Published in final edited form as:

Science. 2012 January 6; 335(6064): 104–108. doi:10.1126/science.1211600.

Langerhans Cells Facilitate Epithelial DNA Damage and Squamous Cell Carcinoma

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Abstract

Polyaromatic hydrocarbons (PAHs) are prevalent, potent carcinogens, and 7,12-dimethylbenz[*a*]anthracene (DMBA) is a model PAH widely used to study tumorigenesis. Mice lacking Langerhans cells (LCs), a signatory epidermal dendritic cell (DC), are protected from cutaneous chemical carcinogenesis, independent of T cell immunity. Investigation of the underlying mechanism revealed that LC-deficient skin was relatively resistant to DMBA-induced DNA damage. LCs efficiently metabolized DMBA to DMBA-*trans*-3,4-diol, an intermediate proximal to oncogenic *Hras* mutation, and DMBA-treated LC-deficient skin contained significantly fewer *Hras* mutations. Moreover, DMBA-*trans*-3,4-diol application bypassed tumor resistance in LC-deficient mice. Additionally, the genotoxic impact of DMBA on human keratinocytes was significantly increased by prior incubation with human-derived LC. Thus, tissue-associated DC can enhance chemical carcinogenesis via PAH metabolism, highlighting the complex relation between immune cells and carcinogenesis.

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Supporting Online Material

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Materials and Methods

Figs. S1 to S4

References (35–37)

Movie S1

Epithelial tissues, including skin, are situated at critical junctures with the environment and repeatedly exposed to chemical toxins and mutagens. In humans, ~90% of cancers arise in epithelial tissues. Such tissues are commonly replete with associated dendritic cells (DCs), for which the prototype is the epidermal Langerhans cell (LC) network. DCs have long been viewed as the primary means by which peripheral tissue neo-antigens are internalized, processed, and presented to antigen-specific T cells that may then mount and coordinate immunoprotection. Consistent with this, LCs induce contact hypersensitivity (CHS) responses and are considered, along with other tissue-resident DCs, to be well-placed to limit carcinogenesis through presentation of tumor-associated antigens to T cells. Recent studies of several LC-mutant mouse strains, however, have collectively argued for a reevaluation of the major functional contributions of LCs to epidermal biology (1–3). For example, CHS responses are augmented in mice where Langerin⁺ LCs are selectively deleted (2).

To investigate the potential of immune cells to protect against carcinogenesis, a two-stage cutaneous chemical carcinogenesis model is commonly used wherein single exposure of FVB mouse skin to the “initiator” 7,12-dimethylbenz[*a*]anthracene (DMBA), followed by repeated application of tumor “promoter,” 12-*O*-tetradecanoylphorbol 13-acetate (TPA), induces papillomas, some of which develop into squamous cell carcinomas (SCCs) (4, 5). An activating codon 61 mutation of the *Hras* proto-oncogene within affected keratinocytes characterizes >90% of DMBA-induced SCCs (6). DMBA is representative of mutagenic polyaromatic hydrocarbons (PAHs) to which humans can be exposed, and similar mutations are common in human carcinomas. Although TPA is pleiotropic, its proinflammatory effects are crucial to tumor promotion (7), consistent with common associations of inflammation with carcinogenesis. Thus, two-stage chemical carcinogenesis mimics many molecular and etiological aspects of human cancer. By applying two-stage carcinogenesis to gene-knockout mice, we and others have identified nonredundant host-protective roles of discrete lymphocyte subsets, including epidermal $\gamma\delta$ T cells (1, 5) and cytolytic $\alpha\beta$ T cells (8). Conversely, carcinogenesis may be enhanced by immunosuppressive CD4⁺ “T-reg” (9) and noncytolytic, interleukin (IL)–17–producing CD8⁺ $\alpha\beta$ “T-pro” cells (10).

Unexpectedly, LC-deficient huLangerin–diphtheria toxin A (Lang-DTA) mice showed almost complete resistance to DMBA-TPA-induced cutaneous carcinogenesis (1). Unaware of another example of such a profound cancer-protective effect afforded by removal of a single cell type, we sought to better characterize this finding and investigate the underlying mechanism (11). Because LCs can express T cell suppressive cytokines such as IL-10, we first considered that their absence might confer protection by augmenting antitumor potentials of $\gamma\delta$ and $\alpha\beta$ T cells. However, the marked resistance to carcinogenesis was comparable in Lang-DTA (1) and *Tcr β* ^{–/–} *Tcr δ* ^{–/–} Lang-DTA animals (Fig. 1A). Hence, tumor resistance conferred by loss of LCs is T cell-independent. Moreover, resistance was even seen under high-dose protocols in the selective absence of immunoprotective $\gamma\delta$ T cells, a combination shown (5) to induce the highest rate of cutaneous carcinoma (Fig. 1, B and C). This profound impairment of tumor formation on the highly susceptible FVB background suggested that LCs impart an early, requisite influence on keratinocyte transformation. Notably, LCs are located adjacent to both inter- and intra-follicular basal keratinocytes (12), and hair follicle bulge cells have been strongly implicated as targets of cutaneously applied chemical mutagens (13).

K5Hras transgenic mice express a constitutively active HRAS protein within basal keratinocytes (14) and, on the FVB background, show early onset cutaneous SCC (15) (fig. S1A). Crossing the Lang-DTA transgene onto K5Hras transgenic mice did not afford protection from tumor formation (fig. S1, B and C). Because the transgenic provision of mutated *Hras* overcame the effect of LC deficiency, we reasoned that generation of cells

with *Hras* mutations may ordinarily be fostered by LCs. Therefore, we assessed DNA damage in keratinocytes 24 hours after cutaneous application of DMBA. Phosphorylated histone H2A variant H2AX (γ H2AX), which is recruited to sites of DNA damage, showed more than threefold higher frequency staining within basal keratinocytes of DMBA-exposed skin of normal littermate controls (NLCs) relative to Lang-DTA mice (Fig. 2, A to C, and movie S1). Although some DNA damage was observed in Lang-DTA skin, this level is seemingly insufficient qualitatively and/or quantitatively to overcome the threshold required for tumor development. In contrast, application of the mutagenic DMBA metabolite DMBA-*trans*-3,4-dihydrodiol (DMBA-t-3, 4-diol) induced comparable levels of DNA damage in both strains, implicating LCs in the metabolism of DMBA to its mutagenic forms (Fig. 2C).

The carcinogenic potential of DMBA is unleashed by bioactivation via cytochrome P-450 enzymes (e.g., CYP1A1, CYP1B1) and microsomal epoxide hydrolase (EPXH1) after engagement of the aryl hydrocarbon receptor (AHR). In epithelial cells, including keratinocytes and hepatocytes, PAH engagement of the AHR markedly up-regulates the *Cyp1a1*, *Cyp1b1*, and *Epxh1* genes as part of the so-called transcriptional xenobiotic response. Although this response may have evolved for detoxification of potentially harmful natural exogenous aromatic compounds, accumulating evidence suggests roles for AHR in activation and differentiation of cells in response to endogenous ligands, such as the tryptophan photooxidation product 6-formylindolo[3,2-b]carbazole (16). In this regard, AHR has been implicated in LC development, differentiation, and activation (16–18). Murine LCs reportedly do not express *Cyp1a1* (18), however, and when we examined the interaction between DMBA and the Langerin⁺ MHC-II⁺, Birbeck granule⁺ LC line, XS106 (Fig. 2D), or freshly isolated murine epidermal LCs (Fig. 2E), neither baseline nor DMBA-inducible expression of *Cyp1a1* was observed. Rather, primary murine LCs showed modest, transient increases in *Cyp1b1*, *Epxh1*, and the *Ahr* repressor, *Ahr*r. This is important because previous studies in vitro using purified recombinant murine enzymes clearly show that metabolism of DMBA to the mutagenic DMBA-t-3,4-diol occurs preferentially via CYP1B1 (19–21), whereas CYP1A1 metabolism is biased toward nonmutagenic detoxification (fig. S2) (22, 23). DMBA-t-3,4-diol is converted to DMBA-3,4-diol-1,2-epoxide (DMBADE), which is immediately proximal to induction of the *Hras* codon 61 “signature” point mutation (24, 25).

To pursue the impact of LCs on *Hras* mutation in vivo, we designed a quantitative genomic DNA (gDNA) polymerase chain reaction (PCR) assay that selectively amplifies the DMBA-induced *Hras* codon 61 mutation (fig. S3A) (26). We generated plasmids containing either wild-type *Hras* (*Hras* WT) or codon 61 mutant *Hras* (*Hras*-mut61) and demonstrated the efficiency of this assay in *Hras*-mut61 quantification (fig. S3B). Using this assay, relative to primary murine keratinocytes (mKC), *Hras*-mut61 was found at levels exceeding 10⁵-fold in the SCC line CarC (100% *Hras*-mut61 mutant) and exceeding 10²-fold in skin of DMBA/TPA-treated FVB mice (fig. S3C). Serial dilution of CarC gDNA into aliquots of mKC gDNA showed a capacity to detect one mutant allele against a background of >10⁵ normal alleles (fig. S3D). When skin was assayed after a single cutaneous DMBA application followed by weekly TPA, significantly fewer *Hras* mutations in the skin of Lang-DTA mice, relative to NLCs, were apparent after only 2 weeks (Fig. 3), approximately 6 weeks before any palpable signs of tumor formation. During the precancerous period, the differential frequency of *Hras*-mut61 in Lang-DTA mice became more apparent over time and was again unaffected by the presence or absence of T cells (Fig. 3). High-throughput next-generation sequencing (Illumina HiSeq 2000) likewise demonstrated the accumulation of substantially more *Hras* codon 61 mutations in DMBA-treated LC-intact skin versus LC-deficient skin (fig. S4). Taking the data together, we hypothesized that the very high CYP1B1:CYP1A1 ratio in LCs favors metabolism of DMBA to mutagenic DMBA-t-3,4-

diol that then may be available for subsequent conversion to mutagenic DMBADE within neighboring keratinocytes.

To test the potential of LCs to metabolize DMBA, we analyzed lysates of XS106 cells 24 hours post-DMBA exposure by high-performance liquid chromatography (HPLC). Internalization of DMBA was evidenced by a single spike (Fig. 4A), whereas the supernatant from such cultures markedly revealed an additional cluster of spikes, consistent with DMBA metabolites (Fig. 4B). The concentration of XS106-internalized DMBA increased with increasing initial DMBA concentrations (Fig. 4C). Notably, there was a very rapid accumulation of DMBA metabolites in the medium of XS106 cells, which also increased over time and with the concentration of DMBA (Fig. 4, D and E). These data are consistent with the cells' uptake and metabolism of DMBA and the rapid release of metabolites. Liquid chromatography/ tandem mass spectrometry (LC/MS/MS) readily identified the mutagenic metabolite DMBA-t-3,4-diol in the cultures (Fig. 4F) at mean concentrations of 29.2 nM in 32 μ M-DMBA-exposed XS106 samples and 42.4 nM in 64 μ M-DMBA-exposed samples and 42.4 nM in 64 μ M-DMBA-exposed samples. No DMBA-t-3,4-diol was detected in cultures lacking either XS106 or DMBA. These data evoke another study which showed that the LC line, XS52, could metabolize the PAH benzo[a]pyrene, to diol, quinone, and phenol metabolites (27). To further test the hypothesis that the profound contribution of LCs to carcinogenesis reflects the cells' release of mutagens formed by uptake and metabolism of DMBA, we applied synthesized DMBA-t-3,4-diol as the initiating agent in two-stage carcinogenesis of Lang-DTA and NLC mice. In contrast to the markedly reduced tumorigenesis observed in DMBA-treated Lang-DTA mice, DMBA-t-3,4-diol initiated comparable tumor development in the two strains (Fig. 4G). Thus, by artificially providing a mutagenic metabolite of DMBA, the substantial contribution of LCs to carcinogenesis was bypassed.

Given the implication that murine LCs contribute to cutaneous chemical carcinogenesis via nonimmunological properties, it was appropriate to determine whether human LCs might display equivalent properties with the potential to promote disease. We therefore examined the response to DMBA of primary human LCs from several donors and their consequent effects on primary keratinocytes. The data were consistent with the murine data sets. After DMBA treatment, human LCs expressed substantially more *Cyp1b1* than *Cyp1a1* (of which most donors' cells express negligible levels), whereas human keratinocytes expressed an excess of *Cyp1a1* (Fig. 4H). For most donors, *Cyp1b1* expression by DMBA-treated LCs was ~20-fold higher than that in keratinocytes, whereas *Cyp1a1* expression was substantially higher in keratinocytes. To determine whether human LCs are more proficient than keratinocytes at metabolizing DMBA to a mutagenic product(s) that they then export, primary human LCs or keratinocytes were cultured with DMBA for 24 hours, and supernatants were harvested and then transferred onto new primary keratinocyte cultures. After an additional 24-hour incubation, the keratinocytes were assayed for DNA damage by γ H2AX staining. Because keratinocytes were grown in different media (EpiLife) than LCs [tryptophan-deficient RPMI: RPMI^{W(-)}], controls included each medium cultured with DMBA for 24 hours (but without LCs or keratinocytes present). The supernatants from DMBA-treated LCs, but not those from identically treated keratinocytes, provoked a significant increase in DNA damage above baseline (Fig. 4, I and J). Hence, although DNA damage can result from the cell-autonomous breakdown of DMBA by keratinocytes, it is substantially increased by the actions in trans of human LCs.

LCs survey the epidermis via both locomotion and repetitive extension/contraction of their dendritic processes and migrate to draining lymph nodes where their main function has been viewed as initiating and/or regulating adaptive immune responses (28). Here, however, a major pathophysiologic role for LCs is described that is independent of adaptive immunity.

Instead, it highlights the tissue-scavenging functions of LCs, by which they take up and metabolize chemical contaminants of the epidermis. Although this capability may powerfully attenuate the potency of natural toxins, it may be confounded by industrial PAHs such as DMBA where the detoxified metabolites that are released are more mutagenic than the starting compound. Thus, this innate action of LCs increases DNA damage and specific *Hras* mutations in neighboring keratinocytes. Although further studies are necessary to determine the precise mechanism by which LCs transfer DMBA metabolites to keratinocytes, the proximity of LCs to basal keratinocytes is evident. Given that PAHs are highly prevalent in industrial pollution and that extracts of airborne particles topically applied to mouse skin results in SCC development that is *Ahr* dependent (29), PAH-containing particulate matter might represent an underappreciated environmental factor in human skin cancer. Activating *Ras* mutations are found in ~50% of human epidermal SCCs (30), and in xenografting experiments, the activation of *Hras* signaling (plus inhibition of NF- κ B) was entirely sufficient to transform primary human keratinocytes into SCCs (31).

Although the capacity of keratinocytes to metabolize DMBA (32) and express CYP1A1, CYP1B1, and EPXH1 enzymes (33) has clearly been demonstrated previously, this is nonetheless insufficient to induce substantial tumor formation in the absence of LCs. Others have revealed the potential for nonepithelial stromal cells to activate PAH mutagens (34). The marked resistance of LC-deficient skin to chemical carcinogenesis, in an experimental system optimized for tumor formation, markedly establishes the capacity of LCs to substantially enhance the toxicity of environmental agents. Collectively, our data are consistent with a cooperative carcinogenicity scenario in which LC CYP1B1 and EPXH1 preferentially metabolize DMBA to DMBA-t-3,4-diol, which is subsequently delivered to adjacent keratinocytes wherein CYP1A1 converts the DMBA-t-3,4-diol to mutagenic DMBADE (fig. S2). Furthermore, our findings also provoke the possibility that locally resident DC populations may enhance PAH-induced mutations and tumor development within other epithelial tissues, contributing to the risk of lung, colon, and genitourinary carcinomas.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding for this work was provided by the NIH grants R01CA102703 (to M.G.), T32 (to J.N.), RO1-AR044077 (to M.S.), and RO1-AR056632 (to D.H.K.) and the Richard K. Gershon Research Fellowship (to B.G.M.). This work was supported by resources of the National Cancer Institute (grant P30 CA016359) of the Yale Comprehensive Cancer Center, the Wellcome Trust (to A.H.), Cancer Research UK (to A.H.), and the Department of Health via the National Institute for Health Research (NIHR) Comprehensive Biomedical Research Centre award to Guy's and St. Thomas' NHS Foundation Trust in partnership with King's College London (KCL) and King's College Hospital NHS Foundation Trust (to A.H. and E.B.). This paper is not an official guidance or policy statement of the U.S. FDA, and no official support or endorsement by the FDA is intended or should be inferred. We thank E. Gulcicek for HPLC support and M. Choi for sequence analysis support; M. Udey (NIH), A. Balmain [Univ. of California San Francisco (UCSF)], A. Behrens (CRUK), F. Nestle (KCL), and R. Groves (KCL) for protocol advice; O. Sobolev (KCL), J. Strid (KCL), F. Geissmann (KCL), K. Golubets (Yale), D. Smith (Yale), and G. Tokmoulina (Yale) for assistance; and A. Takashima (Univ. of Toledo) for XS106 cells and A. Balmain (UCSF) for CarC cells. The data tabulated in this paper are reported in the main text and in the supporting online material. Illumina sequence data are available from the National Center for Biotechnology Information Sequence Read Archive, accession no. SRA048406. Sites of experiments: New Haven, CT, USA, and London, UK.

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Figure 1.

Resistance to two-stage chemical carcinogenesis is $\alpha\beta$ and $\gamma\delta$ T cell-independent. Chemical carcinogenesis was induced in (A) $Tcr\beta^{-/-} Tcr\delta^{-/-}$ Lang-DTA mice using 50 μ g DMBA to initiate and 6.25 μ g TPA/week to promote, as well as in (B) $Tcr\delta^{-/-}$ Lang-DTA mice using 100 μ g DMBA to initiate and 25 μ g TPA/week to promote. The numbers of tumors per mouse (left) and individual tumor area (right) 14 weeks postinitiation are shown (mean $\pm$ SEM). *** $P < 0.0001$ (Student's t test); $n = 8$ to 12 mice per group. NS, not significant. (C) Representative images from (B).

Figure 2.

LCs enhance DMBA induced DNA damage. Confocal microscopy of epidermal sheets isolated from the ears of (A) NLCs or (B) Lang-DTA mice 24 hours post-DMBA application shows LCs (CD207+) in blue and nuclei containing DNA damage (γ H2AX+) in red. (C) The ears of NLCs and Lang-DTA mice were treated with 8.75 μ M DMBA or DMBA-t-3,4-diol and γ H2AX⁺ cells enumerated 24 hours post treatment. Each dot represents an individual mouse. ** $P < 0.001$ (Student's t test). Similar results were obtained from vehicle (acetone)-treated and -untreated mice. (D) XS106 was treated with 64 μ M DMBA and changes in gene expression monitored by quantitative real-time fluorescence PCR (qPCR). *Cyp1a1* expression is not shown because it was undetectable at all time points. (E) NLC mice were untreated or treated with 100 μ M DMBA, and, 24 hours later, epidermal Langerhans cells were purified by cell sorting and analyzed for the expression of the indicated genes by qPCR. Purified LCs did not express *Cyp1a1* either before or after DMBA treatment (n.d., not detected). All graphs depict mean $\pm$ SEM.

Figure 3.

Reduced levels of cutaneous *Hras* codon 61 mutations in LC-deficient mice detected by qPCR of gDNA. Lang-DTA, $Tcr\beta^{-/-} Tcr\delta^{-/-}$ Lang-DTA, and appropriate NLC animals were treated with DMBA [200 μ g for D+T(2), 100 μ g for all others], followed by TPA (25 μ g per week, number of weeks in parenthesis). *Hras* codon 61 mutations in the treated skin were quantified by qPCR of gDNA. * $P < 0.01$ (Student's *t* test), $n = 5$ to 6 mice per group. Error bars indicate SEM.

Figure 4.

LCs have the capacity to metabolize DMBA to DMBA-t-3,4-diol, and initiation with this metabolite abrogates the tumor resistance seen in LC-deficient mice. XS106 cells were cultured with 64 μ M DMBA for 24 hours, the supernatant was then collected, and cells were pelleted to prepare a lysate. (A and C) HPLC analysis of the lysate. (B,D, and E) HPLC analysis of the supernatant. (F) LC/MS/MS analysis of XS106 cells in the presence or absence of DMBA. (G) Tumor induction after treatment with DMBA (12.5 μ g) or DMBA-t-3,4-diol (100 μ g). 25 μ g/week TPA promotion was used in both groups, and initiator doses were chosen on the basis of predicted diol instability, reduced cutaneous penetrance, and

preliminary studies. Each dot represents an individual mouse. **(H)** Human keratinocytes or LCs were cultured in the presence (right) or absence (left) of 64 μ M DMBA for 24 hours, then *Cyp1a1* (1A1) and *Cyp1b1* (1B1) expression was quantified relative to β -actin by qPCR. **(I and J)** Human keratinocytes (in EpiLife medium) or LCs [in RPMI^{w(-)}medium] were cultured 24 hours in the presence (or absence, not shown here) of 64 μ M DMBA. Supernatants (sup.) were then harvested and transferred onto fresh keratinocyte cultures for an additional 24 hours, then DNA damage was assessed by γ H2AX staining. Confocal microscopy (I) and quantification of the data (J). ** $P < 0.001$, *** $P < 0.0001$ (Student's t test).