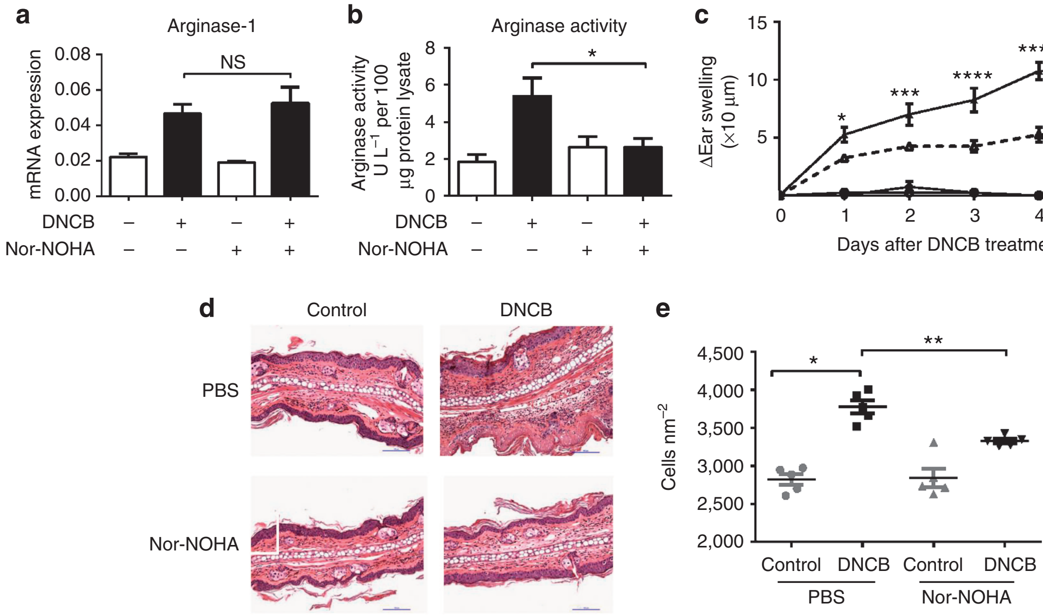

Figure 5. Inhibition of arginase ameliorates the ear swelling of dinitrochlorobenzene (DNCB)-treated K14.E7 mice. Mice were injected with 500 µg of arginase inhibitor (N^{ω}-hydroxy-nor-L-arginine (nor-NOHA)) or saline buffer 1 day before DNCB treatment and daily for 5 days. Arginase-1 messenger RNA (mRNA) expression (**a**) and arginase activity (**b**) in saline or nor-NOHA-treated K14.E7 mice after 24 hours of exposure to DNCB, determined by real-time PCR and arginase assay, respectively. Ear swelling of K14.E7 mice treated with saline (solid line) or nor-NOHA (dashed line) monitored during 5 days after DNCB treatment (**c**). Histological sections (**d**) and quantification of cell numbers infiltrating into the dermis from Nor-NOHA or saline-treated K14.E7 skins 1 day following DNCB application (**e**). Means ± SEM, data show results from two independent experiments ($n = 5$). NS, not significant; PBS, phosphate-buffered saline. *$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$.

DISCUSSION

DNCB triggers a T-cell–independent inflammatory response through the activation of the NALP-3 inflammasome in keratinocytes (Schuepbach-Mallepell *et al.*, 2013), and it has been used to treat HPV-associated genital warts. Despite its efficacy, this chemical is potentially mutagenic and carcinogenic (Black *et al.*, 1985). Therefore, we sought to understand the influence of the expression of the major oncogenic protein of HPV16, E7, on the inflammatory mechanism induced by DNCB using mice transgenic for the E7 protein expressed in keratinocytes. Here, we show that the acute response to DNCB in previously unexposed mice is significantly higher in K14.E7 transgenic mice than in nontransgenic mice. In response to DNCB-induced inflammasome activation, keratinocytes have been shown to produce a wide range of proinflammatory cytokines including IL-6 and IL-1β (Enk and Katz, 1992; Rambukkana *et al.*, 1996), which are known to be responsible for the recruitment of polymorphonuclear cells (Zohlnhofer *et al.*, 2000; Fielding *et al.*, 2008). We indeed found an increased number of inflammatory myeloid cells in the dermis and enhanced mRNA expression levels of IL-6 and IL-1β, in DNCB-treated K14E7 transgenic mice, and show further that DNCB-induced IL-1β and IL-6 production was greatly enhanced in K14E7 skin when compared with non-transgenic skin. We could not detect significant induction of IL-6 in nontransgenic skin. Therefore, we hypothesize that this cytokine might be a regulator of inflammatory cell infiltration in K14.E7 skin and its ear swelling. Premalignant skin of HPV16.E7 transgenic mice is characterized by hyperplastic epidermis and infiltration of innate immune cells of myeloid origin. The presence of these cells in K14.E7 skin might be responsible for the marked inflammatory response to short-term DNCB treatment and inflammasome activation. Interestingly, we were not able to detect these responses to DNCB in other transgenic skin including K14.hGh and K5.OVA, suggesting that they are unique to K14.E7 mice, a consequence of the presence of HPV.E7 oncoprotein in the skin.

DNCB treatment of K14.E7 mice significantly induced mRNA expression of Th2 cytokines IL-4 and IL-10, when compared with wild-type mice. This finding is consistent with the finding in a mouse model of allergic skin inflammation, in which Th2 cytokines are responsible for the amplification and chronicity of allergic skin inflammation (Masuoka *et al.*, 2012), and with a study in which IL-4-deficient mice displayed an impaired ear swelling response to DNCB application (Traidl *et al.*, 1999). Furthermore, we show that DNCB-treated K14.E7 skin exhibited an accumulation of $CD11b^{+}$ myeloid cells, but not of non-myeloid bone marrow–derived cells, which are mainly lymphocytes. Th2 cytokines induce arginase activity in myeloid cells including macrophages and dendritic cells (Munder *et al.*, 1999; Barron *et al.*, 2013). Arginase-2 can be expressed by macrophages, and it has structural and enzyme characteristics similar to arginase-1 (Khallou-Laschet *et al.*, 2010), but it remained unchanged in K14.E7 skin. Thus, the higher level of arginase activity in DNCB-treated K14.E7 mice is mainly contributed by arginase-1, and is promoted possibly by higher levels of Th2 cytokines and by accumulation of myeloid cells.

One predicted source of enhanced Th2 cytokine production in DNCB-treated K14E7 skin would be Th2 $CD4^+$ lymphocytes. Indeed, K14E7 skin has a large number of $CD4^+$ lymphocytes (Choyce *et al.*, 2013). However, we demonstrate here that the induction of arginase-1 and enhanced ear swelling response of K14.E7 skin are independent of lymphocytes. Th2 cytokines, which induce arginase-1 expression and hyperinflammatory response in DNCB-treated K14.E7, might therefore be derived from innate immune cells (Bradding *et al.*, 1992; Kuroda *et al.*, 2009) or epithelial cells (Balato *et al.*, 2012). We also detected the induction of prostaglandin E2 synthase in DNCB-treated K14.E7 skin. Our data are consistent with a previous study that HPV16.E7 oncoprotein induced cyclooxygenase 2 transcription and prostaglandin E2 synthase production (Subbaramaiah and Dannenberg, 2007). Furthermore, the cyclooxygenase 2–Pteg2s synthase axis has been shown to induce arginase-1 in myeloid cells in a tumor environment (Rodriguez *et al.*, 2005). However, further studies are needed to address the induction mechanism of arginase-1 in DNCB-treated K14.E7 skin.

Cells producing arginase-1 in K14.E7 skin were positive for F4/80 and Ly6C antigen, and express Gr1 at the intermediate level. This population has been defined as monocytic myeloid suppressive cells or inflammatory monocytes, which appear to adopt an immune stimulatory or suppressive function depending on the local environment (Kallberg *et al.*, 2012). Indeed, $CD11b^+Gr1^+$ cells during the early phase of polymicrobial sepsis exhibit proinflammatory phenotypes, whereas this cell population becomes immature and immune suppressive in the late phase (Brudecki *et al.*, 2012).

To understand the role of arginase in the hyperinflammation in K14.E7 skin, we used the compound nor-NOHA, which efficiently suppresses arginase-1 and arginase-2 activity in *in vitro* and *in vivo* studies (Tenu *et al.*, 1999; Bratt *et al.*, 2009). Nor-NOHA abrogates the function of arginase by modifying the structure of the enzyme, and it does not affect the transcription of the arginase gene, consistent with an unaltered arginase mRNA level (Krotova *et al.*, 2010). Arginase inhibitor treatment decreased the arginase activity and the level of leukocyte infiltrate and ear swelling in DNCB-treated K14.E7 mice. This suggests that enhanced arginase activity is critically required for the strong and sustained inflammatory response and that arginase inhibitor alleviates DNCB-induced inflammation by decreasing the recruitment of leukocytes in the skin. Arginase efficiently competes with inducible nitric oxide synthase for the common substrate L-arginine, leading to the inhibition of inducible nitric oxide synthase expression and NO production. As endothelium-derived NO is reported to suppress the expression of adhesion molecules such as vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 (Peng *et al.*, 1998), limiting of NO bioavailability through arginase results in enhanced vascular inflammation (White *et al.*, 2006). In a mouse model of atherogenesis, arginase-2 but not arginase-1 enhances monocyte adhesion to endothelial cells and triggers the production of proinflammatory cytokines through mitochondrial reactive oxygen species (Ming *et al.*, 2012). Liu *et al.* (2013) show that infiltrating myeloid cells produce arginase-1, which promotes angiogenesis and further recruitment of monocytes in a laser-induced injury murine model.

Taken together, our findings demonstrate that HPV16.E7 oncoprotein–expressing skin develops a hyperinflammatory response to DNCB via an arginase-1-dependent mechanism. These findings provide insights into the proinflammatory role of arginase-1 in HPV16.E7-expressing skin in response to immunostimulation by DNCB.

MATERIALS AND METHODS

Mice

K14.HPV16E7 (K14.E7) mice were generated from inbred strain C57BL/6 (Narayan *et al.*, 2009). K14.E7, K14.HgH, and $Rag^{-/-}$, all on a C57BL/6 background, were purchased from Animal Resources Center (Perth, Australia). K5.mOVA mice on a C57BL/6 background were kindly provided by H. Azukizawa (Azukizawa *et al.*, 2003). $Rag^{-/-} \times$ E7 mice were generated by crossing male K14.E7 with female $Rag1^{-/-}$ knockout C57BL/6 mice; heterozygous K14E7 mice were crossed and then backcrossed with homozygous $Rag1^{-/-}$ mice to an F2 generation (Narayan *et al.*, 2009). All mice were maintained under specific pathogen-free conditions at Princess Alexandra Hospital Biological Research Facility. Experimental mice were sex-matched and used at 6–9 weeks of age. All animal procedures complied with guidelines approved by the University of Queensland Animal Ethics Committee.

DNCB treatment

DNCB (Sigma, Sydney, New South Wales, Australia) was dissolved in vehicle (acetone: olive oil (4:1)) immediately before use. Six- to nine-week-old mice were treated with 20 µl of 1% DNCB or vehicle on the left ear and right ear, respectively. After 24 hours, the ear tissues were collected for mRNA and protein analysis. Ear thickness was measured by using the digital caliper, and change in ear swelling was determined by calculating the mean increase in ear thickness compared with untreated ears.

Histological analysis

Ear tissues were fixed using 4% paraformaldehyde. Tissues were embedded in paraffin and 7-µm sections were prepared and stained with hematoxylin and eosin. Immune cell infiltration was evaluated by light microscopy and quantified by using the Nis-elements Br 3.2 software (Nikon Instruments, New York, NY).

Real-time PCR

RNA was isolated from homogenized tissues by using the RNaeasy Mini kit (Qiagen, Melbourne, Victoria, Australia). RNA extracts were quantified using absorption of light at 260 and 280 nm (A260/280). Details of the procedures and primers used for the quantitative real-time PCR are described in Supplementary Methods online.

Arginase activity

Arginase activity was measured by colorimetric determination of urea formed from L-arginine, as previously described (Chang *et al.*, 2000). Details of the procedures are desbribed in Supplementary Methods online.

Flow cytometry and cell sorting

Flow cytometry staining was performed as previously described (Mattarollo *et al.*, 2010). Details of flow cytometry and cell sorting are described in Supplementary Methods online.

Statistical analysis

Each data point represents the mean ± SEM and is representative of two independent experiments with at least four mice per group. Prism (Graph pad Software, La Jolla, CA) was used for graphs and statistical analysis: *$P<0.05$; **$P<0.01$; ***$P<<0.001$; ****$P<0.0001$. Multiple comparisons of ear swelling data were derived by two-way analysis of variance. For other data, statistically significantly differences between groups were analyzed by nonparametric *t*-test (Mann–Whitney test).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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