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Investigating The Influence Of Langerhans Cells On Keratinocyte Proliferative Response To Chemical And Ultraviolet B (uvb) Exposure

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Investigating the influence of Langerhans cells on keratinocyte proliferative response to chemical and ultraviolet B (UVB) exposure

A Thesis Submitted to the Yale University School of Medicine in Partial Fulfillment of the Requirements for the Degree of Doctor of Medicine

by
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MD Candidate, 2011
INVESTIGATING THE INFLUENCE OF LANGERHANS CELLS ON KERATINOCYTE PROLIFERATIVE RESPONSE TO CHEMICAL AND ULTRAVIOLET B (UVB) EXPOSURE.

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Initially Girardi lab demonstrated that LC facilitate chemical carcinogenesis by showing lower levels of proliferation in LC-deficient (Langerin-dipheria toxin A transgenic) mice relative to LC-intact littermate controls. Thus, we sought to explore the breadth of potential influences of LC on keratinocyte responses (hypertrophy, apoptosis, and clonal expansion) in two classic models of carcinogenesis (chemical and UVB), by using immunohistochemical (IHC) and immunofluorescent (IF) methods of cell identification. At baseline, LC-deficient compared to LC-intact mouse skin showed comparable proliferation levels as measured by Ki-67 IHC (13.6±2.6 vs 12.7±2.8 Ki-67+ cells, NS) and epidermal thickness (7.9±1.8 vs 6.9±2.8 µm, NS). After a single application of mutagen DMBA, followed by repeated applications of tumor promoter TPA (x 5.5 wks), LC-depleted skin showed smaller increase in epidermal proliferation (31.5 ± 7.7 vs 25.7 ± 7.4 # Ki-67+ cells, P=0.007) and minimal epidermal thickness (59.2 ± 28.4 vs 25.8 ± 8.9 µm, P<0.0001), as compared to LC-intact skin. There was a similar trend of decreased epidermal proliferation and decreased minimal epidermal hypertrophy in LC-depleted (Lang-DTA) skin after chronic TPA-alone (x 4 wks, P=0.0002 and P<0.0001, respectively), or DMBA-alone application (P=0.0001 and P<0.0292, respectively). To address the potential influence of LC on acute UVB-induced keratinocyte damage, mice were irradiated with a single dose of 3,360J/m². There was a trend toward increased epidermal apoptosis (cleaved caspase-3, P< 0.0001 and sunburn cells). Chronic low-dose UVB (500-1500 J/m², 5d/wk x 5-9wks) induced mutant keratinocyte islands over-expressing p53 (CM5) in both LC-intact and LC-deficient skin, with a statistical trend towards greater levels within LC-intact skin. After 5 and 9 weeks of chronic UVB exposure Lang-DTA mouse skin, compared to NLC, had a smaller number of mutated clones per mm² (P=0.0489 and P=0.004, respectively). After 5 weeks of chronic UVB followed by 3 weeks of no UVB (and DT injection at weeks 6, 7 and 8), there was a smaller number of mutant clones per mm² in Lang-DTR mouse skin versus NLC (P< 0.0001). For this group, statistical significance was also reached for mean number of mutated clones/mm² per group in Lang-DTR vs NLC mice (P=0.0106). After 5 weeks of chronic UVB, ‘replacement’ LC are associated with mutant clones in Lang-DTA mice, as compared to areas without clones (P< 0.0001). Statistical significance was also reached for mean density per mouse (P=0.0106). A similar association was seen in NLC mice after 5 weeks of chronic UVB, where LC were associated with mutant clones in NLC mouse skin as compared to areas without clones (P= 0.0047). After chronic UVB, GL3+ dendritic cell numbers were decreased, but still present, in Lang-DTA and NLC mice, many located within or near hair follicles and some seen interacting with LC. After 5 weeks of chronic UVB, Lang-DTA mice have approximately 12 times less langerin+ DC compared to NLC mouse skin. In summary, LC exert diverse, measurable influences on chemically induced keratinocyte proliferation and acute and chronic UVB-induced keratinocyte apoptosis.
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INTRODUCTION

Langerhans cells (LC) are dendritic cells (DC) of the epidermis whose exact functions are not completely understood. LC constitute a subset of DC that reside in the stratified squamous epidermal layer of the skin, as well as in the mucosal epithelia lining the ocular, oral and vaginal surfaces (1). LC were first described by Paul Langerhans in 1868 in Berlin, as dendritic cells in the human epidermis and were mistaken for nerve cells. Not until 1985 were LC accepted as part of the DC family by Schuler and Steinman (2). In 1992 Jacques Banchereau and Christophe Caux were the first to generate LC with Birbeck granules from human hematopoietic progenitor cells (CD34+ cells) (3), a cell line model still used widely today. In 1999 Sem Saeland and Jenny Valladeau identified the langerin /CD207 marker, which facilitated further study of LC biology. Miriam Merad in 2002 revealed that in the steady-state Langerhans cells remained in the epidermis for virtually the entire life, and that they slowly renew themselves in situ by proliferation (4). In 2003 Allan et al. revealed the surprising findings that LC are not the only APC for pathogen-presentation in vivo, as they were not involved in herpes viral presentation (5). Just when it seemed that generation of mouse models around 2005-2006 to selectively deplete LC in vivo would clarify LC role in immune regulation, they created even more controversy by showing heterogeneous findings in contact hypersensitivity (CHS) reactions. In 2007 it was shown that langerin was no longer a unique marker of epidermal LC, but was also shared with several other DC of the dermis, lymphoid tissues, and various other organs of the body outside the skin.
**LC Characteristics and Other Langerin**^+** DC**

In humans, the LC population can be characterized as co-expression of langerin (a C-type lectin receptor), CD1a (which plays a role in presentation of lipid microbial antigens), major histocompatibility complex (MHC) class-II, E-cadherin (which mediates LC attachment to keratinocytes), the membrane ATPase (CD39), the chemokine receptor CCR6, the adhesion molecule Ep-CAM, and the integrin CD11b (6-8). LC in steady state are known to be very long-lived and re-populate within the epidermis. However, during inflammation (hapten sensitization, tape stripping, UV irradiation), monocyte-derived progenitors from the blood (characterized by high expression of the Gr-1 molecule) enter the LC-depleted epidermis in a CCR2-dependent way and give rise to LC (9).

Although langerin was initially described as a specific and exclusive marker for LC (10,11), it was recently revealed that other DC outside the epidermis can also express langerin (12-15). Over the years and through the use of LC ablating models, it had become apparent that langerin^+ DC exist in epithelia outside the skin’s epidermis and constitute various functional DC subsets. Originally, it was presumed that the recently observed langerin^+ DC in the dermis (dDC) were just migratory epidermal LC. However, new evidence has revealed the former to be a separate subtype of dermal dendritic cells (dDC) (13,14). Langerin^+ dDC are CD11b^lo, CD103^+, Ep-CAM/CD326^lo, while LC are CD11b^hi, CD103^-, Ep-CAM^hi (1,16). Moreover, dDC seem to originate from different precursors and have different characteristics. dDC seem to come from a radio-sensitive blood-derived precursor recruited to the dermis, while LC are
radioresistant and in steady state conditions originate from stem cells in the epidermis and seem to have a significantly lower BrdU incorporation (14). LC are dependent on TGF-β, colony-stimulating factor (M-CSF)CSF-1 and interleukin (IL)-34, while langerin+ dDC are dependent on the cytokine Flt3 ligand for development ((1).

The majority of blood CD8+ DC in the thymus, spleen, cutaneous lymph nodes (LN) and mesenteric LN are langerin+ as well (17-19), and can be distinguished from dermal langerin+ DC (dDC), by the former being CD8+ and CD11c^high (1). Langerin+ DC have identified in various organs of the body, including the bronchial epithelia and arterioles of the lung (20), throughout the GI tract, and in the cornea.

**THE LC PARADIGM**

The classically accepted ‘LC paradigm,’ a term coined by Wilson and Villadangos in 2004 (21), included three key functions of LC established mainly from *in vitro* studies. Firstly, immature LC residing in steady state were regarded as highly specialized in antigen uptake and ideally placed in skin to survey the environment with their dendrites (22). Second, LC transport antigen to skin draining LN to present to naïve T cells that recognize rare epitopes. To do this, LC are activated by cytokines and pathogen products (23,24), down-regulating E-cadherin and expressing CCR7, a chemokine receptor that homes LC to T cell areas in the LN. Third, during migration LC ‘maturate’ into a stimulatory phenotype, during which processed antigen is presented on major
histocompatibility complex (MHC)-class I/II and co-stimulatory molecules are upregulated. This LC paradigm was extended to explain peripheral self-tolerance. LC were presumed to be the key players in presenting self-antigen in both chronically inflammatory (25,26) and steady-state environments (27,28). DC (presumably LC) have been shown to transport epidermally restricted antigens (29), present them via the endocytic molecule CD205 (30), and establish CD4 and CD8 T cell tolerance (31), which may occur via induction of regulatory T cells (32,33), with involvement of E-cadherin (34).

However, with the advent of increasing in vivo studies in LC research, more and more studies suggest alternative possibilities regarding the relative contributions of skin DC (LC and dDC) in cross-presentation of skin-derived antigens, T cell priming, viral immunity, and contact hypersensitivity (CHS). Early in vivo reports had hinted at LC role in CHS, by demonstrating close apposition of mononuclear cells to LC in contact allergic reactions (35) and adoptive transfers of haptenized LC in naïve animals (36,37), but this role was questioned with the development of LC ablating models.

**LC Ablation Models**

Langerin is a C-type lectin that induces the formation of Birbeck granules (10) and is specific to LC in the epidermis, dermis and lymphoid tissues (as well as other organs as discussed earlier). Because its ablation does not interfere with LC function (38), it has been employed as a useful target to deplete LC in in vivo mouse models by three international groups: French, Dutch, and American.
These transgenic mouse models either use diphtheria toxin (DT) subunit A (DTA) to constitutively delete LC from birth or the DT receptor (DTR) to induce LC ablation with DT injection. The following discussion of these models was covered by Dan Kaplan and others in their 2008 review (39).

DT is a heterodimeric protein that inserts its A subunit by binding the B subunit to heparin-binding EGF-like growth factor (HB-EGF) precursor on the surface of mammalian cells (39). Once inside the cell, DTA works by inhibiting protein biosynthesis and inducing cell death. Inserting a human HB-EGF precursor (DTR) into a rodent LC sensitizes the cells to DT, resulting in selective ablation with exogenous injection of DT. Since the rodent homologue of HB-EGF precursor does not bind to the B subunit of DT, DTA is not internalized by rodent cells and remain resistant to exogenous DT injection (40).

The inducible ablation models (initially developed by the French and Dutch groups) (19,41) were generated knock-in mice of the human DTR into the langerin locus (Langerin–DTR–EGFP). The Dutch group targeted a DTR–EGFP cassette into the second exon of the langerin gene (41). The French group generated two separate lines in which either an IRES–EGFP or an IRES–DTR–EGFP cassette was knocked-in to the sixth exon, in the 30-untranslated region of langerin (19).

Thus, systemic intraperitoneal (i.p.) injection of DT depletes epidermal LC within 24-48 hours, which start to repopulate in 2-4 weeks. However, whereas epidermal LC are still absent 10 days after depletion with DT, langerin⁺ dDC start to repopulate by that time (12). Similar kinetic analyses were performed in the
Clausen group Lang-DTR mice (42). Forty-eight hours after DT injection, no LC and no dDC could be detected, but by day 10, around 22% of the dDCs were found, but no LC. Fourteen days after injection, around 25% of the dDCs had reappeared and a few LC could be visualized, as previously reported by others (15,41). dDC reach approximately 50% of their steady-state levels by week 2 post DT injection (39). Of note, a subset of CD8+ DC, which is found throughout the secondary lymphoid tissues (LN, spleen and thymus), also express langerin and are ablated after DT administration (19).

Alternatively, the American group generated bacterial artificial chromosome (BAC)-transgenic mice by inserting DTA (not DTR) into the 3’-untranslated region of the langerin gene contained within a 70-kb fragment of human genomic BAC DNA (43). Unlike Langerin–DTR–EGFP (Lang-DTR) mice, where all langerin+ DC are ablated, in Lang-DTA mice only epidermal LC are absent. This is probably related to altered regulation of expression of the human langerin promoter in Lang-DTA mice compared to the endogenous mouse promoter in Lang-DTR mice (39). The Kaplan group later developed the inducible DTR-model as well (44).

**LC as APC:**

LC have long been thought of as potent initiators of skin immunity. Consisting of only 2% of the epidermal population (22), LC manage to cover more than 25% of the skin’s surface, made possible by laying their disc-like bodies parallel to epidermal surface and extending a network of dendrites (45).
Interestingly, the reduction in LC density per unit area in sun-exposed sites is reported to be compensated for by an increase in the mean cell volume (45). Using their dendrites (22) LC probe their environment for antigens – exogenous and endogenous- to present to the adaptive immune system. Their strategic location in the epidermis and evidence of langerin+ DC localizing to well vascularized marginal zones in the spleen (46) gives them the ideal opportunity to take up antigens trafficking through these locations (47). LC can be induced by topical haptens and perturbations to the skin to undergo phenotypical and morphological changes, and migrate to draining lymph nodes carrying melanosomes and apoptotic bodies (48). LC can cross-present antigens (49), induce expansion of memory CD8+ T cells and are very efficient at priming or inducing CD4+ T cells to secrete Th2 cell- type cytokines, i.e. IL-4, IL-5, and IL-13 (50). LC have also been shown to promote cytotoxic responses against infectious antigens and tumor cells (49).

Most of our earlier knowledge about dermal DC and LC phenotype and function was obtained through the study of these cells cultured in vitro from CD34+ hematopoietic progenitor cell or blood-derived monocytes (51,52), and had shown LC to be potent APC. Epidermal LC were found to be able to present exogenous peptides to T cells (53). Often LC are referred to as prototypic immature dendritic cells, stemming from early reports showing freshly isolated LC as weak stimulators of primary T cell proliferation, but in tissue culture these murine epidermal LC have been shown to mature into potent immunostimulatory DC in vitro (54).
With more advanced technologies of deriving LC from skin explants and freshly isolated from epidermal and dermal suspensions, it has become more evident that a heterogeneous population of DC inhabits the skin and that LC role as APC may be redundant. Analysis using global transcriptional profiling of skin-derived CD1a⁺ dDC and CD1a⁺ LC in resting, non-inflamed skin, found these two DC to be two truly separate DC subsets and not a functional continuum of one subset as thought earlier (8). Moreover, a whole shift in the ‘LC paradigm’ has been occurring, as more evidence is presented on LC role in silencing immune responses against self-antigens and innocuous environmental antigens (55). LC may contribute to immune tolerance, alluding to a possible intrinsic nature to maintain homeostasis of self-tolerance and limit autoimmune reactions.

In murine studies, it has been noted that LC are slower to arrive at draining lymph nodes than Langerin-negative DC, raising the possibility of an immune regulatory role for LC when they enter the lymph nodes (56). During steady state, LC and other DC are thought to take up self-antigen and melanin and migrate to draining LN (4,29,57). However, one recent report comparing gene analysis of LC and their dDC counterparts, found that the population of migrating LC may account for a small fraction of the dermis-derived CD1a⁺ DC and thus may play a smaller role in steady state antigen presentation that previously thought (8). They found that LC residing in the epidermis under steady-state conditions exhibit a non-migratory and non-stimulatory phenotype. Steady state LC expressed molecules involved in cell adhesion (i.e. E-cadherin, ICAM-3, and epidermal surface antigen) or are involved in DC retention such as (junctional adhesion
molecules and CD47), but lacking expression of molecules involved in DC migration (i.e. CCR7). Notably, ligation of CD47 (also known as integrin-associated protein), has been demonstrated to regulate LC maturation and migration, resulting in the suppression of LC function, resulting in decreased T cell priming and consequent immune suppression (58).

Furthermore, Sangoets et al. showed that under steady state conditions LC exhibit a non-stimulatory profile, expressing relatively low levels of co-stimulatory and adhesion molecules (i.e. CD80, CD40, and CD54) or pro-inflammatory cytokines and cytokine receptors and do not express T cell stimulation molecules (i.e. 4-1BB and CD30L). The non-T cell stimulatory profile of LC is further illustrated by the expression of CD43 (also known as leukosialin), a glycoprotein that is only expressed on immature DC and implicated in the inhibition of nonspecific T cell contacts. In addition, under these steady state conditions, LC do not exhibit competent B cell stimulatory capacity, as illustrated by the absence of BAFF, a TNF family member, which is known to play an important role as a co-stimulator of B lymphocyte proliferation and function (8). Still, the authors admit that although these LC isolated from steady state skin are quiescent and sedentary, it is still possible that a fraction of LC mature and migrate to regional LN under steady-state conditions.

LC continue to surprise researchers with regard to their key functions in immune regulation. For example, it was recently found that LC were not important in cutaneous antigen presentation in herpes virus infection, a lytic process that involves apoptosis (5). Other studies of infectious immunology have
revealed that LC don't play a key role in immunity against Leishmania and Candidal infections, and may even be immunoregulatory (59,60). For example, a report using both models of LC ablation to study Candida infection has demonstrated increased immune responses pointing to LC role in immune suppression. More recently, Hogquist and colleagues showed that radioresistant langerin⁺ DCs (presumably LC) are dispensable for peptide antigen presentation in their K14-OVAp/K14-mOVA/Lang-DTR mouse model in which an MHC class I-restricted peptide is expressed under the K14 promoter (61).

**LC Role in CHS: A Prototype Assay of Antigen Presentation and T Cell Induction**

Although, much evidence points to LC ability to be potent APC *in vitro*, several studies in mouse models of LC depletion allude to the fact that LC role may be redundant and dispensable in antigen-presentation. LC role in the prototypic T cell response to haptens painted onto the skin as measured by murine contact hypersensitivity (CHS) reactions remains inconclusive. In this assay, hapten-specific T cell responses are measured in mice sensitized by cutaneous application of hapten on abdomen or back, which are then challenged several days later by painting the same hapten onto the ear. Inflammation is measured by ear thickness (62). Based on an earlier study, which found that the density of epidermal LC played a key role in induction of either CHS or tolerance after skin sensitization with DNFB (63), one would predict that CHS reactions would be absent or greatly attenuated in the absence of LC (64,65). However,
things in the world of LC ablation and CHS are not that simple.

Reports employing LC depleted mouse models are heterogeneous. The French group, who sensitized Lang-DTR mice with DNFB and treated with DT 3 days prior and 1 day after sensitization, showed no effect on CHS (19). The Dutch group sensitized their Lang-DTR mice with trinitrochlorobenzene (and in later study with oxazolone, (12)) 3 days after DT injection and showed slightly diminished but not absent CHS (41). Whereas, the American group who used Lang-DTA (constitutively depleted mice), developed an increased CHS reaction to DNFB and oxazolone as shown by Dan Kaplan group (43). Moreover, an even more recent report by Kaplan and colleagues using a Lang-DTR mouse model showed that acute inducible ablation of LC similarly results in increased CHS reactions (44).

It appears that the timing of DT administration in Lang-DTR mice can affect the outcome, since interestingly when the French mice were treated with DT only on day -1, they developed diminished CHS responses similar to the Dutch mice (16,66). The reason could be that the timed DT treatments make sure all dermal langerin+ DC were gone. CHS responses were indeed decreased when all langerin+ skin DC were absent (by sensitizing closer to DT injection), but restored to wild type levels once the langerin+ dermal DC subset had returned (sensitization 7 or 13 days after DT) (12). This suggested that langerin+ dDC exerted an essential immunogenic function in CHS, but still contradicted the reduced CHS observed by the Dutch group (41).

As hypothesized by Clausen and colleagues (42), the above discrepancy in
the Dutch group may be explained by the amount of hapten used in sensitization. It is possible that when limited amounts of antigen was applied (as by the Dutch group) langerin$^+$ DC were present, but antigen did not reach them, resulting in a decreased CHS response. Thus, antigen dose, application site and thickness of skin, which limits diffusion of antigen, may be important factors that will determine the efficiency of epicutaneous immunization and which skin DC subset will induce the immune response. This was recently supported by the Clausen group, who verified this hypothesis by demonstrating attenuated CHS in the absence of all langerin$^+$ cells applying both a low and a higher dose of hapten onto the skin. In contrast, in the selective absence of epidermal LC, CHS was only diminished at a low hapten dose, but restored to wild type levels at a higher concentration, that is when enough hapten penetrated through the epidermis and across the basement membrane to reach the dDC (42).

In parallel to the above findings, in bone marrow chimera experiments selectively depleting dermal langerin$^+$ DC, Honda et al. found CHS responses similar to wildtype when epidermal LC and langerin-negative dermal DC were present (1,60). The type of hapten does not seem to matter, since when the Clausen group compared the two Lang-DTR mice (19,41), using two different haptens (Oxazolone and DNFB) and controlling for other variables, they saw no differences in their CHS responses (42). In summary, it seems that antigen dose and the number of skin DC that have access to the hapten, which subsequently prime antigen-specific T cells in skin-draining lymph nodes, determine whether or not reduced CHS develops as compared to wild type mice.
Until recently, there were no other reports of the inducible Lang-DTR models to have enhanced CHS as observed in Lang-DTA mice with constitutive LC deficiency. Some hypothesized that in the acutely inducible depletion the still reduced levels of dDC and/or CD8+ langerin+ DC prevented what otherwise would have been an enhanced response in the absence of LC (39). Moreover, given other reports of spontaneous autoimmunity in mice constitutively lacking DC (67), others thought that it is possible that Lang-DTA mice have altered responses to hapten sensitization or different T cell properties, involving LC expression of MHC class II and IL-10 in controlling immune responses (60,68). The latter speculation becomes obsolete since Kaplan and colleagues recently demonstrated that acutely ablated LC (in a Lang-DTR model that does not affect langerin+ dDC) had increased CHS responses similar to the group’s earlier findings in their constitutively ablated LC model (44). Thus, this finding in a model what has intact langerin+ dDC again reinforces the possibility that attenuated levels of langerin+ dDC in other acutely ablating LC models may be responsible for previous reports of decreased CHS responses. Some may question how LC could induce a regulatory function in the inflammatory milieu of hapten sensitization (69), but there are reports showing LC role in T cell regulation in chronic inflammatory responses.

**LC Role in Tumorigenesis**

LC role in cancer has long been studied and heterogeneous findings have been reported regarding LC role in tumors of many organ systems. Initially, LC
were thought to play an important role in tumor antigen presentation to induce antitumor immune responses, but the LC paradigm has shifted to include LC as possible immune regulators. Conflicting data exist on the number of LC in SCC, BCC, cervical lesions and other cancers. No straightforward association with tumors exist and both a decrease and increase in LC density have been reported. For example, LC number may have been found to be decreased in malignant lesions, showing as inverse relationship between LC and melanoma thickness (70), or increased in lesions with better prognosis (71). However, this seeming decrease in LC may have been related to the method of identification of LC, back in the day using ATPase and S-100 or changing character of LC. For example, Connor et al. has demonstrated decreased number of S-100 positive LC associated with HPV peptide, but numbers of LC measured by CD1 were not altered (72).

Maturity of LC in tumors have also been briefly addressed. Giannini et al. report that LC are immature in squamous intraepithelial lesions (SILs) of the cervix and are deficient in their presentation of alloantigens to T cells (73). Golcalves et al. suggested that the increased presence of immature LC in cervical intraepithelial neoplasia could be responsible for deficient presentation of alloantigen to T cells (74). LC-depleted epidermal cells from melanoma patients failed to augment lymphocyte responses to PHA and tetanus toxoid (75). Guess et al. showed that HPV-16 E6 and E7 inhibit MIP-3α (Macrophage Inflammatory Protein 3α/CCL20) transcription, resulting in suppression of the migration of immature LC precursor-like cells (using CCR6), thus possibly inhibiting the
immune alert systems and oncogene silencing induced LC migration (76,77).

Even \textit{in vivo} mouse models fail to show a consistent role of LC in tumorigenesis. Prior studies have shown that epicutaneous immunization of DC with tumor material generates protective antitumor responses (78). A more recent report by Stoitzner et al. followed up on that phenomenon \textit{in vivo} and demonstrated that LC played a critical role in the immunization process. Depletion of LC by DT treatment (Lang-DTR mice) at the time of skin immunization dramatically reduced the tumor-protective effect. The topically-applied antigen was presented by skin-derived LC in draining lymph nodes to CD8 T cells. (79). Contrary to these findings, the Girardi group found that in Lang-DTA mice (constitutively lacking LC), the absence of LC has a protective effect on tumor development after two-stage chemical carcinogenesis. This resistance to tumorigenesis of Langerhans cell–deficient mice was independent of the presence or absence of either some or all \(\gamma\delta\) T cells (80).

\textbf{Chemical Carcinogenesis and the Potential Tumor-Promoting Role of LC}

For many years, two-stage chemical carcinogenesis model has been used to study stepwise and sequential analysis of tumor development, with the ability to differentiate between tumor initiation and tumor promotion stages (81). The initiation stage is accomplished by applying a single dose of a carcinogen like DMBA, which is a polyaromatic hydrocarbon (PAH) used to model mutagens present in the environment (pollutants, cigarette smoke, etc). It causes a signature A\(\rightarrow\)T transversion mutation at position 2 of codon 61 of the Harvey-ras
protooncogene that accelerates cellular proliferation and papilloma progression (82,83). More than 94% of papillomas develop this specific mutation after the two-stage chemical carcinogenesis protocol (84). The second, or tumor promotion, stage of chemical carcinogenesis involves applying chemical agents (i.e. TPA) or wounding that leads to sustained inflammation. TPA is a phorbol ester that promotes low-grade inflammation observed in association with human carcinomas. Basal keratinocytes are the key targets of carcinogenesis in this model (85).

Chemical carcinogenesis has been classically associated with Ras proto-oncogene, and UVB carcinogenesis with p53 mutations, but the two processes may be linked by sharing similar downstream regulators. For example, the process of apoptosis (linked to reactive oxygen species, ROS) is shared in both processes and can have a dual role in cancer processes (as discussed later). One report found that, although oncogenic Ras has been demonstrated to suppress p53 by enhancing its degradation via MDM2 in a Raf-dependent manner (86), oncogenic Ras signaling has also been found to contribute to apoptosis. A study by Zhao et al. (2006) has demonstrated that DMBA/TPA application induces apoptosis in mouse skin epidermis and that the apoptotic event was associated with increased p53 expression and mitochondrial translocation (87,88). Later the same group demonstrated the role of Ras in this p53-associated apoptotic event in a mutated Hras cell line after TPA treatment. They found increased apoptosis (measured by cleaved caspase-3) and increased upregulation of p53 (measured by a mitochondrial marker) was
mediated by NOX (NADPH-oxidase) of neutrophils. *In vivo* analysis showed similar finding only after DMBA+TPA, not DMBA-alone treatment. Furthermore, another group, Ryu et al., who were investigating UVB-carcinogenesis found that the NADPH oxidase family protein Nox1 lies downstream of BLT2 (a low-affinity leukotriene B4 receptor) and mediates UVB-induced ROS production and apoptosis (89).

Their strategic location in the epidermis and far reaching dendrites that sample microparticulates from the environment allows for the potential role of LC in tumorigenesis by interacting with self-antigens altered in chemical and UVB processes. Preliminary data (Girardi et al., unpublished) and other reports (90) suggest that LC may be involved in metabolism of mutagenic agents to higher mutagenic material. Similarly, LC may be involved UVB carcinogenesis as they are positioned suprabasally, where they may sense UV damage directly, or indirectly through recognition of apoptotic vesicles and soluble mediators derived from surrounding keratinocytes.

**UVR and UVR-Damaged Keratinocytes: Mutated Clones**

In the UVR carcinogenesis model UVR appears to act as a tumor initiator and as a tumor promoter (91), creating mutated cells and then promoting their expansion by UVB-induced apoptosis (92). UV-radiation causes UVR-specific mutations in keratinocyte DNA, most of which arise from errors in repairing cyclobutane pyrimidine dimers (CPD) (93) that form at cytosine (C) molecules, which are highly mutagenic because of error-prone repair that results in thymine
(T) substitutions. These errors are responsible for much of the mutations within the tumor suppressor gene p53 often found associated with human SCC (94) and are prominently formed by DNA absorption of UVB, and to a lesser extent by absorption of UVA. The cutaneous DNA damage action spectrum indicates that the peak damage bandwidth occurs in the UVB spectrum at 300 nm (95). However, UVA still remains a potential key player in skin cancer as it makes up ~95% terrestrial UVR, can penetrate skin more deeply and has less efficient DNA repair mechanism (93). UVA-induced photodamage effects have principally been attributed to the generation of reactive oxygen species (ROS) and the secondary consequences of lipid peroxidation, protein oxidation and DNA oxidation, particularly of guanosine nucleotides to form the “UVA signature” adduct, 8-oxo-7,8-dihydro-2-deoxyguanosine (8-oxo-dG) (96,97).

UVR-induced tumors have been demonstrated by Kripke and colleagues to be highly immunogenic and are rejected when transplanted into unirradiated syngeneic mice (98-100). On the other hand, when the immune system of host is downregulated by chronic UVB irradiation, it suppresses the rejection of these tumors (99). Many biochemical and molecular factors have been identified as important mediators of photodamage-related immune suppression (101,102), such as trans-urocanic (UCA, present in stratum corneum), DNA, and sunburn cells (apoptotic cells). For example, DNA-damage and UVR-transformed cis-UCA have been shown to be involved in impaired APC function after UV irradiation (103,104).

One way we can study progression of UVR carcinogenesis is by visualizing
microscopic premalignant lesions of mutated p53 and aberrantly apoptosing keratinocytes. Select mutant p53 keratinocytes progress to develop into clusters of keratinocytes filled with p53 with altered conformations. These UVB-induced microscopic lesions of mutated clones have been shown to be correlated with subsequent number of tumors (105). Moreover, studies demonstrate that the p53 mutation profile of these UVB-induced skin patches are very similar to those found in SCC, linking the mutated clonal patches as precursor lesions for SCC (106).

Most precursor lesions regress (‘acute regression’) within 2-3 weeks (107). Working on a Rag1 mouse model, Remenyik et al. found that acute regression of UVB-induced p53 mutant clones is not mediated by antigen-specific immunity (cells such as B, αβT, γδT cell); however natural killer (NK) T cells were still present (108). It could be hypothesized that LC can influence mutated clone progression or regression by directly (e.g. cell-cell interaction), or indirectly (e.g. cytokine production) by regulating apoptosis.

**APOPTOTIC SIGNALS IN KERATINOCYTES**

Classically, apoptosis is viewed as a natural defense against cancer. One of the earliest connections between apoptosis and cancer was the discovery of the role of apoptosis regulator protein Bcl-2 in follicular lymphoma (109,110). Moreover, ultraviolet-B (UVB) induced keratinocyte apoptosis has been viewed as prevention of squamous cell carcinoma, by deleting UVB-induced DNA-damaged mutated cells proliferating out of control (94). P53 has a key role
protective mechanism and consistent with its protective role, mutations in p53 are a common finding in human SCC (111), and p53-knockout mice are highly susceptible to both UVB (112) and chemical-induced SCC (113). However, recently several groups have been reporting a paradoxical role for apoptosis in processes involving carcinogenesis.

Genome instability caused by DNA damage requires checkpoints along the cell’s life cycle, primarily regulated by the “guardian of the genome” tumor suppressor gene p53, which serves a dual role of either arresting the cell cycle at G1/S to allow repair, or inducing apoptosis. Keratinocytes respond to extracellular and intracellular proapoptotic and antiapoptotic/survival signals. Extracellularly, death receptors and their ligands activate the apoptotic pathway, i.e. FasL/FasR, TNFα and TNF-R1 (114). Also, abrogation of cell-cell adherence can elicit apoptosis (115,116). Moreover, intracellular signals (DNA damage, oncogene action, survival factor insufficiency, ROS) can activate the proapoptotic cascade (117). Intracellular signals, mediated by members of BCL-2 family proteins can be proapoptotic (Bax, Bak, Bid, Bim, Puma) or antiapoptotic (Bcl-2, Bcl-XL, BcL-W). Recent reports show that signals mediated by p53 and Bax, act primarily via activation of p38 MAPK (118). Intercellular signaling eventually converges on mitochondria activation and release of cytochrome C, whose ultimate effectors are the intracellular proteases called caspases (119).

Similarly, the survival pathway has several ligand/receptor pairs that confer survival of the cell, i.e. IGF1/IGF2 and IGF-1R, IL-3 and IL-3R (120,121). Other prosurvival factors are FLIP, Bcl-xl and IAP molecules (i.e survivin) (122). Two
cell survival pathways have been characterized. First, the MAPK pathway, triggered by interaction of EGF/EGFR. EGF is known to protect against UVB apoptosis and signaling via EGFR, and activates the MAPK cascade, promoting MEKs and ERKs. The second survival path is also activated by EGR, which involves phosphatidylinositol 3-kinase (PI3K), via activation of Akt. Akt in other cell types inactivates Bad and caspase 9 (123), and in keratinocytes also activates NFκB (124). Akt may promote survival in activating NFκB, by upregulating survival factors such as IAPs (i.e. survivin). In addition to EGF, other growth factors contribute to KC survival. For instance, reports show that keratinocytes are protected against UVB-induced apoptosis by maintaining levels of Bcl-2 and Bcl-XL (125), and activating Akt (126), through nerve growth factor and hepatocyte growth (scatter) factor. Di-Poi et al., 2002 show that transcriptional activation of nuclear peroxisome proliferator-activated receptors in keratinocytes can also activate Akt (127).

**Sunburn Cells**

External perturbations, such as UV exposure, may activate both pro- and antiapoptotic pathways, and the skin’s natural extracellular milieu stimuli determine cell’s fate to either survive or apoptose (110). Interestingly, compared to *in vitro* studies, keratinocytes *in vivo* are less susceptible to UV apoptosis. This could be due to the survival factors present in skin *in situ* (and cell matrix interactions mentioned above), such as laminin 5, which is an extracellular matrix protein that binds to integrins and promotes cell survival via MEK/ERK signaling.
path activation (128).

Although a lot of useful data has been gathered using lines of cultured keratinocytes, the study of apoptosis in sunburn cells in vivo after UVB exposure is certainly more applicable to real life. Sunburn cells are UV-damaged cells with hyperpyknotic nuclei and eosinophilic cytoplasm, which can be stained with TUNNEL (129) or cleaved caspase-3 (130), and require activation of p53 (94), which is thought to act via a transcriptional target galectin-7 (131). In addition to upregulation of p53, reports saw upregulation of Bax and downregulation of Bcl-2 in UV-exposed skin (132). Sunburn cell formation seems to require activation of Fas receptor (133-135), as well as UVB-induced DNA damage (cyclobutane pyrimidine dimers, CPD) (136). Interestingly, in addition to activation of apoptotic signals in response to DNA damage, there is also upregulation of E2F1 (shown to be a survival factor in vivo), to balance the apoptosis (137).

**Protective Role of Apoptosis in Cancer**

Several studies hint at the conflicting role of apoptosis in cancer (110,138). They found that neither forced expression of apoptotic resistance nor deletion of pro-apoptotic molecules in these studies is purely oncogenic. There is ample evidence proving that apoptosis reduces mutations of damaged keratinocytes in both chemical and UVR-induced processes. For example, UVB-induced epidermal apoptosis minimizes accumulation of mutant epidermal keratinocytes by several folds (133,139,140). Li et al. showed development of SCC after UVB in p53 deficient mouse (112). Overexpression of MDM2 in the basal layer of
epidermis induced hyperplasia and premalignant lesions in chemical carcinogenesis in K14-MDM2 mice (141). Bcl-x mice showed enhanced chemically induced tumors, and increased malignant conversion (142). Suzuki and others found that Pten activation accelerated tumor onset in chemical tumorigenesis (143). El-Abaseri et al. showed that EGFR inhibitor treatment reduced UVB tumorigenesis (144,145). In other studies, Stat3 deficient mice were resistant to chemical treatment (146), and HK1.bcl-2 mice had increased susceptibility to chemical treatment (147).

**Conflicting Role Of Apoptosis In Cancer**

Greenhalgh (148) and Kemp (113) found that mice with reduced p53 (+/-) resulted in increased resistance to tumor formation in chemical carcinogenesis, but had an increased rate of malignant conversion. Similarly, Chaturvedi and others reported that RNA interference-mediated reduction of p53 levels in keratinocytes surprisingly enhances UVB-induced apoptosis (149). Dajee et al. report that when they blocked NFκB (a survival factor that is activated in many tumor types) keratinocyte transformation and SCC formation is enhanced (150). Moreover, although Rel/nuclear factor κB transgenic mice have increased keratinocyte apoptosis, they also spontaneously develop SCC (151). Mice deficient in Cox-1 had increased keratinocyte apoptosis, but were associated with reduction in UVB- induced tumors (130).

Data from other studies with models of decreased apoptosis or increased survival resistance shows paradoxically decreased susceptibility to skin
carcinogenesis. Such studies showed reduced numbers of papilloma formation after chemical or UVB exposure, in models of K14-Bcl-2 (152) and K14-Survivin (105,153). Moreover, some of these studies report the incidence of hyperkeratosis in their mice (141,147), while others do not (Bcl-xL transgenic, 142). Allen et al. surprisingly found that tumor formation was less frequent and significantly delayed in K14-survivin mice compared with non-transgenic littermates (153). Moreover, these anti-apoptotic transgenic mice had no papilloma regression compared to controls and more conversion to SCC.

One way to reconsolidate the conflicting roles of apoptosis in tumorigenesis, is to think of apoptosis as a necessary process for lateral expansion by a mutated keratinocytes into nearing epidermal proliferating units (92). Zhang et al. showed clones expand without new mutations (107) indicating that UVB is driving clonal expansion by a physiological mechanism rather than by creating additional mutations. As hypothesized by Brash et al., once a cell is mutated, it may become resistant to apoptosis and may use UVB-induced apoptosis to break boundaries of stem cell compartments (105), expanding without new mutations. This group demonstrated that UV-induced apoptosis drives clonal expansion during skin tumor development in their K14-survivin model (105).

This model predicts that in a mouse with reduced apoptotic capacity more mutated cells will appear initially, but that these cells will expand into clones more slowly than in wild-type animals. Survivin-regulated apoptosis appears to suppress two stages that involve new mutations, initiation and malignant conversion, yet drives clonal expansion of existing p53 mutant cells. In this way,
tumor generation could actually be increased by apoptosis if greater clonal expansion offsets the reduced mutation frequency (105). Although, survivin expression was associated with decreased papilloma formation, there was an enhanced rate of malignant conversion from papilloma to SCC in transgenic animals. However, since it has been shown that transition from papilloma to SCC (malignant conversion) involves new mutations, apoptosis would be expected to exert a negative or protective influence at this stage as well.

**LC AFTER UVB IRRADIATION**

There have been discrepancies in reports describing what happens to LC after UV exposure. Some reports demonstrate that LC migrate to draining LN (103,154,155), while others show that they undergo apoptosis and still others show that neither occurs.

Many of the earlier studies showing LC apoptosis after UVR were done *in vitro* conditions. *In vitro* experiments demonstrated that LC went into apoptosis after UVB irradiation and 2 days of culture (156). Another group found that death of cultured murine LC is augmented by UVB irradiation (157). A population of HLA-DR$^+$ cells migrating from cultured, *ex vivo* irradiated skin explants are shown to be apoptotic by binding of annexin V (158). One study showed by electron microscopy that some human LC become necrotic when irradiated with UVB *in vitro* (159). However, one has to interpret *in vitro* evidence with some caution, as LC *in vivo* may be protected from apoptosis by shielding with several layers, but
also by cell-cell contacts, cell surface molecules, and cytokines that are lacking or different in vitro (160,161).

Kolgen et al. demonstrated in humans in vitro studies that only a few (<3%) epidermal CD1a+ Langerhans cells in skin biopsies or blister roofs undergo apoptosis after UVB exposure as detected by cleaved caspase-3 or TUNNEL (162). They did not see evidence of LC necrosis and found cyclobutane pyrimidine dimer (CPD) positive LC in blister fluid assumed to be en-route to dLN. Okamoto et al. reported in mice that after UV radiation only 1.5 cell per 900 Langerhans cells per mm² were apoptotic, believed to be too few to contribute to the observed Langerhans cell depletion (163). McGee et al. show that neonatal mouse epidermis does not have LC apoptosis or migration, instead hypothesizing that LC depletion occurs from restriction of Langerhans cells populating the epidermis (164).

**LC Role in UVR-Induced Immune Suppression**

UVR has long been known to cause immune suppression, either through cytokines or induction of regulatory immune cells. For example, skin-infiltrating monocytes/macrophages were shown to migrate to draining lymph nodes and produce IL-10 after contact sensitizer exposure to UV-irradiated skin (165). UVR-induced T regs (regulatory T cells), which suppress hapten-mediated CHS, have also been implicated in this process. UVR-T regs differ from natural T regs with regard to their antigen specificity (166) and also from immunosuppressive drugs that cause immunosuppression in a general manner (167). They express CD4
and CD25 (168), the negative regulatory molecule CTLA-4 (169), glucocorticoid-induced tumor necrosis factor receptor (170), and bind the lectin dectin-2 (171). In addition, they secrete IL-10 on hapten-specific stimulation (101,172) and may use the apoptosis-related FasL/FasR-ligand system (62).

Earlier studies by Schwarz et al. demonstrated in Lang-DTR mice that migration of UVR-damaged, but still viable, LC into lymph nodes appears to be essential to induce regulatory T cells (Tregs) (168). The UVR-induced Tregs appear to be critically dependent on the migration of LC into the lymph nodes and the presence of UVR-induced DNA damage in the LC (155). This was confirmed by the observation that IL-12, which reduces UVR-mediated DNA damage (104,173,174), prevents the generation of UVR-Tregs (175,176). Langerin+ cells were still detected in the lymph nodes, but did not contain CPD. These findings gave rise to the hypothesis that the appearance of UVR-damaged but still viable LC in the regional lymph nodes is an essential event during the generation of UVR-Tregs. These authors also proved that prevention of UV radiation-induced immunosuppression by IL-12 is dependent on DNA repair of cis-UCA (155). Cis-UCA was found to inhibit the ability of Langerhans cells to present tumor antigens for primary and secondary tumor immune responses, and showed that IL-12 treatment completely prevented the suppression by cis-UCA (104).

In a recent publication, the Schwarz group paralleled their earlier studies above by demonstrating that Lang-DTR mice are resistant to UVR-induced immunosuppression (177). However, in contrast to their findings and almost
simultaneously, another paper published by the Hogquist group reported that LC are not involved in UVR-induced immunosuppression (178). However, several criticisms or limitations of the latter publications should be noted. For example, role of T regs is unclear in the latter paper as only suppression of the induction of CHS by UVR was studied and adoptive transfer experiments were not performed. Another critique is that epicutaneous sensitization against ovalbumin and transfer of OT-I cells were used as an immunological model. There is much evidence that this system cannot be compared with the hapten-induced CHS employed by Schwarz (179). Moreover, lower UVR doses and lower hapten concentrations were applied, which might account for the differences in the results. In addition, the authors claimed that UVR does not deplete LC, in contrast to the literature (162). This alludes to the fact that UV doses used were too low by Hogquist group, especially since the presence of LC was monitored in epidermal cell suspensions, not in ear sheets.

**LC ROLE IN IMMUNE SUPPRESSION**

Extending the LC paradigm of LC maintaining peripheral tolerance via engulfment of self-particles, one can hypothesize that an impaired cell immunity or even induction of tolerance may contribute to inability to prevent tumor formation. Can LC distinguish between transient formations of neo-self antigens from stable expression of neo-antigens that mark an oncogenic event (93)? UV-induced immunosuppression is mediated via FasL/FasR system, an apoptosis mediator (62,101,133). Similarly, Rossiter et al. made the observation that
induced apoptosis resulted in paradoxical retardation of both UV and chemical carcinogenesis (152) (see section Conflicting Role of Apoptosis in Cancer, above). The direct interplay between LC and apoptotic keratinocytes is not well characterized. However, given above conflicting role of apoptosis in carcinogenesis, the controversial reports of LC inducing immune tolerance using T regs and LC role in antigen-presentation, we can try to reconcile these by hypothesizing that apoptosis can induce immune tolerance and LC are somehow involved. The type of antigen and the nature of the surrounding inflammatory environmental milieu may affect the maturation and function of LC.

LC are strategically positioned just above the germinative layer in the epidermis, where they can extend their dendrites and continually sample signals of skin damage. After UVB exposure, LC have been reported to migrate, and langerin$^+ \text{ CPD}^+$ LC can be detected in draining LN 3 days after a 4-day UVB exposure (103,180). LC may carry DNA-damaged, apoptosed cells and present them to immune system (2,181). A report by Pradhan et al., in a mouse knockout model of pro-apoptotic protein Bid (Bcl-2 family member), showed that the absence of Bid-induced keratinocyte apoptosis abrogates UV-induced immune tolerance (182). Moreover, several reports show evidence that the presence of apoptotic cells during monocyte activation increases their secretion of the anti-inflammatory and immunoregulatory cytokine interleukin 10 (IL-10) and decreases secretion of the pro-inflammatory cytokines tumor necrosis factor (TNF$\alpha$), IL-1 and IL-12 (183,184).

Immature DC can induce T regs (185) and reprocessing of dying DC in LN
by recipient DC could lead to tolerance (186). Skin-derived DC can mediate deletional tolerance of class I-restricted self-reactive T cells (28). The role of LC in immune regulation, such as graft rejection, is still controversial. One group demonstrated that LC are not required for graft rejection (187), finding no difference in the number of Foxp3 (T reg) cells. However they do admit that it's possible that LC may promote the development of T reg populations that do not express Foxp3 or that there may be Foxp3 T regs induced in an antigen-specific manner, but which are too few to affect the total number of Foxp3 cells (188). LC may also primarily affect other cell types, such as plasmacytoid DC or NK (natural killer) T cells, that have recently been reported to exert tolerogenic effects in solid organ transplantation (189). Indeed, another DC subset, plasmacytoid DC, has been recently shown to generate regulatory effects in allografted mice treated with CD40L blockade (189). When bone marrow-derived and epithelial DC are stimulated with factors like human thymic stromal lymphopoietin, IL-10, and tissue growth factor-β, they have been shown to promote tolerance to presented antigens (190,191). Keratinocytes are a major source of both human thymic stromal lymphopoietin and tissue growth factor-β, which raises the possibility that LC may be acting similarly (192).
**STATEMENT OF PURPOSE**

**Specific Aims:**

1. To investigate the influence of Langerhans cells on the epidermal proliferative response to DMBA (mutagen) and/or TPA (tumor promoter).
2. To investigate the influence Langerhans cells on the epidermal proliferative response to **acute** and **chronic** ultraviolet B (UVB) light exposure.

**Specific Hypotheses:**

1A: There is decreased keratinocyte proliferation in LC-deficient mice in response to DMBA+TPA, TPA alone and DMBA alone.

1B: There is decreased epidermal hypertrophy (minimal epidermal thickness) in LC-deficient mice to DMBA+TPA, TPA alone and DMBA alone.

2A: There is increased epidermal apoptosis in LC-deficient mice in **acute** UVB-exposure, as measured by cleaved caspase-3 and sunburn cells.

2B: There is a decreased number of mutated islands (mutant keratinocytes over-expressing p53) in LC-deficient mice after **chronic** UVB-exposure, as measured by CM5 antibody by immunohistochemical and immunofluorescent staining.

2C: The area of mutant clones (clusters of mutant keratinocytes over-expressing p53) is decreased in LC-deficient mice in **chronic** UVB-exposure as measured by CM5 antibody by immunohistochemical and immunofluorescent staining.

2D: LC or other langerin+ DC will be present after **acute** and **chronic** UVB exposure.
**MATERIALS AND METHODS:** All experiments were performed primarily by first author, unless otherwise specified.

**Mice**

Transgenic mice that are constitutively depleted of LC (Lang-DTA) and those in which LC ablation is inducible by DT (Lang-DTR) were generously donated by Dan Kaplan (Minnesota, USA); the generation of these mice is described elsewhere (Kaplan et al. 2009). A dose of 400ng of diphtheria toxin (DT) was injected at 400nm/100uL intraperitoneally. All littermates were genotyped for transgene by PCR (by Renata Filler) and used at 7-9 weeks of age. Animal procedures were approved by the Institutional Animal Care and Use Committees at Yale University.

**Chemical carcinogenesis**

For all mouse skin, at seven weeks of age mouse dorsal hair was first clipped (electric shaver), then treated with Nair (2-3 min), then washed off with water, and covered with thin layer of petroleum jelly). A week later, a single dose of DMBA (400nm/100uL, with 200uL tip) was applied and after 3 days we applied TPA (twice-weekly Mon, Thurs, 20nm/100uL, with 200uL tip). Chemical application was aimed at comparable location and area in all mice. During the following weeks (4-5wks) dorsal skin of mice was shaved with clippers prior to each repeated application of TPA. Treated (dorsal) skin was harvested one week after the last TPA application, using a ruler to identify comparable areas and
locations on the body. Skin strips were then either cryofrozen (using upside down can of duster), or skin was formalin-fixed and paraffin embedded (latter part was done by Yale Dermatopatology). For “DMBA only” studies, mice (DOB 5-10-09) were treated with DMBA high dose (400 nmoles) on 07/06/09 and thereafter once every 2 wks (total about 8 times).

*Immunohistochemical Ki67 Staining of Paraffin Embedded or Frozen Sections*

Cryosections were cut onto glass slides by author. Formalin fixed paraffin embedded (FFPE) sections were cut by Dermatopatology. FFPE slides were either baked for 20 min at 85°C or for 1 hr at 65°C, after which they were deparaffinized by two washes of 5 min each in xylene, 100, 95, 70% ethanol and water, citrate buffer in that order. Incubated slides in 3% hydrogen peroxide for 10min (use 500 mL 30%H2O2 in 4.5 mL dH2O, or 250 mL 30%H2O2 in 2.25mL dH2O) and made new dilution every time. Heat induced antigen retrieval (HIAR) was performed on FFPE (not cryosections) by steaming in preheated sodium citrate buffer (pH6) for 10 min, cooling for 30 min and washing in dH2O three times for 5 min. [Another method is boiling slides. Place slides in heated sodium citrate buffer between 40 and 60°C. Once temperature reaches 95°C, heat for 20min between 95-98°C (letting reach 98 a couple times). Then cool to 35-40°C on benchtop]. Wash for 3min in PBS two times. Apply primary antibody to cover tissue, incubate for 30min at room temp on benchtop. [SP6 monoclonal Ki-67 antibody stopped working, so moved on to polyclonal, RB-1510-R7, Thermo Fisher Scientific]. Wash for 5 min in PBS, x2. Applied secondary antibody in
PBS, and incubated for 30min at room temp on benchtop. Mixed 5mL antibody (biotinylated anti-rabbit IgG, made in goat by Neomarkers), 1 mL PBS, 25 mL goat serum (as blocking agent by Vector labs). If using ABC reagent (instead of Strepavidin-HRP), be ready to prepare it 30 min ahead of applying it in the next steps (1 drop A, 1 drop B, in 2.5mL PBS). Washed in PBS two times for 5min. Applied ABC reagent and incubated for 30min at room temp. Washed two times in PBS for 5min. Applied 200-300mL DAB until pigment fully developed, which is anywhere between 1-8min (1 drop buffer, 2 drops DAB, 1 drop H2O2, all into 2.5mL HPLC water, mix well at each step). When pigment developed, quenched by washing in distilled water, prepared in Coplin jar beforehand. Counterstain with Haematoxylin and Bluing reagent. Wash gently three times with dH2O in between and after. Place slides into Coplin jar with dH2O. Dehydrate slides through xylene (30sec dH2O x2, 70% EtOH x2, 100%EtOH x2, xylene x2). Air-dry in hood, coverslip with Permount (avoid bubbles), let dry flat overnight.

**Immunofluorescence staining for p53/CM5, langerin/CD207, GL3 and cleaved caspase-3**

On staining day one (EDTA lysis, fixation, primary) harvested skin, scraped off subcutaneous fat, and cut skin into strips. Incubated for lysis (2hrs) on dermal side in EDTA buffer (pH 7.3) at 37°C in CO2 chamber. Separated epidermis and dermis to obtain the epidermal sheets by scraping off the complete epidermis with the forceps on a plastic plate, while holding and detaching the dermis with forceps. Washed sheets 3 times in PBST (phosphate buffered saline with Tween)
for 15 min each. Next, fixed sheets by placing sheets as flat as possible on cover slips and fix by immersing in cold acetone for 20 min, on ice in the hood. Washed two times in PBS for 10 min. Then, after positioning sheets basal side DOWN, blocked (2% donkey or rabbit serum in 2% BSA) at room temperature on slides. (Note: To position sheets, as you bring microscope stage toward lens, should see stratum corneum first, then hair bulbs. Also, sheets tend to curl towards basal side, but corneum side does tend to stick to itself). If many samples, keep sheets on slides in 2% BSA to keep from sticking to slide, in covered chamber, until last sheet positioned. Pipetted primary antibody on sheets. Primary antibodies were rabbit CM5 (1:2000), rat langerin (1:200), hamster GL3 (1:100) and rabbit cleaved caspase-3 (1:200, Asp175, Cell Signaling Tech, #9661) and mixed at appropriate concentrations cocktail (in 2% BSA) and pipetted under sheets that are basal side DOWN on slides (used hydrophobic pen for borders). Incubated overnight at 4°C.

On staining day two, sheets were washed in 15 mL conicals 2-3 times for 30 min in PBST (PBS with 0.05% Tween-20), on end-to-end rotator. Sheets were then positioned basal side DOWN again (as described above) and pipetted in secondary cocktail. Secondary antibodies were FITC-Donkey-anti-Rabbit (1:500, JacksonImmunoResearch # 711-016-152), Cy5-Donkey-anti-Rat (1:300, JacksonImmunoResearch # 712-175-153), and goat Cy5-anti-hamster, 1:200) and were mixed at appropriate concentrations under sheets positioned basal side DOWN, and incubated for 1 hr and 15 min at room temperature. Washed with 2-3 washes of 30 min, as before in PBST. Positioned sheets basal side DOWN again,
as described above and pipetted in tertiary antibody (1:500, anti-FITC Alexa 488), which was incubated for 1hr at room temperature. Washed off tertiary antibody, as before. Take out ProLongGold to thaw (at least 20 min), during the washes. Mount and coverslip epidermal sheets basal side UP, (in ProLongGold) on slides. (Note: Let edge of coverslip touch the mounting media, and let it fall; don't press too hard to get rid of bubbles.) Tacked with nail polish on all four edges and leave at room temperature in dark slide holder for 24 hrs, before viewing on confocal, to allowed to cure. Before handling slides, sealed all sides with nail polish (to prevent coverslip movement) and kept at -20°C. Pictures were obtained on Confocal (Zeiss, TAC LCM510 meta) or Dr. McNiff’s scope (Dermatopathology, 5th floor) and statistics performed in Prism software.

*Immunochemistry staining of cleaved caspase-3 of Formalin Fixed Paraffin Embedded (FFPE) sections*

On staining day one, incubated sections in 3% hydrogen peroxide for 15 minutes and wash sections in dH2O twice for 5 minutes each. Washed section in wash buffer for 5 minutes. Drew boundaries around samples on slides, with hydrophobic pen. Blocked each section with blocking solution for 1 hr at room temperature. Primary antibody rabbit cleaved caspase-3 (Asp175, Cell Signaling Tech, #9661) diluted in recommended antibody diluent. Incubated overnight at 4°C, with covered plastic containers.

On staining day two, after removing antibody solution, washed in wash buffer three times for 5 minutes each. Applied secondary antibody (biotinylated goat
anti-rabbit, diluted in TBST) to each section. Incubated 30 minutes at room temperature. [If using ABC avidin/biotin method, prepare ABC reagent according to the manufacturer’s instructions and incubate solution for 30 minutes at room temperature.] Remove secondary antibody solution and wash sections three times with wash buffer for 5 minutes each. For the immunoperoxidase reaction, added 100-400 µl ABC reagent (or Streptavidin-HRP) to each section and incubate for 30 minutes at room temperature. Removed ABC reagent (or Streptavidin-HRP) and washed sections three times in wash buffer for 5 minutes each. For chromogen reaction, added 100-400 µl DAB to each section and monitor staining closely. Quenched as soon as the sections develop, by immersing slides in dH₂O. Counterstained sections using Hematoxylin and bluing reagent. Washed sections in dH₂O two times for 5 minutes each. Then, dehydrated sections by incubating sections in 70%, 95%, 100% ethanol and xylene, two times for 30 seconds each. Mounted with toluene-based mounting medium and coversliped (manually or using automated machine).

Quantification of stained cells by immunohistochemistry and immunofluorescence

Counting of immunohistochemically stained cells was done by a blinded observer, by identification of brown stained cells of Ki67 (RM-9106-R7, ThermoFisher) or Cleaved caspase-3 (Asp175, Cell Signaling). Positively staining cells were quantified along a linear 250-µm grid at 400x high power field on Olympus compound microscope (Dr. Bosenberg’s lab, 2nd floor). For
immunofluorescent staining, mutant p53 clones were visualized as green clusters (NCL-p53-CM5p; secondary FITC conjugated donkey anti-rabbit, followed by Alexa488 conjugated anti-FITC). Most p53 mutations increase P53 protein stability and allow antibody detection (ref 13,24). Langerhans cells (LC) or langerin+ DC were visualized as blue cells (eBioRMUL.2; secondary Cy5 conjugated donkey anti-rat). Zeiss confocal microscope (TAC LSM510 meta) was used to capture images, and ImageJ64 for analysis of islands.

**UVB-irradiation in Acute and Chronic UVB Experiments**

The UVB irradiation chamber was generously donated by Dr. Brash and contained a bank of four UVB lamps (FS20T12- UVB; National Biological Corp., Twinsburg, OH) that emit wavelengths between 250 and 420 nm (72.6% UVB, 27.4% UVA, 0.01% UVC), with peak emission at 313 nm, according to the manufacturer. A filter (Kodacel TA422; Eastman Kodak, Rochester, NY) was placed over the cages to block residual UVC. There was a wire mesh to keep mice from standing up. Dosimetry was determined using a calibrated UVB-500C meter (National Biological Corp.). The irradiation rate was ~2.8 J/m²/s. Of note, there is a possibility that UV dose was higher than measured at beginning of experiments. After an acute exposure at the beginning of my time in the laboratory the UVB dose measurement was 2.8 J/m²/s. However, upon repeating the same experiment at the end of my time in the laboratory the dose was 4.2 J/m²/s. Reports of dose measurements checked by other members of the lab
during the year was found to be higher. These inconsistencies may be due to problems with UV box ventilation or leaving the bulbs on for a long time.

For acute UVB experiments, mice were clipped and naired as specified above and one week later were exposed unrestrained to a single acute dose of 3,360 J/m$^2$ UVB applied to dorsal skin, which was harvested and paraffin-embedded 24 hrs later. Similarly, for chronic UVB experiments, mice were clipped and naired as above and chronic UVB (5wk, 9wk) was applied (Monday, Wednesday and Friday) starting at 500 J/m$^2$ then gradually increased to 1,250 J/m$^2$ by week 3. This gradual increase in exposure produced minimal erythema and was not associated with scaling or ulceration. Three days after the last irradiation (to allow sufficient time for wild-type p53 up-regulated by UVB to return to baseline levels, ref Zhang et al.), epidermal sheets were prepared from multiple strips of dorsal skin as described above.

For acute UVB2 experiment actual mouse numbers were (3 Tg/Lang-DTA and 3 WT) were Tg# 19, 20, 21. For Acute UVB2 experiment the actual mouse numbers and dates of birth (7 Tg/Lang-DTA & 7NLC) were Tg#165, 136 (DOB1/15), 143, 145, 148, 152 (DOB 1/12), 157 (DOB1/13), and NLC#137, 138 (DOB 1/15), 144, 146, 147, 153 (DOB 1/12), 158 (DOB 1/13).

9 weeks Chronic UVB1, Method 1: (Lang-DTA n=3, NLC n=3)

After 9 weeks of chronic UVB exposure mouse epidermal sheets were harvested and stained as described above, and images were captured taken on Confocal (Zeiss, TAC LSM510 meta) microscope only from middle strips from dorsal skin.
Measured out, and marked 3 cm on each strip and moved along every 3000 µm, for 10 high power fields (each HPF at 25x magnification is 0.1296 mm²). Scanned at 25x, then zoomed into each clonal island that touched the HPF. For clone size, manually counted every bright cell per clone middle strip, for all samples except 3, 26, 32. Total area covered per mouse was 1.296 mm² (0.1296 mm² times 10 HPFs). **Actual mouse numbers were Lang-DTA # 5, 31, 14 and NLC # 4, 27, 28.**

**9 weeks Chronic UVB1, Method 2: (Lang-DTA n=4, NLC n=4)**

Pictures taken on Confocal (Zeiss TAC LSM510 meta) microscope only from middle strips from dorsal skin. Collecting data from sheets involved scanning 3 big areas (1.8 mm x 1.8 mm, so 3.38 (actually 3.24) mm² per area), by taking 25 HPFs (5 by 5 at 25x magnification). Area per 25x magnification is approximately 0.14 (actually 0.1296) mm². Total area covered per mouse was 9.72 mm² (3 times each area of 3.24 mm²). Actual mouse numbers were Lang-DTA # 3, 5, 31, 14 and NLC # 32, 4, 27, 28.

**5 weeks Chronic UVB2, Method 3: (Lang-DTA n=8, NLC n=7)**

Pictures taken using fluorescent scope in Dermatopathology department (Dr. Jen McNiff), to count the number of clones at 20x or per 0.9 mm² (diameter = 1.07 mm, radius = 0.535 mm, thus Area = πr² = 0.9 mm²). Approximately 70 HPFs were counted per mouse at 20x, which equals about 63 mm² in area examined per mouse. ImageJ64 was used to analyze clone size by outlining each clone and measuring in µm², which was then converted (multiplying by 10⁶) to mm² for
data in graphs. Number of LC in Lang-DTA versus NLC mice after chronic UVB2 were analyzed for mouse numbers (n=4) for Lang-DTA (#5, 10, 12, 13) and for NLC (#1, 2, 3, 6), by scanning three rows of 10 HPFs at 25x, for a total of 30 HPFs (each HPF area= 0.14mm²) on Zeiss confocal microscope. Also, quantified the number of GL3 positive DC per HPF for Lang-DTA mice (#’s 5, 10, 12, 13 and 15). The actual mouse number and dates of birth were Lang-DTA#406, 407 (DOB 1/14), 417, 418, 442, 443, 444, 445 (DOB 1/17) & NLC#199, 200, 404, 405 (DOB 1/14), 409, 410 (DOB 1/14), 416 (DOB 1/17).

**LC Associated with Mutated Clones in Lang-DTA and NLC mouse skin after 5 weeks of Chronic UVB2.**

Data was gathered from mice from 5 weeks of chronic UVB2. Each HPF had two 3-dimentional layers from top of epidermis to bottom, at set distance. The number of langerin+ DC were counted using ImageJ64 software. Badri Modi assisted in counting using ImageJ64 and analyzing data in excel file. Designated mouse numbers were Lang-DTA # 5, 9, 10, 12, 13, 14, 15 and for NLC # 1, 3, 6, 7, 8, 11. Using Zeiss microscope (TAC LSM510 meta), at 25x (HPF area =0.14 (actually 0.1296) mm²) scanned middle edge of skin strip for clones and areas without clones for 10 HPFs. Tried to get a range for clone sizes (20-300 μm in diameter). Initially, scanned 10 HPFs with clones, and another set of data with 10 HPFs scanned without clones (data not shown). However, since it was difficult to find areas without clones near edge (closer to middle of mouse back) there may have been bias with selecting HPF without clones closer to lateral side of mouse
(where more LC may be present sue to less UVB exposure). To avoid the bias, we used the HPFs only with clones (~10 HPFs per mouse) and compared the number of langerin+ DC within or near (3 cells) to clone versus number of langerin+ DC outside the clones in that HPF. For NLC however, scanned images only with mutant clones (30 HPFs total per mouse).

5 weeks Chronic UVB3 and 3 Weeks Regression Method 4: (Lang-DTR n=7, NLC n=7).

Mice were chronically irradiated for 5 weeks, followed by 3 weeks for regression (no UVB exposure) and with DT injection at weeks 6, 7 and 8. Two strips of dorsal skin (right and left) were harvested per mouse. Images of mutant clones per HPF at 20x magnification (each HPF area= 0.9mm$^2$) were taken using fluorescent scope in Dermatopathology department (Dr. Jen McNiff). ImageJ64 was used to measure areas per clones in $\mu$m$^2$, which was then converted (multiplying by $10^6$) to mm$^2$ for data in graphs. Covered approximately 1245 HPFs at 20x for Lang-DTR and NLC, respectively, and thus about 11.2 cm$^2$ in total per group. Clones that were more than 3 cells apart and with different morphology were counted as separate clones.

Cell counting for IHC stained sections (Ki-67, Cleaved caspase-3) and H&E:

All counting was done on Olympus compound microscope at 400x magnification. Each high power field (HPF) was 250 um and was chosen randomly by a blind observer. For Ki-67 (n=2), each slide contained a roll of skin in which the number
of positive cells in the epidermis were counted by sampling 12 HPFs per mouse along the 250 µm ruler. For the pilot experiment for acute UVB cleaved caspase-3 staining and sunburn cell counting was done on the middle strips of dorsal skin was used and 10-30 HPFs were randomly counted. For the repeated acute UVB experiment, cleaved caspase-3+ cells were counted from slides with two strips per mouse collected from the middle of dorsal skin exposed to acute UVB dose (3,360J/m²), along a 250-µm ruler, by randomly sampling 25 HPFs in each strip, from middle of each strip, for a total of 50 HPFs per mouse. Sunburn cells were identified as cells with: (i) pyknotic nuclei and/or (ii) eosinophilic cytoplasm, or cells without nucleus with eosinophilic cytoplasm.

Statistics
For all cell and clone analysis, data derived from multiple animals was subjected to unpaired, two-tailed Student’s t-tests using Prism GraphPad software (Mac OSX version). Analysis of p53 mutant clones was performed using ImageJ64 (National Institute of Health, USA). For analyses of differences in clone density within predetermined size ranges (bins), the assumption was made that the random observed number of clones of a given size should follow a Poisson distribution, with mean proportional to the total area. P values of ≤0.05 were considered statistically significant. Mean values with ± SEM were reported.
RESULTS

CHEMICAL CARCINOGENESIS EXPERIMENTS:

DMBA + TPA, TPA ALONE AND DMBA ALONE

DMBA + TPA: After two-stage chemical carcinogenesis (DMBA + TPA), there was significantly smaller increase in epidermal proliferation and epidermal thickness in Lang-DTA mice vs NLC mice.

At baseline, LC-intact (NLC) and LC-deficient (Lang-DTA) mouse skin showed comparable proliferation levels (Figure 1a) as measured by Ki-67 immunohistochemistry, IHC (13.6 ± 2.6 vs 12.7 ± 2.8 #Ki-67+ cells, P=NS) and comparable minimal epidermal thickness (7.9±1.8 vs 6.9±2.8 µm, P=NS, Figure 1b). After a single application of mutagen DMBA, followed by repeated applications of tumor promoter TPA (x 5.5 wks), LC-depleted skin showed 23% smaller increase in epidermal proliferation (31.5 ± 7.7 vs 25.7 ± 7.4 # Ki-67+ cells, $P=0.007$) and minimal epidermal thickness (59.2 ± 28.4 vs 25.8 ± 8.9 µm, $P<0.0001$), as compared to LC-deficient skin. Minimal epidermal thickness was measured from basal to granular layer of the epidermis.

TPA alone: After 4 weeks of TPA application, there was significantly smaller increase in epidermal proliferation and epidermal thickness in Lang-DTA mice vs NLC mice.

After repeated applications of tumor promoter TPA (x 4 wks), LC-depleted (Lang-DTA) skin showed a smaller increase in epidermal proliferation (16.7 ± 0.4 vs 19.3 ±0.5 # Ki-67+cells, $P=0.0002$, Figure 3a) and minimal epidermal
thickness (5.1 ± 0.1 vs 8.5 ± 0.2 µm, **P<0.0001**, *Figure 3c*), as compared to NLC mice. Statistical significance was not reached for means per mouse for epidermal proliferation (P= NS, 0.3982, *Figure 3b*), but was reached for epidermal hypertrophy as (**P=0.0037**, *Figure 3d*).

**DMBA alone: After 8 times of mutagen (DMBA alone) treatment every 2 weeks, there was a significantly smaller increase in epidermal proliferation and epidermal thickness in Lang-DTA mice vs NLC mice.**

After repeated applications of mutagen DMBA alone, Lang-DTA skin showed a smaller increase in epidermal proliferation (12.8 ± 0.99 vs 22.7 ± 1.4 # Ki-67+cells, **P=0.0001**, *Figure 4a*) and minimal epidermal thickness (14.4 ± 1.08 vs 17.8 ± 1.0 µm, **P<0.0292**, *Figure 4c*), as compared to NLC skin. *Figure 4b and 4d* show corresponding data per mouse.
RESULTS FOR ACUTE UVB EXPERIMENTS

Acute UVB (formalin-fixed epidermal sections): After a single dose of UVB (3,360 J/m²), there was a significantly smaller number of apoptotic cells (cleaved caspase-3 and sunburn cells) in the epidermis of Lang-DTA versus NLC mice.

A pilot experiment (n=3) showed LC-deficient skin (Lang-DTA), as compared to skin of LC-intact normal littermate controls (NLC), to have less epidermal apoptosis after acute UVB irradiation, as measured by IHC on formalin-fixed sections (21.4 ± 1.1 vs 26.4 ± 1.4 # cleaved caspase-3+ cells, \( P=0.0099 \), Figure 5) and on H&E (9.3 ± 0.5 vs 13.9 ± 1.5 # sunburn cells, \( P=0.0069 \), Figure 6a). Statistical significance was not reached for the means per mouse, for either cleaved caspase-3 or sunburn cells (22.57 ± 3.548 vs 28.26 ± 5.441 # cleaved caspase-3+ cells, P=NS, 0.4303, Figure 5b) or sunburn cells (9.2 ± 1.5 vs 14.4 ± 3.8, # sunburn cells, P= NS, 0.2678, Figure 6b), as well as in the combined means per mouse data (n=10) for both pilot and repeated experiment (19.77 ± 1.7 vs 22.7 ± 2.1 # cleaved caspase-3+ cells, P= NS, 0.2877, Figure 8b).

Figure 8a contains combined apoptosis data from pilot acute UVB experiment (n=3) and repeated (n=7) experiment measuring cleaved caspase-3 for Lang-DTA vs NLC irradiated skin on formalin-fixed sections. There was a statistically significant difference in epidermal apoptosis (18.9 ± 0.4 vs 21.1 ± 0.4 # cleaved caspase-3+ cells, \( P< 0.0001 \)). At baseline (Figure 8a), non-irradiated
anterior belly skin showed no difference in apoptosis in Lang-DTA vs NLC skin (0.01 ± 0.0 vs 0.01 ± 0.0 # cleaved caspase-3+ cells, P= NS, 0.3692).

*Figure 7a* shows cleaved caspase-3 data for the first (pilot) experiment (n=3) per each strip 1-3, where strip 2 corresponds to the middle dorsal treated skin, and strips 1 and 3 are side strips. Of note, previously shown data in *Figures 5 and 6* are from middle strips (i.e. strip 2 from each mouse). *Figure 7b* contains all the cleaved caspase-3 data per mouse for both old and repeated experiments.

**Acute UVB (epidermal sheets): After a single dose of UVB (3,360 J/m²), epidermal sheets were co-stained with cleaved caspase-3 and langerin (CD207) by immunofluorescence in Lang-DTR and NLC mice.**

Experiment ‘Acute UVB Sheets1’ found that NLC mouse epidermal sheets show several cells double-stained with cleaved caspase-3 (green) and langerin/CD207 (blue) at 25x magnification in *Figure 9* for NLC and in *Figure 11* for Lang-DTA, and at 63x magnification in *Figure 10* for NLC and in *Figure 12* for Lang-DTA mice.

A follow-up experiment ‘Acute UVB Sheets2’ similarly revealed some co-staining of langerin and cleaved caspase-3 positive cells. Plus, anterior belly skin (unexposed to UVB) was also found to be stained for the apoptotic marker in both Lang-DTA and NLC mice (*Figures 13 a and 13b*). In this follow up experiment ‘Acute UVB Sheets2’ there was a lot of heterogeneity in number of langerin+ cells in both Lang-DTA and NLC. For example, sometimes there was an unusually high number of langerin+ cells in Lang-DTR group, as in *Figure 13b*
(panels 4-6), which may be due to inadequate depletion by DT. On the other hand, sometime there was an unusually low number of langerin+ cells in NLC as in *Figure 13a* (panels 4-6), which could be due to random location on skin. A relatively small area of skin was examined and a repeat experiment needs to be conducted. IgG control for cleaved caspase-3 was not completely clean (see *Figure 14c*, panel 1). IgG control for langerin/CD207 was clean (see *Figure 14c*, panel 2).
RESULTS FOR CHRONIC UVB EXPERIMENTS

3 Weeks (Chronic UVB0) and 9 weeks (Chronic UVB1): Mutant Clones and LC in Skin of Wildtype Mice.

After chronic UVB exposure, NLC mouse skin contains p53-mutant clones and viable (with long dendrites) langerin+ DC by immunohistochemistry (langerin/CD207). These cells also stain for MHC-class II by immunofluorescence (data not shown). Figure 15a shows p-53 mutant clones (CM5, IHC epidermal sheets, brown) after 3 weeks of chronic UVB. Figure 15b shows a larger p53-mutant clone after 5 weeks (courtesy of Wengeng Zhang). Figure 15c shows langerin+ DC (CD 207, IHC epidermal sheet, brown) after 3 weeks of chronic UVB. Our data show that clones increase in number and size with increased time of UVB exposure (see Figure 21). We have also observed variation in morphology of mutant clonal cells (see Figure 28).

Immunofluorescence staining after 9 weeks of chronic UVB revealed large mutant clones surrounded by and permeated by langerin+ DC, as seen in Figure 16a. Velocity software analysis of captured stacks of two-dimensional slices from epidermis to dermis, allows viewing these cells in three-dimensions (see Figure 16b).
After chronic UVB exposure (3x/wk, increasing dose from 750 to 1250J/m² for 9 and 5 weeks) dorsal skin of Lang-DTA mice revealed a smaller number of clones of mutated p53 keratinocytes compared to NLC mouse skin.

9 weeks (Chronic UVB1): Number of Clones in Lang-DTA vs NLC

Method 1 of collecting data involved taking 10 HPF’s at 25x for each mouse (n=3 per group), covering approximately 1.296 mm² per mouse. After 9 weeks of chronic UVB exposure, Lang-DTA mouse skin compared to NLC had a smaller number of mutated clones per mm² (8.0 ± 1.1 vs 15.4 ± 2.3, \(P=0.004\), Figure 17a). Statistical significance was not reached for means per mouse (10.7 ± 2.2 vs 16.9 ± 4.2, \(P=\) NS, 0.2636, Figure 17c). Figure 17b shows data per HPF per mouse.

Method 2 of collecting data involved taking 3 larger areas (3.24 mm² each) for each mouse (n=4 per group), covering 9.72 mm² per mouse. After 9 weeks of chronic UVB exposure, there was a trend toward Lang-DTA mouse skin having a smaller number of mutated clones per mm² compared to NLC (1.7 ± 0.3 vs 2.5 ± 0.5 # mutated clones/mm², \(P=\) NS, 0.1636, Figure 18a), which did not reach statistical significance. Means per mouse also did not reach statistical significance (1.7 ± 0.3 vs 2.4 ± 0.7 mean # mutated islands/mm², \(P=\) NS, 0.3528, Figure 18b).
There is a discrepancy in the number of clones per mm\(^2\) in Method 1 versus Method 2 at 9 weeks of chronic UVB, with the former being approximately 5-6 times greater (represented visually in Figure 21a and 21b). There are two possible explanations for this. One: in Method 1, each clone that touched the edge of the HPF at 25x was zoomed into and also counted, even if it was outside the HPF, but the area was not accounted for. Second: the Method 2 of counting clones may have missed some, since the bigger area (3.24 mm\(^2\) vs 0.1296 mm\(^2\)) was captured in lesser resolution.

**5 weeks (Chronic UVB2): Number of Clones in Lang-DTA vs NLC**

Method 3: After 5 weeks of chronic UVB exposure, Lang-DTA mouse skin had a smaller number of mutated clones per mm\(^2\) compared to NLC (0.9 ± 0.05 vs 1.0 ± 0.1 # mutant clones/mm\(^2\), \(P=0.0489\), Figure 19a). Statistical significance was not reached for means per mouse (0.9 ± 0.1 vs 1.1 ± 0.2, \(P=0.3797\), Figure 19b).

**5 weeks UVB and 3 weeks regression (Chronic UVB3): Number of Clones in Lang-DTR vs NLC**

Method 4: After 5 weeks of chronic UVB followed by 3 weeks of no UVB (and DT injection at weeks 6, 7 and 8), there was a smaller number of mutant clones per mm\(^2\) in Lang-DTA mouse skin versus NLC (0.2 ± 0.0 vs 0.4 ± 0.0, \(P<0.0001\), Figure 20a). Statistical significance was also reached for mean number
of mutated clones/mm$^2$ in Lang-DTA vs NLC per mouse (0.2 ± 0.0 vs 0.4 ± 0.0, $P=0.0106$, Figure 20b).

**Area of Mutant Clones**

Mutant clone size was not statistically different in LC-deficient versus NLC group. However, there seemed to be an overall skew toward smaller area in Lang-DTA mice versus NLC after 9 weeks (34.2 ± 7.4 vs 46.3 ± 9.2 #cells/clone, $P=\text{NS}, 0.3375$, Figure 22a) and 5 weeks of UVB (0.041± 0.00 vs 0.047 ± 0.01 mm$^2$, $P=\text{NS}, 0.2695$, Figure 22b). Interestingly, the general trend almost reversed after mice had 3 weeks time to regress their clones following the 5 weeks of UVB (0.034 ± 0.00 vs 0.031 ± 0.00, $P=\text{NS}, 0.5590$, Figure 22c ) and Lang-DTA mice seemed to have more larger clone sizes (not statistically significant).

Arranging clone areas into bins, as done by Zhang et al 2005 (ref) in Figure 24, whose authors organized area bins by 1-16, 17-32, 33-48, 49 -160, 161-499, with 16 being the number of cells for the fundamental unit of EPU (epidermal proliferating unit). Zhang et al. found that their transgenic mouse model (K14-survivin mice) that was resistant to apoptosis had more clones in the smaller areas ranges (1-16 cells). A similar trend is seen in our Lang-DTA mice (preliminary data showing decreased apoptosis). Possibly, this is due to DNA-damaged cells not being ablated by decreased apoptosis early in the UVB process, leaving behind many more mutant clones as compared to NLC mice.

Thus, in all three of our experiments (9 weeks, 5 weeks, and 5 weeks+ 3 weeks no UVB) there seems to be a slight trend toward a greater number of
smaller clones (<16 cells) in Lang-DTA and Lang-DTR mice. The 9 week experiment is most consistent with Zhang et al. results, perhaps because clone areas were counted manually and also mice were irradiated for longest time.

In experiments that we used to measure area by outlining the clones, the bin with 33-48 cells per clones has a trend toward Lang-DTA mice having greater area per clone versus NLC, as seen in Method 3 at 5 weeks (0.038 ± 0.01 vs 0.011 ± 0.01, \( P=0.0601 \), Figure 23b) and in Method 4 at 5 weeks UVB and 3 week regression (0.0989 ± 0.02 vs 0.0736 ± 0.01, \( P= NS, 0.3038 \), Figure 23c).

Zhang et al. manually counted the cells in each clone, stained by immunohistochemistry. For Method 1, we also counted cells manually. However, for further experiments, it was not feasible for us to count each cell in clones in real-time using a confocal microscope, so instead we captured images by a confocal microscope and measured the area by outlining the clones using ImageJ64 software. Then, the area was converted to cells per clone by dividing by 80 \( \mu \text{m}^2 \), which was the average area per cell accounting for intercellular surrounding space. The fact that we don’t see the same distribution as in Zhang et al. may be due to the fact that the area to cell conversion is not perfect, as each cell varies in size and morphology, as well as spatial arrangement within the clone (see section on Mutant Clone Morphology, below).
**LC Number in Lang-DTA vs NLC after 5 weeks of chronic UVB of dorsal skin:**

After 5 weeks of chronic UVB, Lang-DTA mice have approximately 12 times less ‘replacement’ langerin+ DC compared to NLC mouse skin (23 ± 0.46 vs 278 ± 17 # langerin+ cells/mm$^2$, $P<0.0001$, Figure 26a).

**LC Density Associated with Mutant Clones in Lang-DTA and NLC after 5 weeks of chronic UVB of dorsal skin:**

After 5 weeks of chronic UVB, ‘replacement’ LC (see Figure 25) are associated with mutant clones in Lang-DTA mice as compared to areas without clones (61.0 ± 9.1 vs 19.7 ± 2.7 # langerin+ cells/mm$^2$, $P<0.0001$, Figure 27a). Statistical significance was also reached for mean density per mouse ($P=0.0106$, Figure 27b).

A similar association was seen in NLC mice. After 5 weeks of chronic UVB, LC were associated with mutant clones in NLC mouse skin as compared to areas without clones (242.4 ± 21.4 vs 163.8 ± 15.3 LC density (mm$^2$), $P=0.0047$, Figure 27c). Mean density per mouse missed statistical significance ($P=0.1848$, Figure 27d).

**Mutant Clone Morphology: Epidermal proliferating units (EPUs) expand into clonal islands.** Mutant clones are made up of p53-mutant keratinocytes that fill up the epidermal proliferating units (EPUs) as ~16 cell units, which are hexagonal in shape (Figure 28 a-c). These mutant EPUs coalesce into larger
units, around the hair follicles (Figure 28 d-e). Moreover, these clones are made of keratinocyte cells of different sizes, from small (as seen in clone on bottom half of Figure 28e) to larger sizes (clone on top of Figure 28e). Sometimes, the cells became disproportionately big as compared to surrounding cells in the clone (Figure 28d).

**DETC and LC in Lang-DTA Mice:** There is a drastic decrease in GL3-positive DETCs after 5 week chronic UVB exposure and some were seen interacting with langerin+ DC. Figure 29 a-b is from the skin of a Lang-DTA mouse, which shows DETC (GL3+, red) interacting with langerin+ DC (CD207, blue), next to mutant clone (CM5, green). Figure 29b is a close up of this DETC and LC interaction. Figure 29c shows that there is no significant relationship between GL3+ cell density and presence of clones in HPFs in Lang-DTA mice (3.2 ± 0.7 vs 4.2 ± 1.4 #GL3+ cells/mm², P=(NS) 0.4966). Baseline numbers of GL3+ cells from non-irradiated anterior bellies have not yet been quantified. However, if there is an average of 0.47 GL3+ cells per HPF at 25x, then there seems to be an approximate 80-fold decrease in GL3+ cells as compared to anterior belly control (approximately 40 GL3+ cells/HPF non-irradiated, Figure 29f) in Lang-DTA mouse skin. Many GL3+ cells were located within or near hair follicles, as show in Figures 29 e-f.
DETC and LC in NLC Mice: There is a drastic decrease in GL3-positive DETCs after 5 week chronic UVB exposure and some seen interacting with langerin+ DC. Figures 30 a-d show DETC (GL3, red) interacting with langerin+ DC (CD207, blue) in NLC mouse skin. Figures 30b and 30c different planes of same image. Figure 30c has a scattered clone (CM5, green) in the background. Figure 30e is anterior belly control for NLC (non-irradiated). Many GL3+ cells were located in or near hair follicles.
DISCUSSION

As seen from our preliminary data, there are several postulations that can be made regarding what processes LC may be affecting in the epidermis to cause increased tumorigenesis in chemical models and increased premalignant lesions in UVR models. Previous findings from the Girardi lab revealed that LC have a fundamental role in tumorigenesis, as LC-deficient (Lang-DTA) mice were shown to be significantly resistant to two-stage chemical carcinogenesis. Moreover, our more recent preliminary findings suggest that LC facilitate keratinocyte proliferation and epidermal hypertrophy (Figure 1 a-b). We hypothesized that LC may be affecting the initiation step of carcinogenesis, where a mutagen (i.e. DMBA) causes DNA-damage that results in mutations that cause aberrant cell proliferation. To this effect, our laboratory revealed preliminary evidence that LC may have the capacity to internalize mutagens (i.e. DMBA) and metabolize them to more carcinogenic intermediates (unpublished).

On the other hand, LC may also be acting at the tumor promotion stage, where post-mutational events may contribute to neoplasia, controlling cell cycle dysregulation and apoptosis. For example, the fact that much of LC effect on epidermal proliferation and hypertrophy can be observed with TPA alone (Figure 3) indicates that this effect is not entirely dependent on mutagenesis by DMBA. Thus, since LC absence has a negative effect on proliferation and hypertrophy in DMBA+TPA (Figure 1), TPA alone (Figure 3), and even DMBA alone (Figure 4), it suggests LC may have a role in both initiation and promotion in carcinogenesis. Since the initial DMBA+TPA experiment was only a small sample (n=2), a repeat
chemical carcinogenesis experiment (DMBA and 4 weeks of TPA) has been completed to confirm the findings and is awaiting analysis.

Because we saw such a drastic effect of the presence or absence of LC on keratinocyte proliferation in chemical carcinogenesis, we were interested to study the influence of LC on UVB carcinogenesis under both acute and chronic exposure conditions. It was surprising to find that the presence of LC is correlated with increased epidermal apoptosis (Figure 8), since apoptosis has been classically associated with protective effect in cancer and we have hypothesized that LC somehow promote cancer in the skin of mice. However, as already discussed in the introduction, apoptosis has been more recently described to play an important role in the expansion of a mutated clone into nearing stem cell compartments in the epidermis (105).

After repeating the acute UVB experiment with a greater number of mice, the same trend was observed. There is a question whether the increased levels of apoptosis was secondary to LC themselves apoptosing, thus raising our cell counts in the NLC mouse group. It is not completely clear from the literature what happens to epidermal LC during UVB exposure. Earlier in vitro reports claimed that LC apoptosed after UVB exposure (156-158), however later in vivo studies demonstrated that only a few LC undergo apoptosis and depletion was mainly attributable to migration (162,163). One report examined LC apoptosis more than 48 hours after UV at increasing doses and found LC have delayed apoptosis (182).
In a pilot experiment, we co-stained epidermal sheets of Lang-DTR and NLC mice (*Figure 9*) with cleaved caspase-3 (apoptosis marker) and langerin/CD207 (LC marker) by immunofluorescence. While we did not have enough mice to make a detailed comparison, we could see that not all LC were co-stained with cleaved caspase-3. However, just by looking at co-staining we could not distinguish between LC that may be apoptosing and those that may be engulfing apoptotic keratinocytes. One way to distinguish apoptosing LC from ones that are engulfing apoptosing keratinocytes would be by LC morphology. As described by Pradhan et al. (182), the morphology of wildtype LC differed strikingly from apoptosis resistant KO LC on day 7, most occurring by day 2. They report that LC developed enlarged rounded cell bodies with ‘blebbing’ (at 1000 J/mm$^2$), as well as diffuse smeary (500 J/mm$^2$) or ‘blebby’ dendritic processes (2000 J/mm$^2$), providing morphological hallmarks of apoptosis *in situ* (182). In our preliminary data LC staining did show some blebbing, but that may be due to staining methods. Ideally, this experiment should be repeated with an unirradiated dorsal skin control and the morphology of the LC noted. Our preliminary data suggests that LC can facilitate keratinocyte apoptosis after acute UVB, but more research needs to be done to confirm this finding.

Another reason why we may not be seeing a strong difference in apoptosis marker in LC-deficient versus NLC mice may be due to a possibility that our UV-box produced a supra-physiological dose of UVB. A very high amount of keratinocytes were apoptosing after our calculated dose of 3,360 J/m$^2$
and it’s possible that may have just flooded the system to see any strong differences.

Nevertheless, if we hypothesize that LC presence indeed influences the level of keratinocyte apoptosis in the epidermis and that apoptosis promotes mutant clone expansion, this would be consistent with our findings in chronic UVB experiments, where LC-deficient mice (Lang-DTA and Lang-DTR) were found to have a statistically significant smaller density of p53 mutant clones. Mutant clones have been described to consist of p53-mutant keratinocytes that fill up the epidermal proliferating units (EPU) as 16-cell units, which are confined or “trapped” by hexagonal geometry of an EPU without further UVB irradiation (107). Clonal expansion has been shown to be driven not by new mutations, but by UVB-induced keratinocyte apoptosis (105). Brash group describes this conflicting role of apoptosis in their K-14 survivin transgenic mice, which are relatively resistant to apoptosis compared to wildtype mice. Their transgenic mice had a decreased number of microscopic clones and corresponding decreased numbers of papillomas, but interestingly had increased malignant conversion (tumor transformation) to squamous cell carcinoma.

Thus, if we compared our LC deficient mice to K-14 survivin mice (both have less apoptosis relative to controls), it is not surprising that both transgenic mice have deceased numbers of mutant clones compared to control mice. Moreover, clonal numbers binned by clone sizes followed a similar pattern described in the Brash group’s K14-survivin transgenic mice (Figure 21), especially for our 9 week mice. We saw a similar trend (although not statistically
significant, i.e., \( p=0.06 \) in a two-tailed t-test for bin 33-48 cells) of increased numbers of smaller clones in the mouse group with relatively less apoptosis (K-14 survivin mice and our Lang-DTA/DTR-mice).

Moreover, in the regression experiment of 5 weeks of chronic UVB and then no UVB for 3 weeks (Method 4), mouse groups had the same number of LC for 5 weeks of chronic UVB exposure, but after 3 weeks of no UVB and LC depletion by DT injections Lang-DTR mice had a decreased number of clones. This suggests that either the presence of LC still promotes clonal expansion even without UVB exposure, or LC presence prevents clonal regression. Further studies need to be done, preferably by manually counting the cells per clone.

It is possible that LC may be mediating apoptosis directly or indirectly via immune regulation cells that mediate apoptosis, which in turn affect clonal expansion and regression. UV-induced immunosuppression is mediated via the FasL/FasR apoptosis mediator system and abrogating keratinocyte apoptosis prevents UV-induced immune tolerance (182). Schwarz and colleagues reported that the ablation of LC in their UVR study failed to produce immune tolerance via T regs, which are known to secrete IL-10 on hapten-specific stimulation (101,177) and may use the apoptosis-related FasL/FasR-ligand system. Moreover, as mentioned earlier in the introduction, apoptosis links both chemical and UVR-induced carcinogenesis, as it is related to reactive oxygen species and Ras-induced p53 mediated apoptosis (87,88), both of which may be related to NADPH-oxidase of neutrophils, UVB-induced ROS production and apoptosis (89).
LC may also be using apoptosing keratinocytes to influence the immune system. Viable, but functionally impaired (by UVR) LC that have engulfed apoptotic or photodamaged particles, have the potential to cause tolerance of premalignant lesions. Evidence of delay in LC apoptosis after acute UVR (193) (providing the necessary time to CPD-positive LC to successfully reach draining LN to localize to T cell areas in the inner cortex of the LN, as opposed to their dDC counterpart that localize to B cell areas), supports even further LC role in possible immune regulation of altered self-antigens. Alternatively, LC may themselves indirectly promote regulatory T cell generation by undergoing apoptosis to distribute peripheral “altered self” antigens to resident CD8α+ DC, which have been shown to induce tolerance (194,195).

Another way in which LC may be mediating immune response may be via their interaction with another dendritic population of γδ T cells, also called dendritic epidermal T cells (DETC), which are present in murine (and other mammals, but not human) skin (196). DETC have been shown to cause specific immunologic tolerance in vivo by inhibiting proliferation, via a cytotoxic mechanism, of naïve T cells in response to hapten-bearing dendritic cells in vitro (197). LC and γδ DETC are in contact with the renewing basal keratinocytes, the main cells involved in carcinogenic transformation. Moreover, after specific epidermal stimuli, LC and γδ DETC cell bodies have been shown to become juxtaposed (80). Although some studies have found that DETC were absent during the development of ultraviolet radiation (UVR)-induced skin cancers, consistent with the possibility that this may result in impaired host anti-tumor
immunity (198) our studies have shown that GL3\(^+\) DETC numbers are reduced, but are still present after chronic UVB exposure. Thus, it is possible that LC can interact with DETC to induce tolerance (perhaps via a cytotoxic mechanism) to inhibit proliferation of naïve T cells (197). It would be interesting to study the influence of Langerhans cells on clonal numbers in T cell depleted mouse strains after acute and chronic UVB exposure.

LC have been shown to react to NKG2D-ligand upregulation by keratinocytes in situations of environmental stress and cell damage, with recent evidence showing that LC serve as immunoregulatory cells by activating natural killer (NK) T cells (199). Interestingly, in regard to UVB-studies, Brash and colleagues demonstrated that antigen-specific immunity does not mediate acute regression of UVB-induced p53-mutant clones in immunodeficiency mice that still contained NKT cells (108). This raises the possibility that, in our clonal regression studies, LC induction of tolerance may be mediated via NKT cells. It is also possible that LC may be promoting carcinogenesis by stimulating a recently identified and characterized tumor-promoting CD8\(^+\) T cell population (200,201). Moreover, recent finding of tumor associated macrophages (TAMs) that are involved in tumor initiation, promotion and immune suppression (202), raises the possibility that LC may act in a similar fashion.

Recently, Loser et al. demonstrated that the capacity to induce T reg cells was dependent on receptor activator of NF-κB (RANK) activation on epidermal LC by interaction with its ligand (RANKL, also known as CD254, OPGL and TRANCE) on keratinocytes (203). They reported that RANKL is expressed in
keratinocytes of the inflamed skin and its overexpression resulted in functional alterations of epidermal dendritic cells and systemic increases of regulatory CD4\(^+\)CD25\(^+\) T cells. Because RANK-activated LC have demonstrated potent T reg cell activation *in vitro*, it is possible that UV- damaged RANK-activated LC may similarly activate T reg cells directly, once they reach the draining LN.

LC effect on other immune cells most likely depends on the cytokine milieu, and thus the maturity state of LC, which in turn create a pro-or anti-tumorigenic microenvironment. Lower levels of TNF and MIP3\(\alpha\) (both associated with tumor regression) within the microenvironment induced correlated with increased levels of immature LC, which had decreased production of IL-2 and increased production of immunosuppressive IL-10 \((73,204)\). The increased presence of immature LC in cervical intraepithelial neoplasia is thought to be responsible for deficient presentation of alloantigen to T cells \((74)\). It is quite possible that chronic inflammation of chemical carcinogenesis or immune suppression of chronic UVR exposure maintain a population of immature LC, which create a tolerogenic state either by presentation of tumor antigens without co-stimulatory molecules required for T cell activation, or by stimulating other tolerogenic cells.

Our understanding of Langerhans cells’ role in modulating the immune response in the direction of immunity or tolerance has been a hot topic of discussion and continues to evolve. With recent advances in LC ablating models, photoimmunology and cell identification, as well as continuous development of transgenic mouse models inducing and/or abrogating pro-apoptotic or pro-
survival regulators, we come closer to gaining insight into Langerhans cell influence in cutaneous responses to chemical and UVR carcinogenesis. A better understanding of the molecular and cellular mechanisms of DC (and specifically LC) function may open the door to future therapies in fighting skin and other cancers. Some therapies are already currently under way, from using differentiated DC in extracorporeal photochemotherapy in CTCL treatment (205), to exploiting the potent antigen-presenting function of LC with the right stimulation in epicutaneous immunization (206). At this point, other therapies are still only hypothetical, such as mediating Langerhans cells’ recently discovered immune suppression functions (199,203) by targeting or blocking their RANK-RANKL interaction or NKT cell activation.

In conclusion, LC exert diverse and measurable influences on epidermal proliferative responses to both chemical and UVB exposures in a transgenic murine mouse model. In our laboratory we showed that LC presence in the skin is associated with increased epidermal proliferation and hypertrophy after exposure to a mutagen and/or a tumor promoter. This points to tumor initiation and/or to tumor promotion as being two potential mechanisms of by which LC may induce increased tumor formation after chemical carcinogenesis. In UVB studies, LC presence was found to be associated with increased keratinocyte apoptosis levels after acute UVB exposure, which parallels with our findings of increased numbers of premalignant lesions after chronic UVB treatment in LC intact mice. Moreover, we found that when we exogenously induce depletion of LC after chronic UVB exposure, we see more acute regression of premalignant
mutant clones after several weeks. These preliminary findings reinforce the fundamental role of LC in processes involved in cancer formation. It will be exciting to follow up on these findings with repeat experiments in both chemical and UVB models.
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**FIGURES**

**Figure 1 a-b: (DMBA+TPA)** After two-stage chemical carcinogenesis (DMBA + TPA), there was significantly smaller increase in epidermal proliferation and epidermal thickness in Lang-DTA mice.

*Figures 1a and 1b show Lang-DTA mice displaying less keratinocyte proliferation and epidermal hyperthrophy versus NLC. Figure 1a shows at baseline Lang-DTA and NLC mouse skin showed comparable epidermal thickness (7.9 ± 1.8 vs 6.9 ± 2.8 µm, P=NS) and proliferation rates as measured by Ki-67 immunohistochemistry (IHC) (13.6 ± 2.6 vs 12.7 ± 2.8 # Ki-67+ cells, P=NS). After a single application of mutagen DMBA, followed by repeated promoter TPA (x 5.5 wks), Lang-DTA skin showed substantially smaller increases in epidermal thickness (25.8 ± 8.9 vs 59.2 ± 28.4 µm, P<0.0001) and proliferation (25.7 ± 7.4 vs 31.5 ± 7.7 # Ki-67+ cells, P=0.007) as compared to NLC (Figure 1b).*

**Figure 2: Epidermal sections of Lang –DTA (top) and NLC (bottom) mouse skin, stained with Ki-67 by immunohistochemistry (brown).**

*stratum granulare
stratum basale*
Figure 3 a-d: (TPA alone, 4 weeks) After 4 weeks of TPA application, there was significantly smaller increase in epidermal proliferation and epidermal thickness in Lang-DTA mice vs NLC. After repeated applications of tumor promoter TPA (x 4 wks), LC-depleted (Lang-DTA) skin showed a smaller increase in epidermal proliferation (16.7 ± 0.4 vs 19.3 ±0.5 # Ki-67+cells, P=0.0002, Figure 3a) and minimal epidermal thickness (5.1 ± 0.1 vs 8.5 ± 0.2 µm, P<0.0001, Figure 3c), as compared to NLC mice. Statistical significant was reached for means per mouse for epidermal hypertrophy (P=0.0037, Figure 3d), but not for epidermal proliferation (P= NS, 0.3982, Figure 3b).
Figure 4 a-d: (DMBA alone) After 8 doses of mutagen (DMBA alone) treatment every two weeks, there was a significantly smaller increase in epidermal proliferation and epidermal thickness in Lang-DTA mice vs NLC mice. After repeated applications of mutagen DMBA alone, Lang-DTA skin showed a smaller increase in epidermal proliferation (12.8 ± 0.99 vs 22.7 ± 1.4 # Ki-67+cells, P=0.0001, Figure 4a) and minimal epidermal thickness (14.4 ± 1.08 vs 17.8 ± 1.0 µm, P<0.0292, Figure 4c), as compared to NLC skin. Figures 4b and 4d show corresponding data per mouse. Skin harvested was paratumoral.
**Figure 5 a-c (Acute UVB1): Cleaved caspase-3 by IHC.** After a single dose of UVB (3,360 J/m²) there was a significantly smaller number of apoptotic cells in the epidermis of Lang-DTA versus NLC mice, LC-deficient skin (Lang-DTA), as compared to skin of LC-intact normal littermate controls (NLC), showed less epidermal apoptosis after acute UVB irradiation, as measured by IHC (21.4 ± 1.1 vs 26.4 ± 1 # cleaved caspase-3+ cells, \( P=0.0099 \), Figure 5a). Figure 5c shows representative cleaved caspase-3 IHC stained sections for corresponding mouse groups.

**Figure 5c**

Lang-DTA Non-irradiated anterior belly skin  
NLC Non-irradiated anterior belly skin

Lang-DTA UVB-exposed dorsal skin  
NLC UVB-exposed dorsal skin
Figure 6 a-c: (Acute UVB1) Sunburn cells on H&E. LC-deficient skin (Lang-DTA), as compared to skin of LC-intact normal littermate controls (NLC), showed less epidermal apoptosis after a single dose of acute UVB irradiation (3,360J/m²), as measured by on H&E (9.3 ± 0.5 vs 13.9 ± 1.5 # sunburn cells, $p = 0.0069$, Figure 6a) in the pilot acute UVB experiment (n=3). Mean per mouse did not reach statistical significance (Figure 6b). Figure 6c shows examples of sunburn cells, with pyknotic nuclei and eosinophilic cytoplasm, on H&E sections.
Figure 7 a-b: Raw Data for cleaved caspase-3 after acute UVB exposure.  
*Figure 7a* shows cleaved caspase-3 data for pilot experiment (n=3) per each strip 1-3, where strip 2 corresponds to the middle dorsal treated skin, and strips 1 and 3 are side strips. Of note, previously shown data in *Figures 6* are from middle strips (i.e. strip 2 from each mouse). *Figure 7b* contains all the cleaved caspase-3 data for both pilot or ‘Old Experiment Data’ asparked in graph (n=3) and repeated experiment (n=7).
Figure 8 a-b: Cleaved caspase-3 after acute UVB-exposure. Combined data from pilot and repeated experiment.

*Figure 8a* contains combined apoptosis data from pilot acute UVB experiment (n=3) and repeated (n=7) experiment measuring cleaved caspase-3 for Lang-DTA vs NLC irradiated skin. There was a statistically significant difference in epidermal apoptosis (18.9 ± 0.4 vs 21.1 ± 0.4 # cleaved caspase-3+ cells, *P*< 0.0001). At baseline (*Figure 8a*), non-irradiated anterior belly skin showed no difference in apoptosis in Lang-DTA vs NLC skin (0.01 ± 0.0 vs 0.01 ± 0.0 # cleaved caspase-3+ cells, *P*= NS, 0.3692).

*Figure 8b* represents the means per mouse combined apoptosis data from the pilot acute UVB experiment (n=3) and repeated (n=7) experiment measuring cleaved caspase-3 for Lang-DTA vs NLC irradiated skin. Statistical significance was not reached for the means per mouse (19.77 ± 1.7 vs 22.7 ± 2.1 # cleaved caspase-3+ cells, *P*= NS, 0.2877).
Figure 9 a-c: Acute UVB Sheets Pilot experiment. Co-staining langerin (green) and cleaved caspase-3 (blue) on epidermal sheets by immunofluorescence in NLC Mice, after acute dose of UVB (3,360 J/m²). Magnification 25x.

Figure 9a – NLC Acute UVB (caspase-3 and langerin)

Figure 9b – NLC Acute UVB (caspase-3)

Figure 9c – NLC Acute UVB (langerin)
**Figure 10 a-c: Acute UVB Sheets1: Pilot experiment.** Co-staining langerin (green) and cleaved caspase-3 (blue) on epidermal sheets by immunofluorescence in NLC Mice, after acute dose of UVB (3,360 J/m²). Magnification 63x.

**Figure 10a – NLC Acute UVB (caspase-3 and langerin)**

**Figure 10b – NLC Acute UVB (caspase-3)**

**Figure 10c – NLC Acute UVB (langerin)**
Figure 11 a-c: Acute UVB Sheets: Pilot experiment. Co-staining langerin (green) and cleaved caspase-3 (blue) on epidermal sheets by immunofluorescence in Lang-DTR mice, after acute dose of UVB (3,360 J/m²). Magnification 25x.

Figure 11a – Lang-DTR Acute UVB (caspase-3 and langerin)

Figure 11b – Lang-DTR Acute UVB (caspase-3)

Figure 11c – Lang-DTR Acute UVB (langerin)
**Figure 12 a-c: Acute UVB Sheets1: Pilot experiment.** Co-staining langerin (green) and cleaved caspase-3 (blue) on epidermal sheets by immunofluorescence in Lang-DTR mice, after acute dose of UVB (3,360 J/m²). Magnification 63x.

**Figure 12a – Lang-DTR Acute UVB (caspase-3 and langerin)**

**Figure 12b – Lang-DTR Acute UVB (caspase-3)**

**Figure 12c – Lang-DTR Acute UVB (langerin)**
**Figure 13 a-b: Acute UVB Sheets.** Co-staining langerin (green) and cleaved caspase-3 (blue) on epidermal sheets by immunofluorescence in NLC and Lang-DTR dorsal skin, after acute dose of UVB (3,360 J/m²). Magnification 25x.

**Figure 13a (panels 1-6): NLC Dorsal Skin (UVB Exposed).**

1  
2  
3  
4  
5  
6  

**Figure 13b (panels 1-6): Lang-DTR Dorsal Skin (UVB Exposed).**

1  
2  
3  
4  
5  
6  

**Figure 14 a-c: Acute UVB Sheets.** Co-staining langerin (green) and cleaved caspase-3 (blue) on epidermal sheets by immunofluorescence in NLC and Lang-DTR unexposed anterior belly skin. Figures 14c are antibody controls after acute dose of UVB (3,360 J/m²). Magnification 25x.

**Figure 14 a (panels 1-3): Anterior Belly (NLC)**

1  
2  
3

**Figure 14b (panels 1-3): Anterior Belly (Lang-DTR)**

1  
2  
3

**Figure 14c (panels 1-2): IgG Control for Cleaved caspase-3 (green) and langerin/CD207 (blue)**

1 – Dorsal skin  
2 – Dorsal skin
Figure 15 a-c: (Chronic UVB0, IHC) After chronic UVB exposure, NLC mouse skin contains p53-mutant clones and viable langerin+ DC by immunohistochemistry. Figure Va shows p-53 mutant clones stained by IHC on epidermal sheets (CM5+, brown) after 3 weeks of chronic UVB. Figure 15b shows a larger p53-mutant clone after 5 weeks (courtesy of Wengeng Zhang). Figure 15c shows langerin+ DC (langerin/CD 207+, brown) after 3 weeks of chronic UVB.
Figure 16 a-b: After chronic UVB exposure, NLC mouse skin shows viable langerin+ DC that co-stain with mutated p53-clones by immunofluorescence. Confocal microscopy reveals langerin+ DC (CD207+, blue) coincident with mutant p53 clones (CM5, green) after 9 weeks of UVB, Figure 16a. 3-D view of LC and mutant clones, Figure 16b.
Figure 17a-b: Method 1 - 9 Weeks (Chronic UVB1, n=3, IF). After 9 weeks of chronic UVB exposure, Lang-DTA mouse skin compared to NLC had a smaller number of mutated clones per mm² (8.0 ± 1.1 vs 15.4 ± 2.3, \textbf{P<0.004}, Figure 17a). Statistical significance was not reached for means per mouse (10.7 ± 2.2 vs 16.9 ± 4.2, P= NS, 0.2636, Figure 17c). Figure 17b shows data per HPF per mouse.
**Figure 18 a-b: Method 2 - 9 Weeks (Chronic UVB1, n=4, IF).** After 9 weeks of chronic UVB exposure, there is a trend toward Lang-DTA mouse skin vs NLC having a smaller number of mutated clones per mm$^2$ (1.7 ± 0.3 vs 2.5 ± 0.5 # mutated clones/mm$^2$, $P=\text{NS}$, 0.1636, *Figure 18a*), which did not reach statistical significance. Means per mouse also did not reach statistical significance (1.7 ± 0.3 vs 2.4 ± 0.7 mean # mutated islands/mm$^2$, $P=\text{(NS)}$ 0.3528).
Figure 19 a-b: Method 3 - 5 Weeks (Chronic UVB2, n=8 and 7, IF). After 5 weeks of chronic UVB exposure, Lang-DTA mouse skin compared to NLC had a smaller number of mutated clones per mm$^2$ (0.9 ± 0.05 vs 1.0 ± 0.1 # mutant clones/mm$^2$, $P=0.0489$, Figure 19a). Statistical significance was not reached for means per mouse (0.9 ± 0.1 vs 1.1 ± 0.2, $P=NS$, 0.3797, Figure 19b).
Figure 20 a-b: Method 4 - 5 weeks UVB and 3 weeks regression (Chronic UVB3, n=8, IF): (Lang-DTR vs NLC). After 5 weeks of chronic UVB followed by 3 weeks of no UVB, there was a statistically significant smaller number of mutant clones per mm\(^2\) in Lang-DTA versus NLC mice (0.2 ± 0.0 vs 0.4 ± 0.0, \(P \leq 0.0001\), Figure 20a). Statistical significance was also reached for mean number of mutated clones/mm\(^2\) per mouse in Lang-DTA vs NLC (0.2 ± 0.0 vs 0.4 ± 0.0, \(P = 0.0106\), Figure 20b).
**Figure 21 a-b: Summary of Chronic UVB methods.** Y-axis corresponds to duration of chronic UVB irradiation. Method 1 and 2 correspond to 9 weeks of chronic UVB, with number of clones counted by different methods. Method 3 corresponds to 5 weeks of chronic UVB in Lang-DTA vs NLC mice. Method 4 corresponds to 5 weeks of chronic UVB with 3 weeks of no UVB time in Lang-DTR vs NLC mice. There is a discrepancy in the number of clones per mm$^2$ in Method 1 versus Method 2 at 9 weeks of chronic UVB, with the former being approximately 5-6 times greater (represented visually in Figure 21a and Figure 21b, below), which may be explained by the method of clone counting and resolution used to capture images.
**Figure 22 a-f: Area/Mutant Clone in Chronic UVB.** Method 1, 3 and 4 correspond to data gathered after chronic UVB for 9 weeks, 5 weeks, and 5 weeks with no UVB for 3 weeks, respectively. **Figures 22 d-f** below are the corresponding means per mouse for each method.
Figure 23 a-c: Mutant Clone Area in Lang-DTA vs NLC Mice by Bins. Bins assigned as multiples of EPU (16 cells or keratinocytes). Method 1, 3 and 4 correspond to data gathered after chronic UVB for 9 weeks, 5 weeks and 5 weeks with no UVB for 3 weeks, respectively.
Figure 24: Mutant Clone Bins as described by Zhang et al. (Carcinogenesis 2005).

Fig. 3. Survivin expression enhances creation of new p53 mutations. K14-Survivin (open bars) and non-Tg littermates (filled bars) were UVB-irradiated for (a) 5 or (b) 7 weeks. Skin was harvested and analyzed for p53 mutant clones by immunohistochemistry. Data are expressed as number of clones per cm² of skin for various clone size bins. Bins represent ≤ 1 stem cell compartment (1–16 cells), and multiples of 2, 4, 8, and 16–32 compartments. The P values for comparisons of clonal densities from Tg and non-Tg mice are two-sided and significant values are indicated above the bars. For statistical analysis the assumption was made that the random observed number of clones of a given size follows a Poisson distribution, with mean proportional to the total area.

Fig. 4. Survivin expression impedes expansion of existing p53 mutant clones. K14-Survivin (open bars) and non-Tg littermates (filled bars) were UVB-irradiated for (a) 10 or (b) 13 weeks. Skin was harvested, and p53 mutant clones were analyzed as in Figure 3. Fewer intermediate and large clones are found in Tg skin.
Figure 25: Langerin+ DC Within or Around Mutant Clones in Lang DTA and Lang-DTR mice after Chronic UVB.
Figure 26 a-b: Number Replacement (langerin+ DC) LC in Lang-DTA versus NLC after 5 weeks of chronic UVB. (Data not analyzed from unirradiated dorsal skin).

Figure 26a: 5 Weeks Chronic UVB

Figure 26b: 5 Weeks Chronic UVB

p < 0.0001
Figure 27 a-e: LC Density Associated with Mutant Clones in Lang-DTA mice (n=7) and NLC (n=6) after 5 weeks of Chronic UVB.

Figure 27a: 5 Weeks Chronic UVB Lang-DTA Mice

Figure 27c: 5 Weeks Chronic UVB NLC Mice

Figure 27d: 5 Weeks Chronic UVB2 NLC Mice

Figure 27e – Langerin+ (blue) DC around clone (green)
Figures 28 a-e: Mutant Clone Morphology. Epidermal proliferating units (EPUs) expand into clonal islands.
Figure 29 a-f: DETC and LC in Lang-DTA Mice: Sparse GL3-Positive DETCs were present after 5 weeks of chronic UVB exposure and some were seen interacting with langerin+ DC. Figures 29 a & b show DETC (GL3+, red) interacting with langerin+ DC (CD207+, blue), next to mutant clone (CM5+, green) in Lang-DTA mouse skin. Figure 29b is a close up of DETC and LC interaction. Figure 29c shows that there is no significant relationship between GL3+ cell density and presence of clones after 5 weeks of chronic UVB. Many GL3+ cells were located within or near hair follicles, as show in Figures 29d and 29e. Figure 29f is anterior belly control in Lang-DTA mouse skin (non-irradiated).

Figures 29 a&b: GL3+ and langerin+ DC interaction, with zoomed-in image on right.
Sparse GL3-Positive DETCs were present after chronic UVB exposure and some were seen interacting with langerin+ DC. Figures 30 a-d are of NLC mouse skin, showing DETC (GL3+, red) interacting with langerin+ DC (CD207+, blue). Figures 30b and 30c are different planes of same image, the latter with a scattered clone in background (CM5+, green). Figure 30e is anterior belly control for NLC (non-irradiated). Many GL3+ cells were located within or near hair follicles, as seen in Figures 30 b-d.