Original Article

Early events after skin injury: Observations on changes in Langerhans cells and Birbeck granules with role of phosphorylase kinase in the injury pathway

Madalene CY Heng, Ming K Heng

Department of Medicine, David Geffen School of Medicine, UCLA, California, USA

Abstract

Background and Aim: To evaluate the role of Langerhans cells (LCs) in the early events of the injury pathway, we studied activated and unactivated LCs in sequential biopsies from uninvolved skin of psoriatic and nonpsoriatic individuals using immunocytochemistry and electron microscopy.

Methods: Tape-stripping injury was used for cell activation. Changes suggestive of LC activation were observed as early as 5–30 min following tape-stripping. These consisted of abundant Golgi cisternae, proliferation of rough endoplasmic reticulum consistent with increased protein synthesis, increased folds and waviness of the nuclear membranes, and development of migratory status as shown by shortening of dendritic processes and presence of spaces around the LCs from detachment of the cell-membranes from the adjacent keratinocytes.

Results: There were notable changes in Birbeck granules (BGs) in LCs, with an inverse correlation of BG density with cell activation as early as 5 min after tape-stripping. The BG was most abundant in the unactivated LCs, and least numerous after activation. With cell activation, BG was observed to be few and unstacked, with isolated granules opening to the cell surface. The presence of T6 antigen (CD1a) 30 min after tape-stripping, with loss of epidermal T6+ LCs and presence of T6+ LCs within the dermal blood vessels 24 h after tape-stripping, strongly suggest development of LCs' migratory status, followed by amplification of the immune response 7 days after tape-stripping.

Conclusion: Because BG contain both C-type lectin (langerin) and T6 antigen (CD1a), BG in activated LCs may provide a mechanism whereby presynthesized molecules (langerin, T6/CD1a, toll receptors) necessary for early LC activation and migration may be simultaneously delivered to the cell surface membrane for rapid enhancement of activated cell function by nonpeptide antigens.

Key words: Birbeck granules, injury pathway, Langerhans cells, phosphorylase kinase, psoriasis

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INTRODUCTION

Studies focused on the effects of cellular and tissue changes after injury stimuli have led to an increased understanding of the sequence of molecular events occurring in the injury pathway. In a rat model of traumatic injury, nuclear factor kappa-beta (NF-kB) activation was observed as early as 30 min following induction of injury,[1] identifying the activation of the transcription activator NF-kB as among the earliest molecular events in the injury pathway. The observation that tumor necrosis factor-alpha, interleukin-1β and the adhesion molecule intercellular adhesion molecule-1, were abrogated with inhibition of NF-kB by pyrrolidine dithiocarbamate,[2] indicates that in injured tissues, cytokine and adhesion molecules are secreted downstream of NF-kB signaling.

However, the events induced by injury upstream of these findings described above are less clearly defined. Among the earliest molecules reported to be present after injury is phosphorylase kinase (PhK), which has been found as early

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Address for correspondence: Dr. Madalene CY Heng, Department of Medicine, David Geffen School of Medicine, UCLA, 500 Paseo Camarillo, Suite 100, Camarillo, CA 93010, USA. E-mail: madalene@madalenehengmd.com

as 5 min after skin injury.[3] PhK, while primarily known for its role in the generation of adenosine triphosphate (ATP) through breakdown of cytoplasmic glycogen, is reported to be also involved in the activation of multiple molecules through phosphorylation reactions.[3-6] Because curcumin, a selective and noncompetitive PhK inhibitor,[7] has been shown to suppress NF-kB activation,[8] it is believed that PhK has a role in activating NF-kB.[9] This may be the basis of a number of reports on the potential therapeutic applications of curcumin,[9-13] which are of great clinical interest with respect to the mechanisms underlying skin diseases induced by injury.

At present, it is unclear as to which cells are implicated and involved in the earliest events following skin injury. Activated Langerhans cells (LCs), also called epidermal dendritic cells, have been observed in psoriatic epidermis as early as 5 min following tape-stripping injury.[14] To clarify early events that occur minutes after skin injury, we report observations on certain molecular and cellular changes minutes to hours after tape-stripping injury, focusing on the LC and its possible role in the injury pathway.

Langerhans cells are identified under electron microscopy by the presence of Birbeck granules (BGs), which are cytoplasmic structures composed of pentamellar zippered membranes. BG have been observed to form where langerin, a C-type lectin, accumulates. The function of BG is currently unclear. Although disruption of the langerin/CD207 gene abolishes BG, marked loss of LC function was not observed,[15] and LCs without BG (e.g., langerin negative mice) were able to capture antigen, migrate to regional lymph nodes, and undergo phenotypic maturation. Despite these observations, there is evidence that BG may have an immunological function, since the disappearance of BG has been shown to accompany the loss of antigen processing capacity in cultured epidermal LCs.[16]

Langerin is a cell surface lectin that is thought to induce the formation of BG.[17] It is a C-type lectin with Type II configuration and a single carbohydrate recognition extracellular domain, which oligomerize as trimers, displaying calcium-dependent binding specificity for mannose, N-acetyl glucosamine, and fucose.[18] T6 antigen, a class I major histocompatibility complex molecule, has also been found in BG.[19] Moreover, it has been observed that both T6 antigens (CD1a) and langerin are necessary for LCs to efficiently present nonpeptide antigens to T-cells.[20] Since both T6 and langerin have been found to colocalize within BG, it is possible that BG may yet be found to have an important immunological function. In this study, we investigated BG in both unstimulated and unactivated LCs, and in stimulated LCs activated by tape-stripping, with the goal of delineating its function and role in early injury events in epidermal LCs.

MATERIALS AND METHODS

The study was conducted while the principal investigator (MCYH) was a faculty at the UCLA-San Fernando Valley Medicine Program and approved by the VA Sepulveda Medical Center Institutional Review Board for Clinical Research.[14]

Participants and specimens

After obtaining informed consent, uninvolved skin of psoriatic and nonpsoriatic subjects was tape-stripped using the method previously described.[14] The nontaped-stripped skins of these subjects were used as controls. Four mm punch biopsies were then taken at 5th min, 30th min, 1 h, 24 h, 72 h, 1st week and 4th week after tape-stripping, with the biopsies taken under local anesthesia (1% xylocaine). The wound was closed each time with 50 ethilon sutures and simple repair techniques. The specimens were processed for immunohistochemistry and electron microscopy.

Immunohistochemistry

One-half of the biopsy specimens were processed for immunohistochemistry using standard DAKO OLSAB2 kit and endogenous biotin block technique using the DAKO Biotin Block system (DAKO, Carpentaria, CA, USA). The biotinylated monoclonal antibodies used were: OKT6/CD1a (Pharmingen, San Diego, CA, USA), antiHLA DR/major histocompatibility complex (MHC) class II (Pharmingen, San Diego, CA, USA).

Electron microscopy

The other half of the biopsy was fixed in 2.5% glutaraldehyde buffered to pH 7.3 in sodium cacodylate (0.1 M), postfixed in 1% osmium tetroxide, treated in block with tannic acid, dehydrated in alcohol and propylene oxide and embed in a mixture of Epon 812 (Shell chemical company, New York) and Araldite 502 (Polysciences, Inc., United States). Silver sections were then cut with a Sorval MT 2B ultramicrotome (Dupont, United States), using a diamond knife (Dupont, United States), stained with lead acetate, and examined under a EM201 Electron Microscope (Philips, Netherlands).[14]

RESULTS

The sequence of events that was observed in LC activated by tape-stripping in this study indicated the conversion of unactivated LCs into the activated migratory forms.[14] In unstripped skin of normal and psoriatic individuals, we found abundant BG [Figure 1] in unactivated LCs. In addition, the LC showed absence of increased folding of the nuclear membrane, absence of blebbing (empty spaces) between LC membrane and adjacent keratinocytes, small

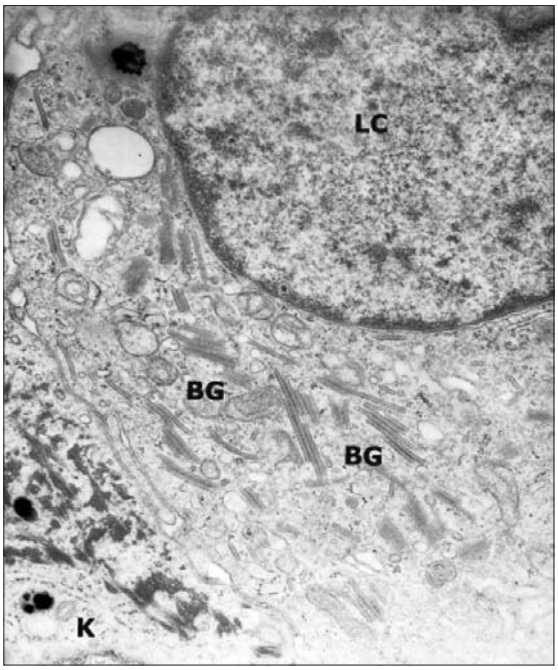

Figure 1: Langerhans cell (LC) in unstripped epidermis showing abundant stacks of Birbeck granules (BG). Note features of unstimulated Langerhans cells in the following: (a) Lack of folds (waviness) in the nuclear membrane; (2) lack of abundant Golgi cisternae; (c) lack of abundant rough endoplasmic reticulum; (d) lack of blebbing and spaces between the LC and keratinocyte (K) (×20,000)

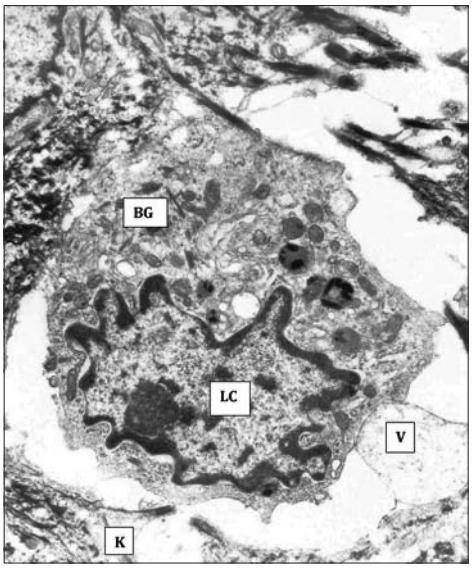

Figure 2: Psoriatic skin 1 hour after tape-stripping showing an activated migratory Langerhans cell (LC) containing residual Birbeck granules (BG). Note the shortened dendritic processes and vacuolated (V) appearance of the tissues surrounding the migratory Langerhans cell as it traverses the epidermis. Note also the increased folds (waviness) of the nuclear membrane of the activated Langerhans cell. Note also the spaces with decreased electron density with depleted glycogen granules in adjacent epidermal keratinocytes (K) (×10,000)

Golgi apparatus, and inconspicuous rough endoplasmic reticulum (RER). Activated LCs in tape-stripped epidermis, on the other hand, were distinguished by increased waviness and folds in the nuclear membranes, and frequently by blebbing between the LCs and the adjacent keratinocytes [Figure 2], a feature indicative of cell mobility. In the tape-stripped specimens, a gradation of LC activity were observed, ranging from the presence of abundant Golgi cisternae [Figure 3] to marked increase in RER with features of protein synthesis [Figure 4] in the 5–30 min biopsy specimens, decreased dendritic processes, presence of vacuolation and blebbing around the LCs, increased folding of the nuclear membrane [Figure 2], and evidence of migratory features in the 30-min to 1-h biopsy specimens. The changes described above are consistent with electron microscopic features of LC activation. In activated LCs, there were decreased numbers of BG, particularly in those migratory LCs 30-min to 1-h after tape-stripping. The migratory LCs show fewer dendritic processes and were detached from the surrounding keratinocytes [Figure 2]. Those migratory LCs that were associated with neutrophils show blebbing [Figure 4], suggesting that proteolytic digestion by the neutrophils contributed to the "spaces" around the migratory cells, possibly to assist in migration of the activated LCs. These activated or migratory LCs contain few or no BG. Early changes in the activated LCs within 5–30 min of tape-stripping also included enlarged Golgi apparatus with prominent cisternae [Figure 3]. The Golgi cisternae may sometimes occupy a significant proportion of cytoplasmic organelles [Figure 3]. The activated status of these cells is indicated by the presence of increased waviness (folds) of the nuclear membrane [Figure 3]. Other early changes in activated LCs of psoriatic epidermis seen within 5–30 min following tape-stripping include evidence of increased protein synthesis within prominent RER [Figure 4]. This is indicated by the observation of prominent Golgi cisternae [Figure 3] as this organelle processes proteins synthesized by the RER.

Few or no BG were noted in activated LCs, whose activation status was suggested by the presence of cellular changes described in detail above. The LCs without BG were recognized by their epidermal location, presence of dendritic morphology, presence of abundant cytoplasm, nuclei with a thin rim of nuclear chromatin, and lack of granules that characterize neutrophils. They differ from T-lymphocytes by the abundance of cytoplasmic organelles, abundance of cytoplasm, and dendritic morphology. In addition, the activated LCs were present in the earliest biopsies (5–30 min), whereas T-lymphocytes were only observed from 24 h onwards, and were only abundant from day 3 onwards [Figures 5 and 6].

In tape-stripped psoriatic epidermis, it was also observed that the epidermal keratinocytes adjacent to the activated LCs contained areas of decreased electron density

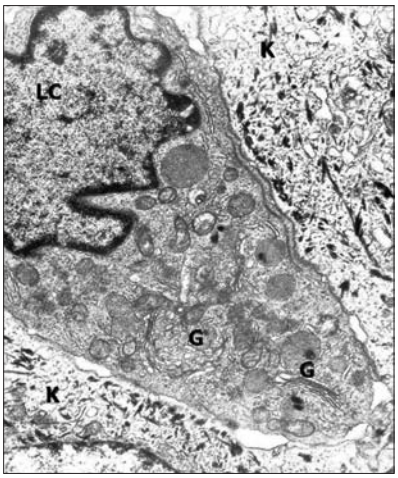

Figure 3: Psoriatic epidermis 5 mins after tape stripping showing an activated Langerhans cell (LC) with increased waviness (folds) of the nuclear membrane, Note abundance of Golgi cisternae (G) and few intact Birbeck granules. Note areas of decreased electron density with depleted glycogen granules in adjacent epidermal keratinocytes (K) (×10,000)

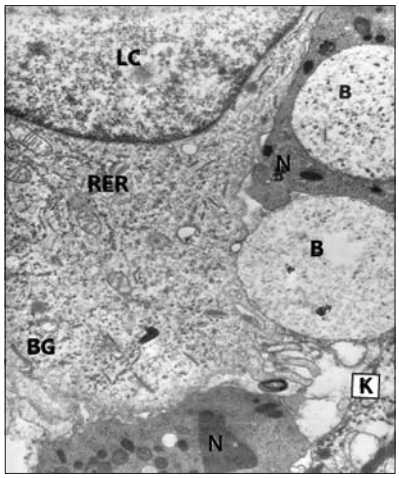

Figure 4: Psoriatic skin 30 mins after tape-stripping showing an activated Langerhans cell (LC) with residual Birbeck granules (BG). Note abundant rough endoplasmic reticulum (RER) showing active protein synthesis. Note also the blebbing (B) in the vicinity of neutrophils (N), easily recognized by the presence of abundant electron dense granules. Note also the areas with decreased electron density within the adjacent keratinocytes (K) containing few glycogen granules (×20,000)

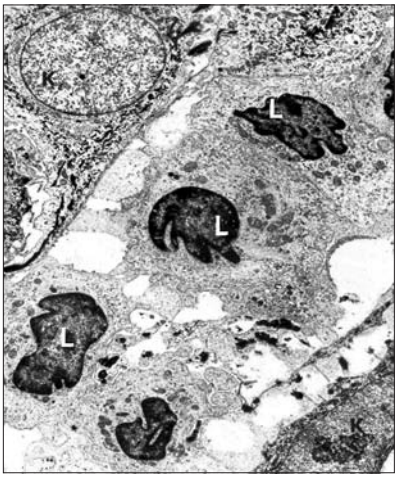

Figure 5: Cluster of T lymphocytes (L) seen within the epidermis of psoriatic epidermis at 72 hours post-tape stripping. This degree of amplification of the immune response was not seen in the 72 hour tape-stripped non-psoriatic (normal) specimens, correlating with less marked Langerhans cell activity of non-psoriatic epidermis (×10,000)

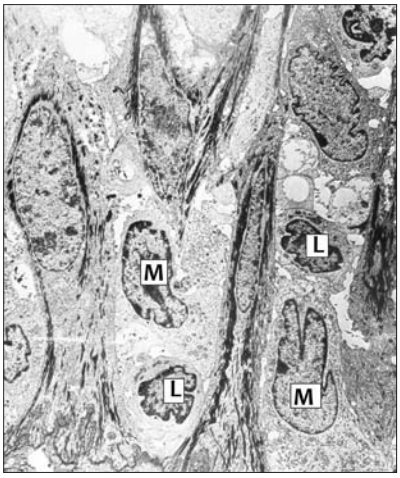

Figure 6: One week following stripping of uninvolved psoriatic skin, abundant infiltration of T lymphocytes (L) and macrophages (M) into the epidermis was observed in tape-stripped uninvolved psoriatic epidermis. This degree of amplification of the immune response was not observed in non-psoriatic tape-tripped skin, corresponding to the lower numbers of activated Langerhans cells (×10,000)

with few glycogen granules [Figures 2-4], confirming observations of depletion of glycogen granules in psoriatic epidermal keratinocytes previously reported.[3] Depletion of glycogen granules is indicative of increased activity of PhK, an enzyme responsible for breaking down glycogen to form ATP for increased cellular metabolic activity.[3-6] Our findings suggest that LCs may be the earliest cells responsible for PhK secretion in tape-stripped psoriatic epidermis.

The loss of BG within activated LCs was associated with increased expression of T6 antigen (CD1a) in the epidermal compartment with abundant expression of $T6^+$ cells in the specimens taken 30 min after tape-stripping [Figure 7a]. In the early stages after tape-stripping, there was no loss of $T6^+$ LCs from the epidermis, suggesting that there was no dermal migration of the stimulated LCs 30 min following tape-stripping. In contrast, in the 24 h posttape-stripping specimens, there was a loss of $T6^+$ LCs from the lower epidermis [Figure 7b], but not from the upper epidermis. The loss of $T6^+$ cells from the epidermis was associated with presence of $T6^+$ LCs in the dermal blood vessels [Figure 7b], and serves as evidence of migratory status of the activated LCs in the 24-h posttape-stripping specimens. This was followed by subsequent observation in the 1-week biopsies of abundant HLA-DR expression in the epidermis (LCs and keratinocytes) and dermis

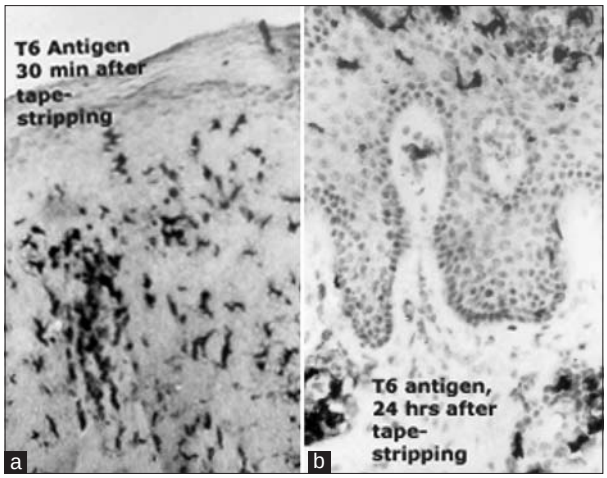

Figure 7: (a) Left panel: Uninvolved skin from psoriatic patient 30 minutes after tape-stripping showing $T6^+$ Langerhans cells (immunohistochemical staining) throughout the epidermis. (b) Right panels: Same patient showing $T6^+$ Langerhans cells in the upper epidermis and within the dermal blood vessels 24 hours after tape stripping, but there was depletion of $T6^+$ Langerhans cells in the lower half of the epidermis (×40)

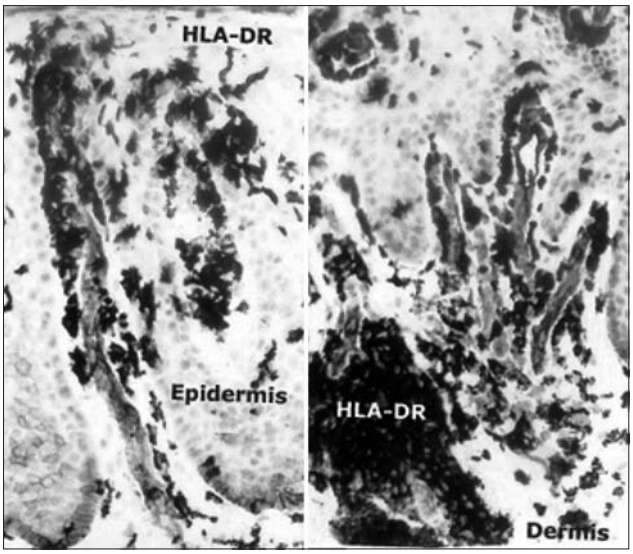

Figure 8: Abundant expression of HLA-DR antigens on dendritic cells/Langerhans cells, T cells and macrophages in the epidermis and dermis, and endothelial cells in the dermis four weeks after tape-stripping of uininvolved psoriatic skin, showing markedly amplified immune response. In non-psoriatic tape-stripped skin, amplification was much less marked than that in psoriatic skin, correlating with lesser (10 to 20%) of Langerhans cell activity at each time point (×40)

(T-lymphocytes and endothelial cells), implying further amplification of the immune response [Figure 8]. HLA-DR antigens were expressed initially on activated LCs, and later on cytokine-activated dendritic cells, keratinocytes, endothelial cells, T-lymphocytes, and macrophages.

DISCUSSION

The activation of LCs within 5–30 min following tape-stripping injury is accompanied by loss of BG and conversion to a migratory status of the cell. The presence of BG at the cell surface of some activated cells suggest their probable function in LC activation. Both T6 antigen (CD1a; class I histocompatibility complex) and langerin (class II lectin/CD207) have been shown to localize in the BG.[19] In addition, both T6/CD1a and langerin/CD207 have been shown to be necessary for efficient presentation of nonpeptide antigens to the LC for its activation.[20] These observations suggest that BG may be involved in the mechanism of early LCs activation. Membranes containing presynthesized lectin receptors (langerin) and activation molecules (CD1a) may be simultaneously and colocally expressed on the cell surface of LCs within minutes of the injury stimulus. This may necessary for $T6^+$ LC to acquire migratory status [Figure 7] in order to activate T-cells in the lymph nodes to amplify the immune response.

Heat shock proteins (HSPs) are secreted minutes after the injury stimulus and have been reported by our laboratory to be expressed after injury in the following tissues – arteries[21] and colon.[22] HSP 60/65 have been shown to be the cognate antigen of a group of dendritic cells with $TCR\gamma\delta$,[23] with lectin binding properties.[24] These HSPs may also bind to toll receptors.[25,26] It has been reported that curcumin, which attenuates acute tissue inflammation, achieves this by inhibiting the toll receptor (TLR4)-mediated NF-kB-dependent pathways.[27] HSPs produced by damaged keratinocytes during tape-stripping injury may also be the cognate antigen activating LCs. It is possible that because the LCs do not express $TCR\gamma\delta$, the colocalization of both T6/CD1a (class I MHC antigen) and the type II langerin/CD207 receptor may be necessary to recognize cognate nonpeptide antigens. Early response requires early delivery of these molecules by BG to the cell surface of the LCs. Early expression of toll receptors on LCs surface may also be achieved through delivery by BG.

Early changes in the activated LCs 5–30 min after tape-stripping include enlarged Golgi apparatus with prominent cisternae [Figure 3]. The Golgi apparatus contain enzymes within the cisternae that function in modifying, sorting and packaging macromolecules, including proteins delivered from the RER, transport of lipids (including cell-membrane lipids), synthesis of glycosaminoglycan through xylose linkage and sulfation, and phosphorylation of molecules by phosphorylating enzymes. One such enzyme, responsible for early phosphorylation events is PhK, which breaks down glycogen to ATP for cellular energy needs.[3-6] It is believed to be responsible for activating transcription activators such as NF-kB through phosphorylation of key sites on the NF-kB molecule. In addition, it also

activates the IkBα kinase subunits responsible for removing the inhibitory molecule from the p50 and p65 NF-kB dimers, so that the dimers can translocate to the nucleus, to bind to the kB site on the DNA.[8-12] This step is necessary to stimulate transcription of over 200 different genes that participate in the injury pathway.[9-12]

A major function of PhK in biologic tissues is the breakdown of glycogen granules to generate ATP for metabolic needs. The association of loss of glycogen granules in the keratinocytes located in the vicinity of activated LCs suggests that PhK released by LCs may be responsible for the depletion of glycogen granules in injured psoriatic keratinocytes. Our laboratory has previously reported that elevated PhK in psoriatic epidermis was found to correlate with increased phosphorylation and psoriatic activity.[3] Additional, we also demonstrated in clinical studies that suppression of PhK activity by curcumin, a selective and noncompetitive PhK inhibitor,[7] resulted in significant improvement of clinical psoriasis.[10] It is believed that the suppression of NF-kB by curcumin,[7-9] achieved through suppression of PhK activity[3,7-10] may play a key role in the resolution of increased proliferative activity in psoriasis.

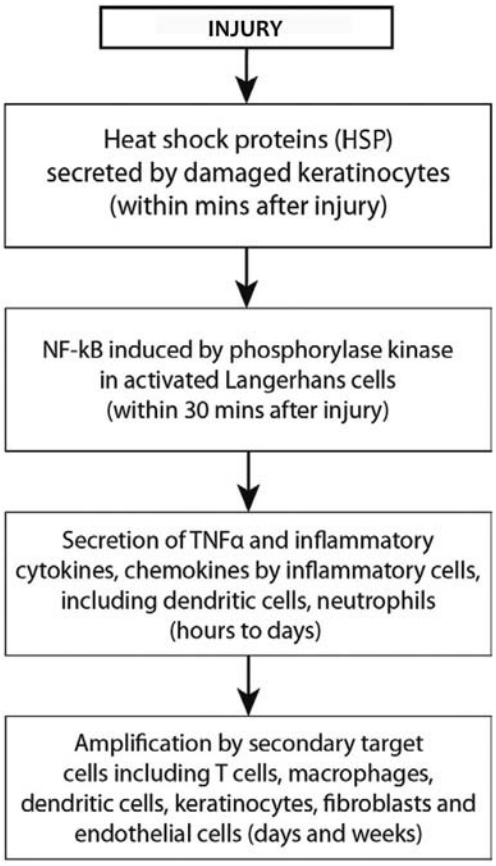

Figure 9: Summary of early sequence of events in the injury pathway

CONCLUSION

Our findings that LCs are the first cell to be activated in the injury pathway [Figure 9], followed by recruitment of other inflammatory cells with amplification of the immune response, are consistent with a previous report[28] of the presence of more than one generation of activated dendritic cells. While investigating why unstimulated LCs are less activated than dendritic cells, these investigators[28] used intradermal immunization with plasma DNA, a system in which the activation of CD8(+) T-cells depends on delayed kinetics of antigen presentation. It was then noted that dendritic cells located in the skin at the time of immunization had limited ability to activate CD8(+) T-cells, while CD8(+) activating capacity was observed in a second generation of in situ epidermal dendritic cells several days after immunization.[28] We have proposed a hypothetical summary of the early events induced by injury followed by amplification of the immune response in Figure 9.

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