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Langerhans Cells Facilitate Mutagenesis And Squamous Cell Carcinoma

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LANGERHANS CELLS FACILITATE MUTAGENESIS AND SQUAMOUS CELL CARCINOMA

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Polyaromatic hydrocarbons (PAHs) are prevalent, potent carcinogens, and 7,12-dimethylbenz[a]anthracene (DMBA) is a model PAH widely used to model epithelial carcinogenesis. To achieve mutagenic effects, DMBA must be activated to DMBA-*trans*-3,4-diol via cytochrome p450 (CYP) 1B1, and this intermediate is the penultimate carcinogen responsible for inducing the signature mutation in the *Hras* oncogene. Recently, we observed that mice deficient in Langerhans cells (LCs), an epidermal dendritic cell (DC), are almost completely resistant to cutaneous chemical carcinogenesis, independent of αβ and γδ T cell influences. Investigations to identify the LC contribution to carcinogenesis revealed that DMBA-exposed LC-intact skin harbor more *Hras* mutations than LC-deficient skin, implying an early effect on tumor initiation. *In vitro* XS106 cells, a cell line derived from murine LCs, efficiently metabolized DMBA to DMBA-*trans*-3,4-diol as detected by liquid chromatography and tandem mass spectrometry. Moreover, initiation with DMBA-*trans*-3,4-diol bypassed tumor resistance and restored tumorigenesis in LC-deficient mice. Consistent with previous reports, murine and human LCs expressed a higher CYP1B1:CYP1A1 ratio than keratinocytes, conferring an advantage in the ability to metabolize to mutagenic intermediates. Thus, our data suggest a cooperative carcinogenesis scenario in which LC generate DMBA-*trans*-3,4-diol which moves into keratinocytes for further metabolism and mutagenesis. Tissue-associated DCs – which are also found in other epithelial surfaces – can enhance chemical carcinogenesis via PAH metabolism, highlighting the complex relation between immune cells and carcinogenesis.

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their love, support and sacrifice.

Acknowledgements

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I. INTRODUCTION

A. EPITHELIAL TISSUES, CANCER, AND THE IMMUNE SYSTEM

Epithelial tissues are situated at critical junctures with the environment and serve as protective barriers from exogenous insults. Such epithelial linings have evolved an organizational structure in which the deepest layer of cells, often regarded as the basal layer and located adjacent to the basement membrane, are regenerative and supply the more superficial layers with differentiated cells that function in secretion, barrier protection, or other tissue specific roles [1]. In this dynamic process, the differentiated cells will ultimately be shed from the surface and replaced by a newly divided cell from the basal layer. With repeated environmental insults to epithelial tissues, perhaps this dynamic organizational structure evolved to enable clearing of damaged cells and maintenance of structural and functional integrity [2]. However, even this dynamic process frequently falls short of protecting epithelial surfaces from aberrant growth and tumorigenesis. In humans, more than ninety percent of neoplasms arise in epithelial tissues [3], which include the skin, gastrointestinal (GI), respiratory, and genitourinary (GU) tracts. The cutaneous epithelial surface (i.e. epidermis) is the site of the two most common human cancers [4] [5] – basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) – and accordingly offers a unique ability to model the biological events that contribute to carcinogenesis more generally.

Many environmental factors are implicated in promoting the development of epithelial-derived tumors. For instance, ultraviolet light exposure is largely considered the major risk factor for cutaneous BCC and SCC. Similarly, polyaromatic hydrocarbons (PAHs) are well recognized for their tumorigenic potential to skin, lung, GI, and GU

tumors. PAHs are ubiquitous chemicals in industrialized societies, present in automobile emissions, tobacco smoke, smoked foods, and ground water [1]. Accordingly, individuals exposed to occupational levels of PAHs experience up to 1.4 times the risk of lung cancer and 2.2 times the risk of bladder cancer compared to the normal population [6]. While perhaps not as important an environmental factor as UVR for the skin, PAHs have been shown to predispose mice to developing UVR-induced tumors. At doses at which neither induce tumors alone, the combination of UVR and PAHs results in a higher rate of tumorigenesis [7]. In addition, the reverse relationship also has been shown, where UV-exposed mice are more sensitive to the PAH effects [8]. Due to its ability to induce tumors, PAHs serve as a means to model both environmental and non-environmental contributions to cancer through the chemical carcinogenesis protocol discussed below.

In addition to the organizational structure that helps to limit epithelial transformation, immune surveillance similarly is an important host protective phenomenon that helps to inhibit carcinogenesis. In support of this, organ transplant patient on immunosuppressive therapy are 65 to 250 times as likely to develop cutaneous squamous cell carcinoma than compared to the general population [9]. Consistent in non-cutaneous epithelial sites, the risk of squamous cell carcinoma of the anal canal is 120 times higher in HIV-positive patients compared to HIV-negative patients [10]. Several models demonstrate that mice lacking effector components of the immune system, including natural killer (NK) cells and natural killer T (NKT) cells, are more susceptible to PAH-induced tumors than their wild type counterparts [11]. Thus, clinical and laboratory observations suggest an anti-tumor role for immune system constituents in cancer biology.

Within the study of non-melanoma cutaneous cancer biology, several immune cells are implicated in affecting the development of tumors. Keratinocytes of the epidermis in mice are in close proximity to two distinct subsets of intraepithelial lymphocytes (IEL), namely the dendritic epidermal T cells (DETCs) [12] and the Langerhans cells (LCs). These two cell types form a network of intraepidermal immature immune cells and their dendritic processes extend to contact keratinocytes, including the epithelial stem cells in the basal layer and near hair follicles [1, 13].

Girardi, Hayday, and Tigelaar have provided insight into the precise functions of the γδ DETC constituency of IELs. Mice lacking TCRδ(-/-) DETCs were highly susceptible to developing chemically-induced tumors compared to wild type mice, implying their role in the down-regulation of cutaneous malignancies [12, 14]. While αβ T cells predominantly circulate in the blood and lymphatics in systemic circulation until recruited to sites of inflammation, γδ DETC have been shown to down-regulate an over-exuberant αβ T cell-mediated inflammatory response [12]. While these studies supported the role of γδ DETCs in regulating carcinogenesis, a similar mouse model of LC-deficiency was not available to examine the role of the Langerhans cell in cutaneous carcinogenesis, until recently.

B. LANGERHANS CELLS BIOLOGY – THE PAST AND PRESENT

Our understanding of the Langerhans cell has evolved in many ways since identification in 1868. Paul Langerhans, a German medical student, mistakenly believed the dendritic processes to suggest he was observing distal nerve cells of the skin [15]. In 1961, Birbeck characterized the tennis racket-shaped organelles present within LC, but he

regarded the cells as worn out, pigmentless melanocytes. Since then, key experiments have shifted our understanding of LC. In 1977, Stingle, Rowden, and Klareskog demonstrated the expression of Fc and complement receptors, and MHCII molecules on the LC surface, which cemented their classification as immune cells [16-19]. LC were found to migrate to the lymph node and have varying states of maturation based on location and antigen stimulation, suggesting a role in antigen presentation. Thus, the "Langerhans cell paradigm" developed and became the prototype for all tissue-resident tantigen presenting dendritic cells: the life cycle consists of residing in the skin (or whichever specific tissue) until encountering an antigen, after which it migrates to a regional lymph node, and matures to upregulate surface molecules critical for presentation to T cells [16, 20].

LC are derived from hematopoietic bone marrow cells [21], and the primary building block of Birbeck granules has been identified as langerin/CD207, a C-type lectin whose exact function is still unclear. The identification of CD207 has advanced the field of LC biology in two important ways [22]. First, it allows for antibody-mediated fluorescent tagging against CD207 which enabled visualization of the cells. Secondly, CD207 represented an LC-specific molecule that could serve as a target for the generation of an LC-deficient mouse model.

One approach to depleting LC is the "huLangerin-DTA" (DTA) strategy and was developed by Dan Kaplan at Yale University. In this strategy, the active subunit of the diphtheria toxin is expressed under the control of the huLangerin promoter, which results in the selective ablation of epidermal langerin^{+}, MHCII^{+} cells [23]. Along with other approaches to ablating LC, these LC-deficient models revealed the existence of two

distinct populations of langerin$^+$ skin cells – one localized to the epidermis and one localized to the dermis [24, 25]. With the advent of LC-deficient models, the understanding of LC-biology has further evolved and become more complex. Although the classic LC-paradigm would predict an LC-deficient mouse to develop a mitigated contact hypersensitivity (CHS) response, the LC-deficient DTA mouse model in fact developed a heightened CHS response as compared to wild type controls [23]. This and other data supported a role of LC in down-modulating the CHS response, while dermal langerin$^+$ DC were important for stimulating the response [16, 22, 26].

C. LC-DEFICIENCY AND CHEMICAL CARCINOGENESIS

Similar to the predictions of the DTA mouse in the CHS experimental model, the classic LC-paradigm would expect the absence of an LC-mediated anti-tumor immune response. Just as tumor burden is higher in the absence of effector γδ DETCs, one might expect greater tumorigenesis in the absence of LC-mediated immune induction. The Girardi lab performed the first investigations of chemical carcinogenesis in LC-deficient mice. Strikingly, they observed the near elimination of tumors in the DTA cohort compared to wild type. Under low dose chemical protocols, wild type mice harbored many tumors by week 14 while more than 50% of DTA mice remained tumor free even at 16 weeks. The tumor-protective effect in the absence of LCs persisted even at high dose chemical protocols [27].

Invoking the latest developments in our understanding of the role of dermal and epidermal langerin$^+$ DC in adaptive immunity induction, one might predict that the absence of LC would substantially decrease LC-mediated immune tolerance and increase dermal DC-mediated anti-tumor immunity induction. It would follow, then, that DTA

mice may be more effective at eliminating early transformed cells through heightened immune surveillance. However, the Girardi lab also demonstrated that even in the absence of αβ T cells – the effector arm of an adaptive immune response against transformed cells – LC-deficiency nearly eliminated tumorigenesis [27]. Thus, the tumor protective effect afforded by LC-deficiency in the DTA mouse occurred independent of an anti-tumor immune response.

These findings raised a fundamental question about the role of LC in carcinogenesis, particularly when induced by PAH. If not an effect on the induction of anti-tumor immunity, then what other roles could LC play that might affect carcinogenesis? These studies suggested an under-emphasized role of LC early in carcinogenesis, likely during tumor initiation. In order to further understand the LC-effect on tumorigenesis, we must first take a closer look at the experimental model in which this finding occurs.

D. TWO-STAGE CHEMICAL CARCINOGENESIS

By employing the tumorigenic influences of PAHs, the chemical carcinogenesis experimental model has yielded many insights into the fundamental influences on cancer development [28]. Wild type mice on the FVB background are highly susceptible to chemical carcinogenesis in a reproducible fashion [29]. As such, these rates can be compared to the level of tumor development in a genetically altered mouse with a specific phenotype undergoing identical chemical treatment. Varying rates of tumorigenesis implicates the phenotype of influencing cancer development.

One major limitation to the complete carcinogenesis model is the inability to deduce the mechanism of the effect. A variation called two-step chemical carcinogenesis circumvents this issue by temporally separating the multi-stage process of carcinogenesis into tumor initiation and tumor promotion. Rather than application of a single carcinogenic dose to induce tumors, sub-carcinogenic doses of PAHs can be used for tumor initiation followed by subsequent treatment with a pro-inflammatory agent for tumor promotion. Thus, experiments may be designed to measure effects on initiation only, promotion only, or both [30-32].

7,12-dimethylbenz[*a*]anthracene (DMBA) is a very commonly used and well described tumor initiating agent. As a result of treatment with DMBA, critical genes in epidermal keratinocyte oncogenes acquire mutations, and such cells are considered "transformed". They will not go on to form tumors without a stimulus for clonal expansion, which will be provided by the tumor promoting agent. Tumor initiation has several prerequisites: DMBA must diffuse through the stratum corneum, enter into keratinocyte stem cells that are thought to be the primary tumor cell, be bioactivated to the downstream intermediate called DMBA-3,4-dihydrodiol-1,2-epoxide (DMBADE) which is ultimately mutagenic [33], and interact with critical regions of DNA. The *Hras* oncogene appears to be DMBA's primary target gene and results in an activating mutation that can be detected as early as 3-4 weeks after DMBA [34] exposure as well as in the majority of papillomas. After bioactivtation, DMBA intermediates induce a signature A → T transversion mutation in codon 61 [35, 36]. Assays have been developed to quantify initiation by measuring the level of DNA-DMBA adducts, DNA damage, *Hras1* mutations, and many others.

The molecular effects of tumor promoting agents such as TPA are more pleiotropic. In general, treatment leads to sustained epidermal hyperplasia as evidenced by increased number of nucleated cell layers and increased thickness, accompanied by an enhanced cell turnover rate [37]. Importantly, initiated cells – that is, those that have acquired the critical mutations – are thought to have a survival and proliferative advantage that results in the selective expansion of the transformed clone while non-initiated cells undergo a normal keratinocyte life cycle of division, differentiation, and death [28].

E. DMBA METABOLISM

As mentioned previously, DMBA is a parent compound that must be metabolized to the ultimate carcinogen DMBADE in order to induce the critical *Hras* mutations. While DMBA at high concentrations can be toxic to cells, it is not mutagenic without metabolic activation by inducible enzymes including cytochrome p450 (Cyp) family members and microsomal epoxide hydroxylase (mEH). Upon entering a cell, DMBA and other PAHs are high-affinity ligands for the cytosolic aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor that can enter the nucleus and activate the expression of xenobiotic response elements (XRE). These include enzymes important for DMBA metabolism such as *Cyp1a1*, *Cyp1b1*, *mEH*, and *aryl hydrocarbon receptor repressor (AhRR)*.

Diagram 1 shows the pathway of DMBA metabolism to DMBADE and the important enzymes. The XRE enzymes are responsible for either unleashing the toxicity of DMBA by converting to pro-mutagen, or conversely protecting the cell by

metabolizing to other inert intermediates [38]. Accordingly, the collective efforts of several investigations demonstrate that mutagenicity and toxicity require a three-step reaction to generate DMBADE [38-41].

CYP1A1 and CYP1B1 are expressed in varying amounts in different tissues, and are very efficient at metabolizing PAH [42]. The development of gene knockout mice in which single XRE enzymes are eliminated has facilitated a clearer understanding of the DMBA metabolism pathway [43]. Heidel et al investigated the susceptibility of *Cyp1B1*-null mice to mutagenesis, in which *Cyp1A1* expression remained intact. After systemic DMBA treatment, they observed that bone marrow cells did not generate the DNA-adducts seen in wild type mice. CYP1A1, in fact, metabolizes DMBA at a higher rate than CYP1B1. However, given the lack of tumors with sustained *Cyp1A1* activity, it appears that CYP1A1-mediated DMBA metabolism plays no role in mutagenesis as quantified by DNA adducts [38, 44].

Furthermore, Buters et al demonstrated that *Cyp1B1*-null mice were almost completely protected from the acute bone marrow cytotoxic and leukemic effects of DMBA, despite the continued metabolism by hepatic CYP1A1. Thus, collectively, it follows that early conversion by CYP1B1 was a requirement for DMBA-induced bone marrow toxicity and mutagenisis [44-46]. Kleiner et al recapitulated these findings in mouse epidermis. Direct application of DMBA to a *Cyp1B1*-null mouse resulted in 64% reduction in DNA-adduct formation, with similar results seen in the AhR-knockout mouse [47]. *Cyp1B1*-null mice also developed no chemically-induced squamous cell carcinoma in contrast to wild type mice [38].

After the initial CYP1B1-mediated conversion to DMBA-3,4-epoxide, mEH immediately converts it to the penultimate carcinogen DMBA-*trans*-3,4-diol [48]. Miyata et al demonstrated that embryonic fibroblast from *mEH*-null mice are unable to generate DMBA-*trans*-3,4-diol and are more resistant to DMBA toxicity than fibroblasts from wild type mice. By showing that *mEH*-null mice are almost completely resistant to developing DMBA-induced tumors, they demonstrated that mEH is required for DMBA carcinogenesis for its role in generation of the proximate carcinogen [49]. The final conversion from DMBA-*trans*-3,4-diol to DMBADE is catalyzed by either CYP1B1 or CYP1A1. The bay region of DMBADE is the ultimate carcinogen that interacts with DNA [33, 38].

Diagram 1: **DMBA metabolism pathway.**

F. SITE OF DMBA METABOLISM IN THE EPIDERMIS

Stem cell keratinocytes of the basal layer adjacent to hair follicles are implicated as the cell of origin for skin cancer [50]. These cells express CYP1B1, CYP1A1, mEH, and other XRE enzymes, all of which are important to unleash the mutagenicity of DMBA. Because downstream metabolites were shown to be extremely reactive, it was the widely held view that DMBA's intermediates could not exist long enough to diffuse out of cells [51]. Thus, XRE within keratinocytes were implicated in activating DMBA. Kuroki and colleagues supported this hypothesis by demonstrating that primary human keratinocytes incubated with the PAH benzo[a]pyrene (BP) could generate HPLC-identified metabolites of BP. The addition of V79 Chinese hamster cells to this culture formed their mutagenesis assay, which correlated ouabain resistance in previously sensitive hamster cells as a proxy for tumor initiation. Hamster cells incubated with BP and keratinocytes resulted in greater ouabain resistance than hamster cells with just BP [52, 53].

However, there are significant concerns in accepting the hypothesis that keratinocytes are the major contributor to DMBA activation. First, the assumption that activated intermediates are too unstable to exist for a prolonged period of time has not been supported. DMBA-*trans*-3,4-diol is available in a stable form for experimental use. In addition, the concept of intercellular metabolite transfer has been proposed in a number of experiments investigating the effects of DMBA on bone marrow cells. Supernatant from AhR^+ bone marrow stromal cells cultured with DMBA can induce apoptosis in bone marrow B cells, while directly incubating B cells with DMBA cannot [54]. This finding suggests the stability of the intermediate over time and during

intercellular transfer. Furthermore, the direct application of DMBADE in the absence of stromal cells was sufficient to induce an apoptosis profile in B cells, providing evidence that the stromal cells metabolize DMBA and transfer a stable intermediate to the B cells [55].

The XRE expression profile of the DMBA-relevant enzymes has been reported in a cell type specific fashion of human epidermal cells. After isolating keratinocytes and LC from the epidermis of six human donors, the expression of CYP1A1 and CYP1B1 mRNA relative to GADPH control (expressed as +, ++, or +++) was determined for each cell type. Each enzyme was detected in both cell types, however keratinocytes expressed greater levels of Cyp1A1 (+++) than Cyp1B1 (++) while the LC expressed greater levels of Cyp1B1 (+++) than Cyp1A1 (+, almost undetected) [56]. Taken together, these data suggest that CYP1B1 is required for generation of the penultimate mutagen. It also follows that LC might be better-equipped enzymatically to facilitate DMBA-mutagenesis than keratinocytes.

II. Statement of Purpose and Hypothesis

Our understanding of LC biology has undergone significant changes since identification of the cell in the mid-1850s. The discovery that LC-deficient DTA mice are resistant to two-stage chemical carcinogenesis offers the possibility for yet another advancement to our understanding of LCs and cutaneous biology. Exclusive of immune surveillance, a mechanism by which LCs influence chemical carcinogenesis has never been described and is precisely the major aim of this investigation. **We hypothesize that the LC effect will localize early in tumor initiation and that, because of the high ratio of *Cyp1B1:Cyp1A1* expression, LCs are better equipped to perform the requisite DMBA bioactivation necessary for cutaneous chemical carcinogenesis**. The investigations detailed herein should demonstrate the following:

1) LC-deficiency abrogates chemical carcinogenesis in the absence of both αβ and γδ T cells, thereby excluding enhanced T cell mediated tumor immune surveillance to explain tumor resistance.
2) With the use of a K5Hras transgenic mouse, we observed that the protective effect seen in LC-deficiency likely localizes to tumor initiation rather than tumor promotion.
3) To show directly that LC-deficiency influences tumor initiation, we employed a novel real time PCR assay to demonstrate that DMBA-exposed LC-deficient mice harbor fewer *Hras* mutations than DMBA-treated wild type mice.
4) When cultured with DMBA in vitro, the Langerin$^+$ / MHCII$^+$ / Birbeck granule$^+$ LC line, XS106, can generate DMBA-*trans*-3,4-diol.

5) With data suggesting that LCs contribute to tumor initiation by converting DMBA to its mutagenic metabolites, we initiated DTA mice with DMBA-*trans*-3,4-diol followed by several weeks of TPA promotion and observed a restoration of tumorigenesis.
6) We examined the XRE mRNA expression profiles present in murine and human keratinocytes and LCs after exposure to DMBA in culture, and with the support of existing data in the literature, suggest a mechanistic rationale for why LC metabolism is required for and keratinocytes metabolism is insufficient for carcinogenesis.
7) LC-deficiency does not confer resistance to UVB-carcinogenesis, however these mice develop tumors later than LC-intact mice.

III. METHODS

A. ANIMALS AND HOUSING

All of the in vivo studies were approved by the Yale Animal Care and Use Committee. FVB/N mice were purchased from The Jackson Laboratory (Bar Harbor, ME). FVB/N Langerhans cell deficient (Lang-DTA) mice were generated with a bacterial artificial chromosome-based transgene containing human Langerin which was modified to drive expression of diphtheria toxin subunit A from an internal ribosome entry site that was inserted in the 3' untranslated region of the *langerin* gene (*2*). *TCR*δ*-/-*, *TCR*β*-/-* *TCR*δ*-/-*, and K5*Hras* transgenic (K5HrasTg) were backcrossed onto the FVB/N background for >10 generations and were previously described (*15*). *TCR*δ*-/-* Lang-DTA, *TCR*β*-/-TCR*δ*-/-* Lang- DTA, and K5*Hras*Tg Lang-DTA mice were generated by intercrossing the respective mutants. Mice were maintained under specific pathogen-free conditions and food and water provided *ad libitum*. The animal facility is accredited by the Association for Assessment of Laboratory Animal Care.

Renata Filler, Jason Neustadter, and Julie Lewis managed mouse colonies and directed generation of the various strains. **Badri Modi, Swapna Reddy, and Bernice Kwong, assisted in house keeping issues and participated in care of animals.**

B. MICE SORTING AND GENOTYPING

Approximately 7-12 days after birth, mice were numbered via toe clipping and tails were harvested for DNA extraction and subsequent PCR-based genotyping. DNA was isolated from tail specimens using the QIAGEN DNeasy® Blood and Tissue kit and according to protocol for purification of DNA from animal tissues on page 28 of DNeasy

Blood & Tissue Handbook (07/2006). DNA was amplified with a commercially available PCR master mix and 5', 3' primers specific for the DTA transgene and TSH transgene. After 30 cycles of PCR, DNA was mixed with ethydium bromide dye and run on a 3% agarose electrophoresis gel along with a 1kb ladder and a positive control sample (DNA from known huLang-DTA). Thus, DNA samples, and their corresponding mice, were identified as transgenic^{+} (tg) or normal littermate control (NLC). Mice were subsequently sorted to house roughly equal numbers of Tg and NLC per cage.

Badri Modi, Renata Filler, Jason Neustadter, Swapna Reddy, and Julia Lewis all participated in sorting and genotyping of mice for the various experiments. Badri Modi was involved in data analaysis and figure representation for all experiments.

C. CHEMICAL CARCINOGENESIS:

All mice undergoing two-stage chemical carcinogenesis were treated as following, according to previously published protocols [57]. At seven weeks of age, the hair of a 4x3cm rectangular area on the dorsal surface was clipped with an electric shaver, being careful not to cause trauma to the skin. The area was treated with Nair® for approximately one minute, which was washed off with water and paper towel. Dorsal skin was dried and mice were placed back into cage. One week later, at eight weeks of age, each mouse was treated with initiating agent – either DMBA or DMBA-*trans*-3,4-diol – reconstituted in acetone. For each experiment, equimolar amounts of initiating agent were applied to clipped and naired dorsal skins of NLC and DTA cohorts. At 9 weeks of age, the mice received twice weekly treatment with equimolar amounts of TPA reconstituted in ethanol for the duration of the experiment. The dorsal skin was observed for tumor development once a week, and tumors counted, measured for area, and

clinically scored as papillomas (well demarcated, symmetrical, pedunculated, or dome-shaped papules without erosion or ulceration) or carcinoma (poorly demarcated, assymetrical, sessile or dome-shaped with erosions or ulcerations). At the end of the experiment, representative tumors were harvested from each mouse for subsequent histologic analysis.

Badri Modi did the two-stage carcinogenesis experiments with D-3,4-tD as initiating agent, including the mouse sorting, genotyping, chemical treatments, tumor charting, and tumor harvesting. Other two-stage carcinogenesis experiments were carried out by Jason Neustadter, Renata Filler, and Julia Lewis. Badri Modi was involved in data analaysis and figure representation for all experiments.

D. CELL CULTURE

RPMI 1640, DMEM, and media supplements were from Invitrogen, Carlsbad, CA unless otherwise noted. CRPMI media was made by supplementing RPMI 1640 with 10% heat- inactivated FBS, 10mM Hepes, 1% non-essential amino-acids, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.05 mM ®-mercaptoethanol, and antibiotics). Mouse keratinocyte (mKC) cells were provided by Yale Dermatology Cell Culture Facility. CarC cell line (kindly provided by Dr. A. Balmain, UCSF, CA), homozygous for *Hras* codon 61 CAA->CTA mutation, was maintained in DMEM media supplemented with 10% FBS, 10mM Hepes, 2mM L-glutamine and antibiotics. XS106 and NS46/NS47 cell lines were kindly provided by Dr. A. Takashima, U of Toledo, OH. NS46/NS47 cells were grown in CRPMI, and the media supernatant was used to supplement media for XS106 cells. XS106 cells were grown in "XS106 media" (CRPMI supplemented with 5% NS46/NS47 supernatant and 0.5 ng/ml mouse GM-CSF (PeproTech, Rocky Hill, NJ.)

Julia Lewis and Renata Filler did the experiments assessing *in vitro* XS106's ability to metabolize

DMBA. Badri Modi was involved in data analysis and figure representation.

E. ISOLATION OF GENOMIC DNA

Genomic DNA (gDNA) was isolated from cells using DNeasy Blood & Tissue kit (Qiagen, Valencia, CA) as per manufacture's protocol. Mice were sacrificed at a specific time point after dorsal skins received chemical treatment. Using blade and scissors, full thickness and area of chemically treated murine dorsal skin was excised and the subcutaneous fat was scraped off. The gDNA of the remaining dermis and epidermis was isolated using the Gentra® Puregene Tissue Kit (Qiagen) as per manufacture's protocol. Each sample underwent further purification via phenol-chloroform-isoamyl extractions followed by precipitation with ethanol and 3M sodium acetate. Isolated cell and tissue gDNA was analyzed with a NanoDrop (Thermo Scientific, Wilmington, DE) for purity and concentration.

Renata Filler, Jason Neustadter, and Badri Modi isolated genomic DNA for experiments presented in this manuscript.

F. *HRAS* CLONING AND MUTANT SPECIFIC REAL-TIME PCR

To quantify the number of DMBA-induced codon-61 *Hras* CAA->CTA mutations, mutant-specific primers 5'-CTAAGCCTGTTGTTTTGCAGGAC and 3'-CATGGCACTATACTCTTCTA [34] and a custom TaqMan probe 5'6FAM-CGGAAACAGGTGGTCAT-MGB3' were designed as part of a novel real-time PCR assay. TaqMan real-time PCR was run on ABI 7500 using SDS 1.4 and SDS 2.0 software and TaqMan Gene Expression Master mix (all from ABI Life Technologies Corporation, Carlsbad, CA) as per manufacture's protocol with validation slope of ΔCt vs. log input of

0.078 (ABI Guide to Performing Relative Quantitation of Gene Expression Using Real-Time Quantitative PCR). Obtained Ct values were normalized against β-*actin* and expression difference was calculated using the equation $RQ=2^{-\Delta\Delta Ct}$. Serial dilution of gDNA from the CarC into aliquots of mKC gDNA provided additional validation of TaqMan assay sensitivity. For further verification and validation of the assay, *Hras* 240bp PCR product containing codon 61 was cloned from CarC and mKC gDNA and singly inserted into pcDNA6.2TM/GW/D-TOPO® expression vector (Invitrogen, Carlsbad, CA) and sequenced (W.M. Keck BRL, Yale University). Serial dilutions of a known number of plasmids and TaqMan real-time PCR were conducted to generate standard curve. Once assay was validated for specificity in *Hras*-mutant detection, DNA from DMBA-treated skins were used in the assay to determine number of *Hras* mutations present in an equimolar amount of DNA.

Renata Filler, Julia Lewis, Jason Neustadter and Bernice Kwong were involved in the development and verification of the assay. Badri Modi used this assay to run other key experiments for development of concepts and was involved with data analysis / representation in figure.

G. GENE EXPRESSION ANALYSIS

For murine cell line and freshly isolated Langerhans cells:

Real- time quantitative RT-PCR (ABI 7500) using SDS 2.0 software was performed on RNA isolated using RNeasy Micro kit (Qiagen) from XS106 cells and purified Langerhans cells. DNA-free RNA was transcribed using High-Capacity cDNA Reverse Transcription kit as per manufacturer's protocol and used in downstream TaqMan assays with TaqMan Gene Expression Master Mix (all from ABI). Obtained Ct values were normalized against β-*actin* and expression difference to untreated samples

was calculated using $RQ = 2^{-\Delta\Delta Ct}$ equation.

For human keratinocytes and Langerhans cells:

RNA was isolated with RNeasy (Qiagen) kit and was treated with Ambion Turbo DNase and then RT Mastermix (ABI) to generate cDNA.Quantitative RTPCR was performed on an ABI Prism 7900HT cycler with SYBR Green. Quantitect Primer assays from Quiagen were used for quantification of *Cyp1a1*, *Cyp1b1* and β-*actin*.

Experiments were carried out by Renata Filler and Julia Lewis. Badri Modi and Julia Lewis developed the hypothesis that Cyp1B1 to Cyp1A1 ratio explains why LC-specific metabolism is required for DMBA mutagenesis and carcinogenesis.

H. HPLC AND LC/MS/MS.

For HPLC XS106 cells were incubated with varying DMBA concentrations of 0, 4, 16 or 64 μM at 37°C for 24 hr. An additional cell sample containing 16 μM DMBA with no cells was incubated under identical conditions. Another sample with XS106 cells and 16 μM DMBA was incubated for only 1 hour. HPLC studies were performed by Yale W.M. Keck BRL. In brief, to calibrate HPLC equipment and determine standard detection times and peak detection heights for DMBA and (+/-)-anti DMBA-3,4-dihydrodiol-1,2-epoxide (DMBADE), obtained from the NCI Chemical Carcinogen Reference Standard Repository, standard concentrations of DMBA and DMBADE at 4, 8, 16, 32 and 64 μM were prepared in 30% methanol. 50 μl of each was injected onto the HPLC system in an identical manner as for the experimental samples. For LC/MS/MS detection and quantification XS106 cells were incubated for 24 hr in F10 media (which has reduced tryptophan concentration) with 10% dialyzed FBS (both from Sigma), 10mM Hepes, 1% non-essential amino-acids, 2mM l- glutamine, 1mM sodium pyruvate, 0.05

mM β-mercaptoethanol, antibiotics, 5% NS46/NS47 supernatant, and 0.5 ng/ml mouse GM-CSF. The following day 32 μM or 64 μM of DMBA was added to the cultures and allowed to incubate for additional 24 hr after which media and cells were harvested and sent to Lining Cai and Peter Fu (Biotranex, FDA). Following extraction, samples were analyzed using LC/MS/MS methods developed by Biotranex to quantify the amount of DMBA-*trans*-3,4-diol.

Experiments were carried out by Renata Filler, Julia Lewis, and Jason Neustadter. Badri Modi was involved with data analysis and figure representation.

I. CHRONIC UV EXPERIMENT

Ten huLang.DTA.FVB and ten NLC.FVB mice at 8 weeks of age were shaved and sorted for this experiment. Three days later, mice were exposed to UVB from three broadband FS20T12-UVB lamps filtered through a Kodacel filter (Eastman Kodak) to remove wavelengths below 290nm [58]. Mice were exposed to 500 J/m^2 for three days during the first week. During the second week, mice were exposed to 750 J/m^2 for two days and then 1000 J/m^2 for three days. Then, mice were exposed to 1250 J/m^2 for five days per week, for the next 29 weeks.

Badri Modi carried out all aspects of this experiment, with planning and guidance from others.

Michael Girardi designed and directed most of the planning, troubleshooting, and analysis of all experiments.

J. STATISTICS

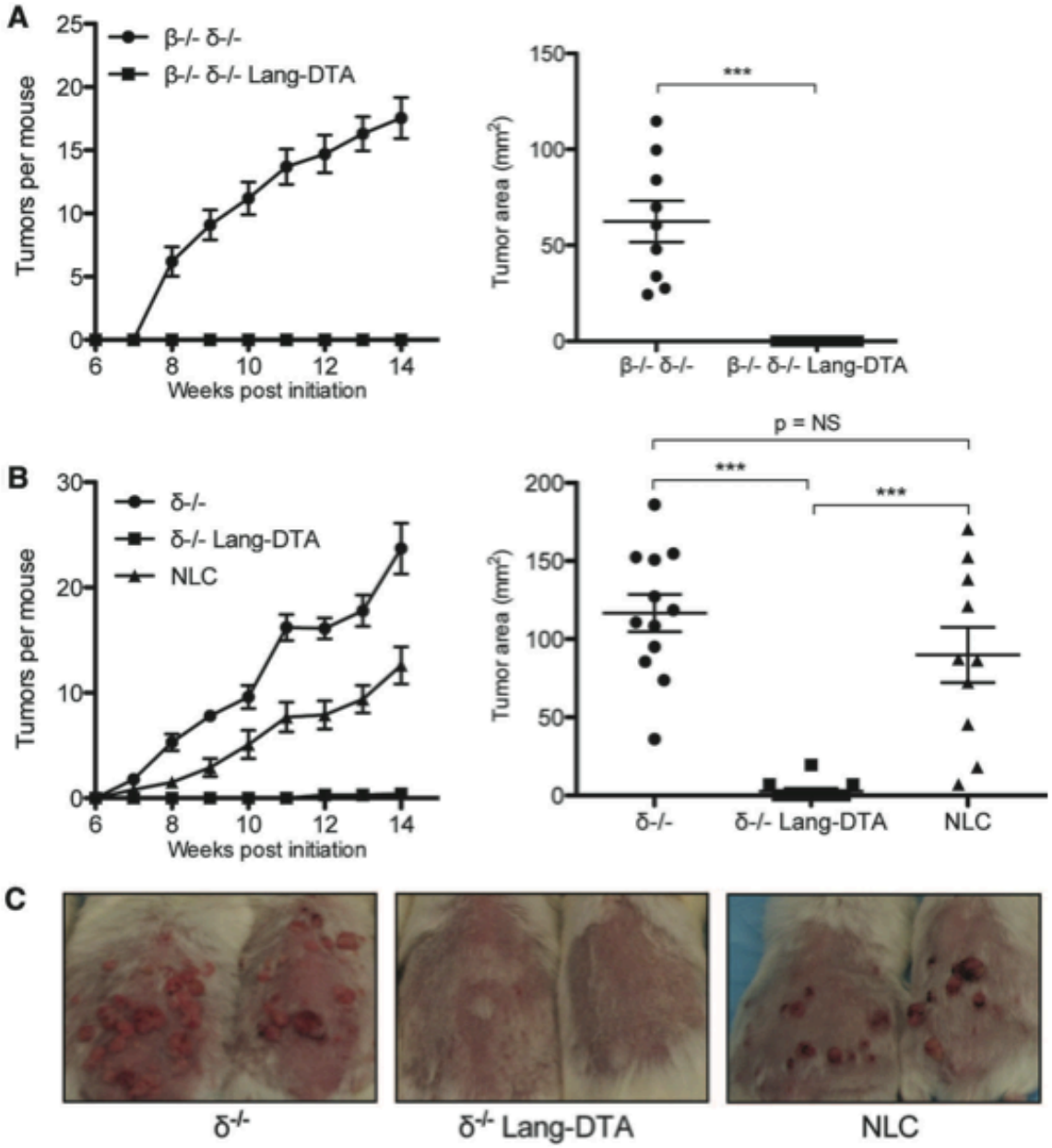
Statistical significance was evaluated by the two-tailed, unpaired Student's t-test. Graphical data is shown with bars indicating standard errors of the mean.

IV. Results

A. Resistance to two-stage chemical carcinogenesis is αβ and γδ T cell independent

The dramatic tumor resistance observed in TCRβ(-/-).huLang-DTA mice provided early evidence that the impact of LC-deficiency are independent of adaptive immunity. However, because LC can express T cell suppressive cytokines such as IL-10 [59, 60], we still considered the possibility that the absence of LC led to an augmented T-cell mediated anti-tumor immune response and, accordingly, abrogation of tumors. γδ T cells are intraepithelial lymphocytes, unique from the systemically circulating αβ T cells, that have been shown to regulate chemical carcinogenesis [14]. TCRδ−/− developed more tumors after DMBA/TPA than their counterparts. To further examine the role of the adaptive immune response's effector T cells in LC-deficiency, chemical carcinogenesis was induced in (**Figure 1A**) Tcrβ−/− Tcr**δ**−/− Lang-DTA mice using 50 mg DMBA to initiate and 6.25 mg TPA/week to promote, as well as in (**Figure 1B**) Tcrδ−/− Lang-DTA mice using100 mg DMBA to initiate and 25 mg TPA/week to promote. Even in the absence αβ and γδ T cells, LC-deficiency conferred a dramatic resistance to two-stage chemical carcinogenesis.

Figure 1:

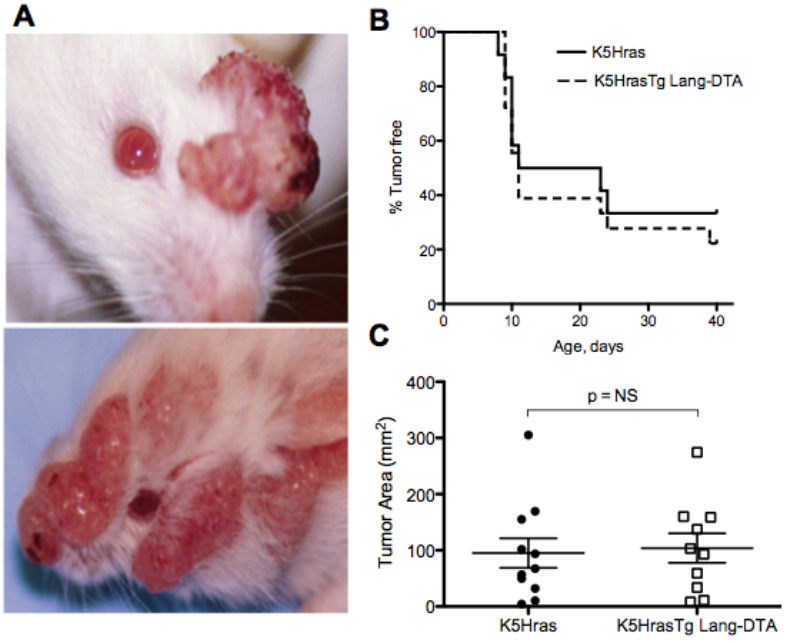


B. LC-deficiency does not prevent tumorigenesis in K5Hras mice

The K5Hras transgenic mouse expresses a constitutively activated *Hras* gene within basal keratinocytes. When expressed on the FVB background, many of these mice will be tumor-positive within three weeks after birth and develop early-onset squamous

cell carcinoma [61], in the absence of any chemical exposure. Since these mice are effectively already initiated, we investigated if the absence of LC would eliminate tumors. Therefore, we crossed huLang-DTA.FVB x K5Hras.FVB and compared rates of tumorigenesis in the double transgenic mice with the K5Hras.FVB mice. Both cohorts developed tumors at a similar rate with a non-significant difference in percentage of tumor-free mice and average tumor area per animal (**Figure 2**). The huLang-DTA transgene had no significant effect on rate of tumorigenesis in the K5Hras.FVB mouse. Thus, the tumor-protective effect seen in LC-deficiency could not overcome the tumorigenic fate of an "initiated" background, suggesting that LC influence acquisition of *Hras* mutations rather than tumor promotion.

Figure 2:



C. DMBA induces fewer *Hras* mutations in LC-deficient mice than in wild type mice

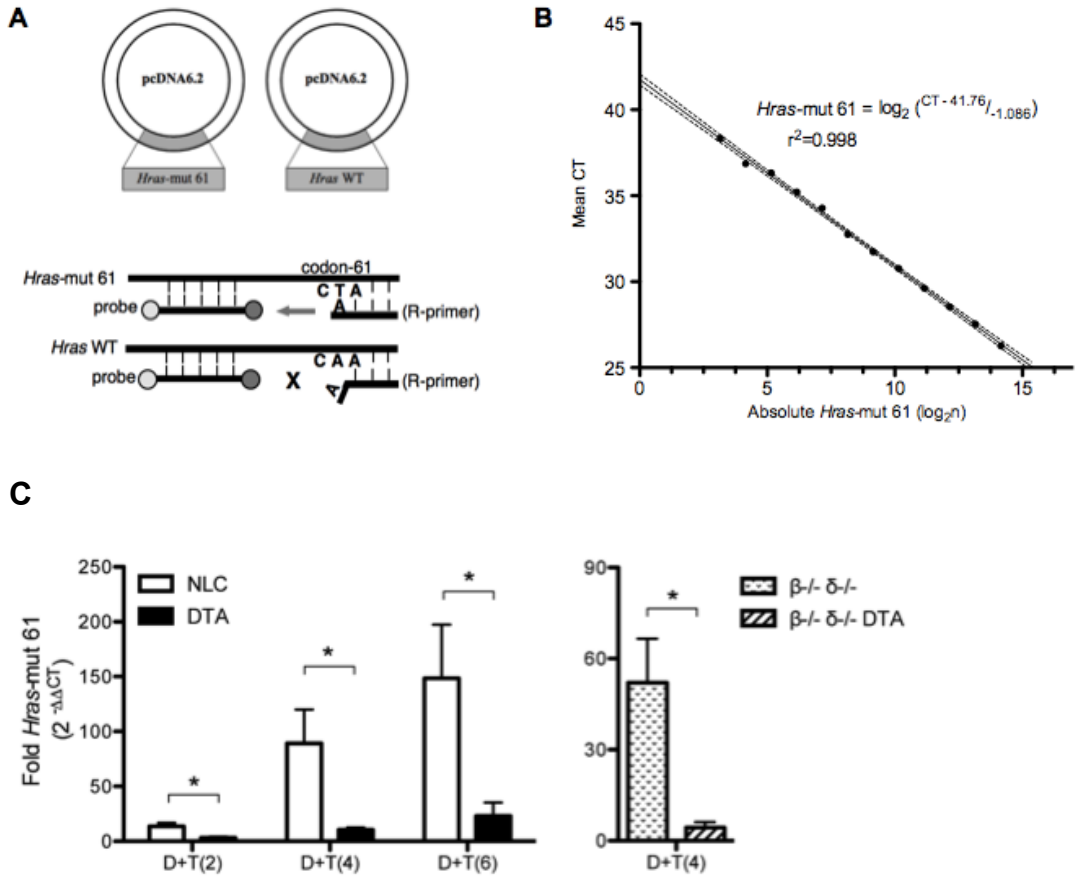
At this point, we strongly believed that the LC-effect occurred early in carcinogenesis, yet we lacked direct evidence demonstrating this. Thus, our lab devised a novel TaqMan real time polymerase chain reaction (RT-PCR) based mutagenesis assay – modified from a previous report [34] – that would enable the relative quantification of *Hras* mutations in DMBA-treated DTA and WT mice. The assay utilized TaqMan probes and primers that would selectively amplify and quantify only the alleles harboring the signature DMBA induced codon 61 *Hras* mutation.

We first verified the validity of the mutagenesis assay using plasmids and DNA from the CarC cell line, which harbors two mutant *Hras* alleles. *Hras* 240bp PCR product containing codon-61 was cloned from CarC (mutant *Hras*) and mKC (WT *Hras*) cell gDNA and inserted into pcDNA6.2TM/GW/D-TOPO® expression vector and sequenced for verification (**Figure 3A**). Knowledge of the plasmid sequence enabled derivation of plasmid mass (1plasmid = 5.486E-18 grams, or 18227 plasmids in 0.1pg). Thus, by running RT-PCR on serial dilutions of known number of mutant plasmids mixed, we were able to determine the lower limit of mutant allele detection. The assay could detect as few as 8 mutant plasmids (**Figure 3B**). The sensitivity of the assay was not affected by the presence of wild type *Hras* sequences. Thus, the novel mutagenesis assay was effective at detecting and quantifying the relative number of *Hras* mutants present in a given sample.

With the assay validated, the focus shifted back to examining DTA and WT mice for relative amounts of mutant *Hras* present in their DMBA-treated epidermis. This would directly investigate if LCs have an effect on *Hras* mutation acquisition. Both

cohorts of mice were treated with DMBA, followed by 2 weeks, 4 weeks, or 6 weeks of TPA. Previous attempts at identifying *Hras* mutant alleles in non-promoted skin were unsuccessful (data not shown); therefore, minimal and equal amounts of TPA were used. DNA was harvested from the DMBA-treated regions of each group and purified for analysis in the mutagenesis assay. For each TPA cohort, significantly fewer *Hras* mutations were detected in DTA mice compared to WT mice, and this result was unaffected by the absence of T cells (**Figure 3C**). Thus, in a T-cell independent fashion, LC-deficiency protected against the acquisition of DMBA-induced epidermal *Hras* mutations.

Figure 3:

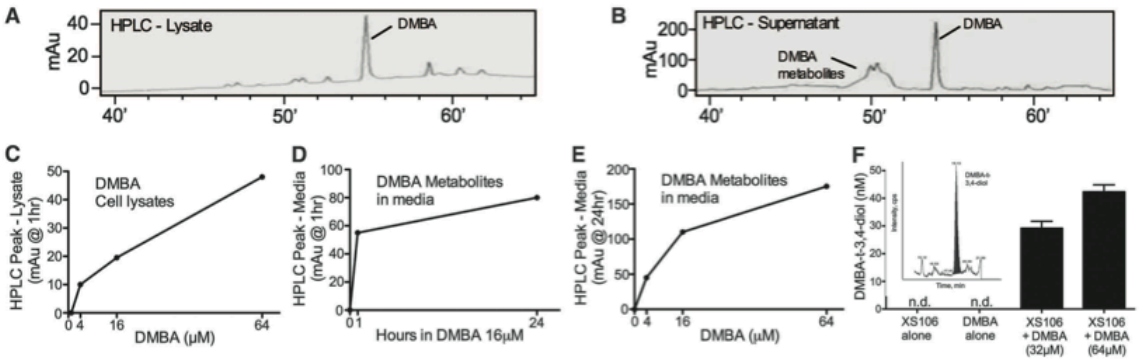


D. XS106 cells cultured with DMBA generate DMBA-*trans*-3,4-diol

Considering the events that must take place prior to acquisition of *Hras* mutations during chemical carcinogenesis, the major events include DMBA diffusion through the stratum corneum, metabolic activation to mutagenic intermediates, and interaction with DNA. Previous reports examining the DTA mouse epidermis revealed no structural differences compared to a wild type mouse except the absence of LC [23]. Pre-treatment epidermal and stratum corneum combined thickness is largely equivalent between DTA and NLC mice [27]. Thus, we focused our efforts at assessing the possible role LC may play in the bioactivation of DMBA.

In order to investigate the capacity for LC to generate mutagenic intermediates, XS106 cells, which are stable cell lines derived from murine LC [62], were cultured with DMBA for 24 hours. High-performance liquid chromatography (HPLC) analysis of cell lysates revealed internalization of DMBA, while analysis of supernatant identified DMBA metabolites (**Figure 4, A and B**). The concentration of XS106-internalized DMBA increased with increasing initial DMBA concentrations (**Figure 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 (**Figure 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) identified the mutagenic metabolite DMBA-*trans*-3,4-diol in the cultures, at mean concentrations of 29.2nM and 42.4nM when initial DMBA concentrations were 32μM and 64μM, respectively (**Figure 4F**). Thus, the LC-derived XS106 cells are able to generate the specific intermediates required for mutagenesis.

Figure 4:

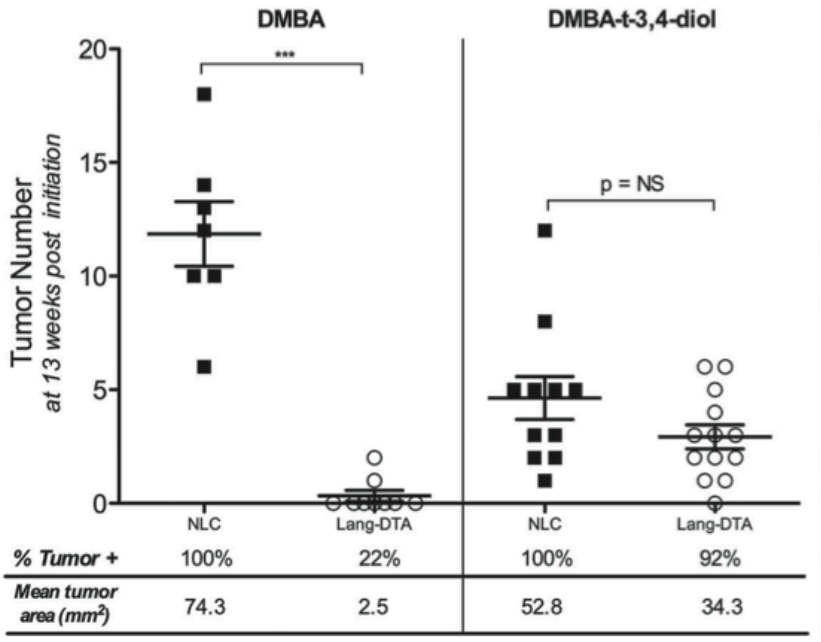


E. Initiation with DMBA-trans-3,4-diol restores tumorigenesis in the DTA mouse

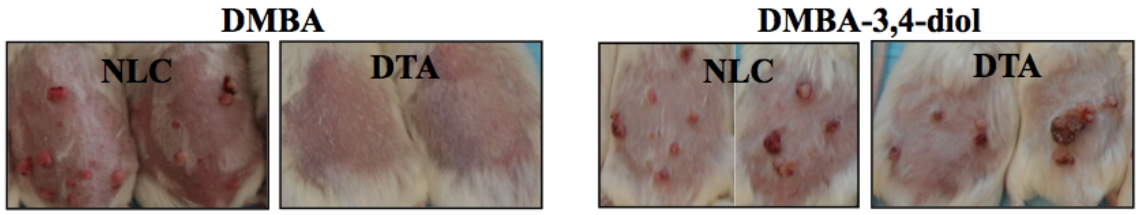
After observing that *in vitro* LC can generate mutagenic intermediates, we aimed to investigate directly whether the LC-requirement for tumorigenesis reflected the cells' release of DMBA metabolites. We initiated WT and DTA mice with 100μg DMBA-*trans*-3,4-diol followed by standard promotion with TPA. Unlike the resistance observed after DMBA initiation, DTA mice were susceptible to tumorigenesis at a comparable level to WT when initiated with the DMBA-*trans*-3,4-diol (**Figure 5**). The restoration of tumors with metabolite initiation demonstrated that the LC-requirement for chemical carcinogenesis can be bypassed by artificially providing metabolism. Not only do these findings demonstrate the importance of LC-specific metabolism for carcinogenesis, but they also highlight the inadequacy of KC metabolism for DMBA tumorigenesis.

Figure 5:

A



B **C**



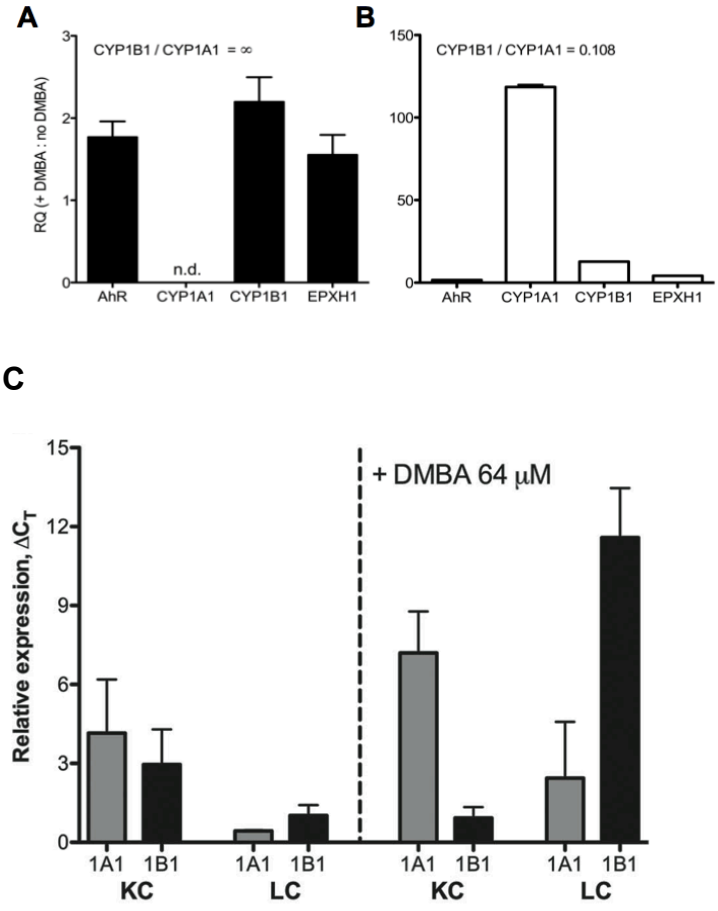
F. Keratinocyte XRE expression favors *Cyp1A1*, while LCs expression favors *Cyp1B1*

In considering the ability of keratinocytes and LCs to metabolize DMBA, we investigated the XRE expression profile of primary murine keratinocyte and primary murine LCs before and 24-hours after DMBA exposure. After creating epidermal cell suspensions of DMBA-unexposed and -exposed skin, keratinocytes were sorted based on $CD45^+$ and LCs were sorted based on MHC^+. Real time PCR of RNA isolated from each

cell type revealed that keratinocytes and LCs have a different expression profile in response to DMBA. LCs did not express *Cyp1a1* either before or after DMBA, while there was a two-fold increase in *AhR*, *Cyp1b1*, and *EPXH1* after DMBA. Overall enzyme expression levels were greater in keratinocytes than LCs, however keratinocytes heavily favored *Cyp1a1* expression over *Cyp1b1* (**Figure 6, A and B**). Thus, the *Cyp1b1* to *Cyp1a1* ratio was 0.108 in keratinocytes, whereas it approached infinity LCs.

Given that these data indicate the major contribution of murine LCs to cutaneous chemical carcinogenesis is mediated by non-immunological properties, it was appropriate to determine whether human LCs might display equivalent properties with the potential to facilitate disease. We therefore examined the response to DMBA of primary human LCs from several donors, and their consequent effects on primary keratinocytes. The data are consistent with the murine data sets and previous reports in the literature [56]. Following DMBA treatment, human LCs express substantially more *Cyp1b1* than *Cyp1a1* (of which most donors' cells express negligible levels), whereas human keratinocytes express an excess of *Cyp1a1* (**Figure 6C**). For most donors, *Cyp1b1* expression by DMBA-treated LC was ~20-fold higher than that in keratinocytes, whereas *Cyp1a1* expression was substantially higher in keratinocytes.

Figure 6:



G. LC-deficient mice develop UVB-induced tumors at the same rate as wild type mice

The data presented thus far implied that the LC contribution to carcinogenesis is unique to the chemical carcinogenesis model. We questioned if the profound resistance seen in chemically treated LC-deficient mice would be observed in UVB-carcinogenesis protocols. In order to investigate the impact of LC-deficiency on UVB-induced tumors, huLang.DTA mice and NLC mice underwent daily UVB treatment five days per week for

thirty weeks. Four out of ten NLC mice harbored tumors by week 24 of treatment, whereas all LC-deficient mice remained tumor-free (**Figure 7A**). NLC mice harbored significantly more tumors and a significantly larger tumor area early during tumor development. On week 24, average tumor number per mouse was 0.5±0.2 for NLC and 0 for DTA mice (p-value = 0.019). Average tumor area was 5.6mm^2±2.8 for NLC and 0 for DTA mice (p-value = 0.031). With continued UVB treatment, however, DTA mice began developing tumors. At week 29 (**Figure 7B**), there was no longer a statistically significant difference between the two cohorts. Average tumor number per mouse was 2.5±0.69 for NLC and 1.6±0.27 for DTA mice (p-value = non-significant). Thus, LC-deficiency did not protect against UVB-induced tumors, although it did delay onset of tumors by a short period of time.

Figure 7A: Tumor number and tumor area per mouse at 24 weeks of UVB treatment.

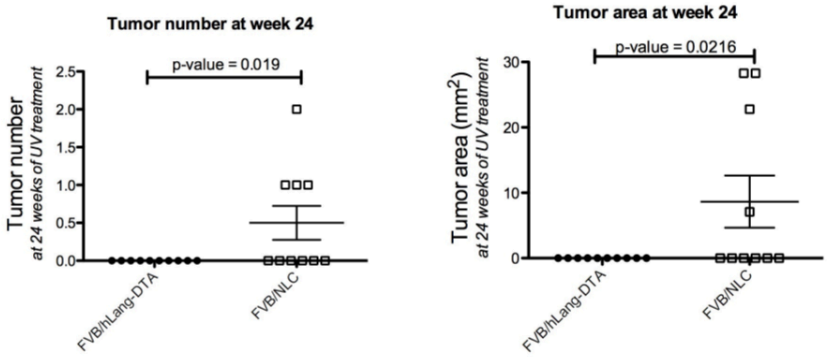
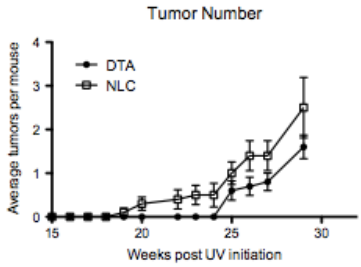
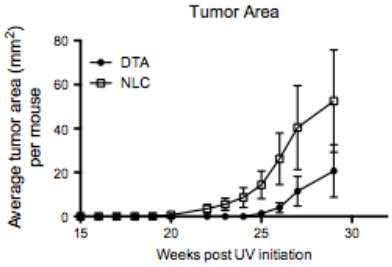
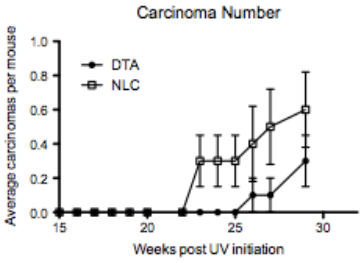
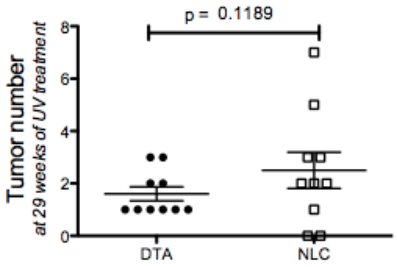


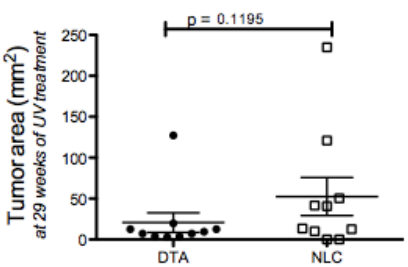
Figure 7B: Tumor number and tumor area per mouse at 29 weeks of UVB treatment.

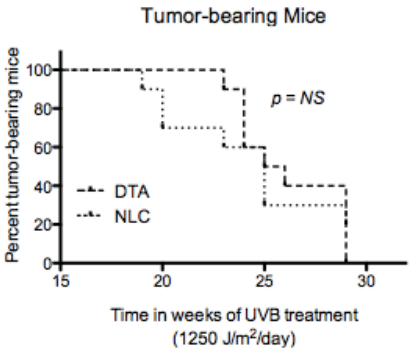












V. Discussion

The immunological influences on cancer biology elucidated to date traverse a wide range of effects. Clinical evidence definitively demonstrates that the immune system is intimately related to cancer in both enhancing and inhibiting capacities. As the major immune constituents present in mouse epidermis, γδ DETCs and LCs represent the interface of the immune system and cutaneous epithelial tumors. Furthermore, the skin epithelial surface faces with an ever-growing level of mutagenic PAHs found in the environment. While γδ DETCs provide an anti-tumor effect on chemical carcinogenesis in which their absence leads to higher rates of tumors [14], LCs contribute an effect so critical to tumorigenesis that their absence results in nearly complete elimination of DMBA/TPA-induced cutaneous papilloma and squamous cell carcinoma [27].

With dendritic processes extending in all directions, LCs are strategically positioned to interact with carcinogenic compounds in the environment and the keratinocyte stem cells of the epidermis [1]. Until the development of LC-deficient mouse models, our understanding of LC biology has primarily centered on its role in antigen presentation and regulating an adaptive immune response [22]. Here, we shift the LC-paradigm further and describe a novel pathophysiologic role for LC in the development of cancer that is independent of αβ and γδ T cell-mediated immunity (**Figure 1**). LC-deficient epidermis harbored fewer *Hras1* mutations than wild type skin (**Figure 3**); *in vitro* LC generated the proximate carcinogen DMBADE (**Figure 4**); and direct initiation with DMBA-*trans*-3,4-diol restored tumorigenesis on DTA mice (**Figure 5**). LC-deficiency did not eliminate UVB-carcinogenesis, although tumors developed later than LC-intact mice (**Figure 7**). Thus, our data collectively demonstrate that LCs

facilitate carcinogenesis in neighboring keratinocytes through inherent scavenging properties and enzymatic processing that generates the critical mutagenic metabolites.

A. Ratio Hypothesis

Our findings further suggest that keratinocytes, by themselves, are inadequate in unleashing DMBA's mutagenic potential, and investigations into the cell-specific expression of XRE enzymes reveal a possible explanation. In both mice and humans, we observed a preferential expression of *Cyp1B1* > *Cyp1A1* in LC and *Cyp1A1* > *Cyp1B1* in keratinocyte (**Figure 6**). Invoking the conclusions of Kleiner and Buters that *Cyp1B1* is required for generation of mutagenic intermediates [47] and that *Cyp1A1* has a faster pharmacokinetics profile [46], it follows that the low *Cyp1B1* to *Cyp1A1* ratio in keratinocytes would skew metabolism towards generation of non-mutagenic metabolites mediated predominantly by *Cyp1A1*. The high ratio of *Cyp1B1* to *Cyp1A1* in LCs enables these cells to preferentially generate the proximate carcinogen DMBA-*trans*-3,4-diol. Others have revealed the potential for non-epithelial stromal cells to activate PAHs [63, 64]. The observation of decreased *Hras1* mutations in LC-deficient mice, in which keratinocytes are normal and abundant, offers *in vivo* evidence to reject that keratinocyte XRE enzymes are the metabolic machinery catalyzing DMBA activation. The lack of tumors in LC-deficient mice further recapitulates this notion.

The relevance of *Cyp1B1* to cancer biology has been investigated from many aspects. For instance, it is expressed at higher levels in tumor samples compared to normal counterparts [65]. Individuals who smoke cigarettes, which contains the PAH BP, preferentially express *Cyp1B1* in their oral mucosa [66]. However, while *Cyp1B1*-

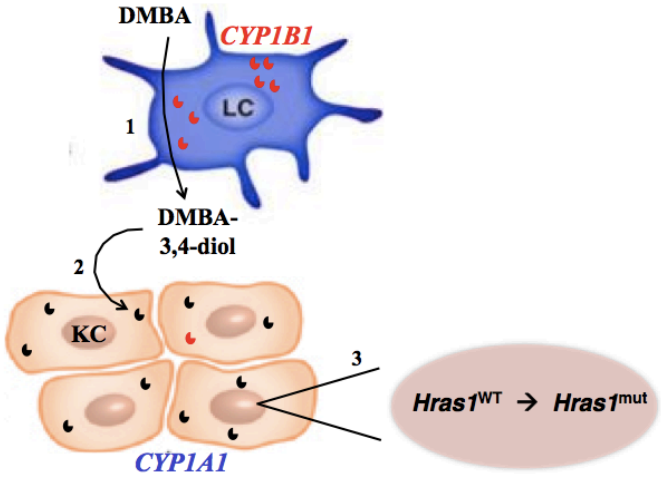
mediated metabolism contributes to PAH-carcinogenesis [44, 46], data derived from colonic adenocarcinoma models suggest that *Cyp1B1* is protective against endogenous carcinogenic substrates [67]. Thus, these data offer a teleological explanation for why LC may have evolved a "cancer-promoting" *Cyp1B1*:*Cyp1A1* ratio. The increased interactions between PAH and the epidermis in industrialized societies perhaps facilitate the hijacking of a protective effect that LC *Cyp1B1* evolved to perform. Future investigations should pursue the development of an LC-specific *Cyp1B1* knockout mice. The use of this animal model would not only confirm the findings presented herein if resistance to DMBA carcinogenesis is observed, but it would also facilitate investigations into the positive roles of LC *Cyp1B1*.

B. Cooperative Carcinogenesis

LCs are located adjacent to both inter- and intra- follicular basal keratinocytes [68], and hair follicle bulge cells have been strongly implicated as targets of cutaneously applied chemical mutagens [69]. Given LC and keratinocyte stem cell proximity, our data imply a cooperative carcinogenesis scenario in which keratinocyte mutagenesis requires neighboring LC-mediated DMBA activation (**Diagram 2**). In this proposed model, LC must first internalize DMBA (1), perhaps via simple diffusion or through a more sophisticated process such as endocytosis. Next, LC-specific *Cyp1B1* and *EPXH1* generate DMBA-*trans*-3,4-diol (1), which then traverse out of LC and into adjacent keratinocytes (2) for further metabolism to DMBADE. Within keratinocytes, the ultimate carcinogen displays a well-described, strong affinity for the *Hras* gene at codon 61 (3) [35]. Intercellular metabolite transfer has been proposed in other models examining

DMBA's effects, which corroborate that non-epithelial "stromal" cells are critical for DMBA-activation [54, 55].

Diagram 2: Cooperative Carcinogenesis Scenario



C. IMPLICATIONS FOR OTHER CUTANEOUS AND NON-CUTANEOUS TUMORS

LCs neighbor other epidermal cellular constituents including melanocytes. Their proximity, coupled with the increasing body of evidence that activation of the *ras* family of oncogenes is necessary for human melanoma development [70], implicates LC-mediated cooperative carcinogenesis and PAH in melanoma biology. Although patients with human familial melanoma syndromes – alterations in *Cdkn2a* and/or *Cdk4* – exhibit a higher incidence of clinical melanomas, mice genetically engineered to mimic these syndromes result in a very low incidence of spontaneous melanoma [71]. In these animals, the high melanoma incidence is restored by inducible expression of *Hras* [72]. Removal of the inducible signal, and thus *Hras expression*, resulted in regression of pre-

existing melanomas [72]. Indeed, in mice with low melanoma incidence despite expressing alterations in *Cdk4*, DMBA treatment also restored heightened melanoma development [73, 74]. The melanocytes of human Spitz nevi have been shown to possess mutations and copy number amplifications of the *Hras* gene [75]. Thus, these findings warrant further investigation into the contribution of LC-mediated metabolism of PAH to pigment cell *ras* activation.

Furthermore, our findings hold implications for carcinoma of non-cutaneous epithelial tissues, such as the GI and respiratory tracts. Arguably, these epithelial surfaces are exposed to some of the highest levels of PAHs secondary to their presence in certain foods and cigarette smoke. The presence of intraepithelial immune cells in these non-cutaneous epithelial surfaces raises the possibility that tissue specific immune cells might have similar non-immunologic contributions to PAH-mutagenesis. Our findings justify future investigations to evaluate XRE expression profiles of other tissue-specific dendritic cells.

Our findings offer a novel target for the reduction in PAH-induced *ras* mutations in non-melanoma skin cancers, and possibly for melanoma and epithelial transformations throughout the body. With therapies targeting LC-specific *Cyp1B1* metabolism, perhaps the mutagenic effects of PAH found in the environmental, cigarette smoke, and certain foods may be mitigated. Nevertheless, these findings demonstrate that tissue-associated DC can enhance chemical carcinogenesis via PAH metabolism, highlighting the complex relation between immune cells and carcinogenesis.

VI. Figure Legends

Diagram 1. DMBA metabolism pathway. The metabolism of DMBA to the principle proximate carcinogen DMBA-*trans*-3,4-diol and the ultimate mutagen DMBADE is depicted as adapted from Miyata, et al. [49]

Figure 1. Resistance to two-stage chemical carcinogenesis is αβ and γδT cell–independent. Chemical carcinogenesis was induced in **(A)** Tcrβ−/− Tcrδ−/− Lang-DTA mice using 50 mg DMBA to initiate and 6.25 mg TPA/week to promote, as well as in **(B)** Tcrδ−/− Lang-DTA mice using 100 mg DMBA to initiate and 25 mg TPA/week to promote. The mean numbers of tumors per mouse (left) and individual tumor area (right) 14 weeks post-initiation are shown. ***P < 0.0001 (Student's t test); n = 8 to 12 mice per group. NS, not significant. **(C)** Representative images from **(B)**.

Figure 2. LC-deficiency does not prevent tumorigenesis in K5Hras mice. K5*Hras*Tg-Lang-DTA and K5*Hras*Tg littermates were monitored for spontaneous tumor development. LC-deficient and LC-intact mice had equivalent tumor incidence and individual tumor area.

Figure 3. DMBA induces fewer *Hras* mutations in LC-deficient mice than in wild type mice. (A) *Hras1* 240bp PCR product containing codon-61 was cloned from CarC (mutant) and mKC (WT) cell gDNA and inserted into pcDNA6.2TM/GW/D-TOPO® expression vector and sequenced for verification. **(B)** Real time PCR using mutant-specific primers was done with a known absolute number of plasmids per reaction in

order to determine the lower limit of mutant copy detection. As few as 8 mutant plasmids were detected in this system, while normal plasmids were not detectable with mutant primers. (**C)** DMBA was applied to the dorsal skin of both NLC and DTA mice, followed by application of 2, 4, or 6 weeks of TPA (twice/week). DNA was harvested for mutation quantification. DNA from LC-intact skin harbored more *Hras*-61 mutants than DNA from LC-deficient skin *P < 0.05 (Student's t test), in a TPA-dose dependent manner. LC-deficiency protected against acquiring Hras-61 mutation even in the absence of αβ and γδ T cells *P < 0.05 (Student's t test).

Figure 4. XS106 cells cultured with DMBA generate DMBA-*trans*-3,4-diol. XS106 cells were cultured with 64 mM 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.

Figure 5. Initiation with DMBA-trans-3,4-diol restores tumorigenesis in the DTA mouse. (**A**) Tumor induction was measured after treatment with DMBA (12.5 mg) or DMBA-*trans*-3,4-diol (100 mg). 25 mg/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. ***P < 0.0001 (Student's t test). (**B and C**) Representative images from (**A**).

Figure 6. KC XRE expression favors *Cyp1A1*, while LC expression favors *Cyp1B1*.

Primary murine LCs (**A**) and keratinocytes (**B**) were cultured with and without DMBA for 24 hours, after which *Cyp1B1* to *Cyp1A1* expression was quantified relative to β-actin by qPCR. (**C**) Human keratinocytes or LCs were cultured in the presence (right) or absence (left) of 64 mM DMBA for 24 hours, then *Cyp1a1* (1A1) and *Cyp1b1* (1B1) expression was quantified relative to β-actin by qPCR.

Figure 7. LC-deficient mice develop UVB-induced tumors at the same level as LC-intact mice. Tumor induction was measured as tumor number and tumor area at (**A**) 24 weeks and again at (**B**) 29 weeks post-initiation of 5-time weekly UVB treatment.

Diagram 2. Cooperative chemical carcinogenesis scenario. Illustration of proposed mechanism by which LCs contribute to chemical carcinogenesis. (**1**) LCs internalize DMBA, where LC-specific CYP1B1 and mEH convert DMBA to DMBA-*trans*-3,4-diol. (**2**) This metabolite moves out of LCs and into keratinocytes where it undergoes further metabolism to DMBADE. (**3**) The ultimate mutagen exerts effects on keratinocyte *Hras* gene.

Image 1. LC internalize DMBA. Confocal image depicting $MHCII^{+}$ (green) cells internalizing DMBA (blue). Taken by Julia Lewis.

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