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A Critical Role for the Proapoptotic Protein Bid in Ultraviolet-Induced Immune Suppression and Cutaneous Apoptosis

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Apoptosis plays an important role in eliminating UV-damaged keratinocytes, but its role in UV-induced immune suppression is not clear. Langerhans cells (LCs) may function as inducers of immune suppression. We have shown that LCs derived from mice deficient in the proapoptotic Bid (BH3-interacting death domain protein) gene (Bid KO) resist apoptosis and induce amplified immune responses. In this report, we examined responses in Bid KO mice to UVB exposure. Acute UV exposure led Bid KO mice to develop fewer apoptotic cells and retain a greater fraction of LCs in the epidermal layer of skin in comparison to wild-type mice. Bid KO mice were also markedly resistant to local and systemic UV tolerance induction to hapten sensitization and contact hypersensitivity responses. Elicitation responses and inflammation at skin sensitization sites in UV-treated Bid KO mice were equal to or greater than nonsuppressed control responses. In Bid KO mice, LCs accumulated in lymph nodes to greater numbers, demonstrated longer lifespans, and contained fewer DNA-damaged cells. These studies provide evidence that Bid activation is a critical upstream mediator in UV-induced keratinocyte and LC apoptosis and that its absence abrogates UV-induced immune tolerance. The Journal of Immunology, 2008, 181: 3077–3088.

The epidermis is our first line of defense against environmental damage, including highly mutagenic UVB irradiation (290–320 nm). Epidermal cells have evolved a highly regulated set of UV damage responses that initially serve to both protect against the carcinogenic effect of UVB, by eliminating highly damaged cells through apoptosis, but at the same time retain and repair damaged cells to maintain skin barrier function (1). In addition to eliminating damaged cells, some molecular pathways of apoptosis are necessary for normal keratinocyte differentiation (2). Those that are not essential for homeostatic keratinocyte differentiation may be critical for certain stress-induced skin responses. One such molecule is the Bcl-2 family member Bid (BH3-interacting death domain protein). While Bid protein expression is reportedly high in differentiated epidermis (3), Bid knockout (KO) mice manifest normal keratinocyte differentiation (4).

Apoptosis1

1 Abbreviations used in this paper: Bid, BH3-interacting death domain protein; CHS, contact hypersensitivity; CPD, cyclobutane pyrimidine dimer; DAPI, 4′,6′-diamidino-2-phenylindole; DC, dendritic cell; DNFB, 2,4-dinitro-1-fluorobenzene; KO, knockout; LC, Langerhans cell; LN, lymph node; RANK, receptor activator of NF-κB; TRANCE, TNF-related activation-induced cytokine; Treg, regulatory T; UVR, ultraviolet radiation; WT, wild type.

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However, since Bid is a potent proapoptotic molecule, it may be an important regulator of certain photodamage-induced responses.

As part of the “BH3-only” domain subset of proteins, Bid activates the intrinsic mitochondrial pathway of apoptosis (5). However, Bid is unique in that it links “extrinsic” death receptor signals to the “intrinsic” mitochondrial pathway. Bid activation results from death receptor-induced caspase-8-mediated cleavage, producing a truncated form (tBid) that translocates to mitochondria, where it efficiently activates the downstream pathways of apoptosis (6–8). Since death receptor activation plays a prominent role in developing “sunburn” apoptotic cells (9, 10), and since Bid is highly expressed in differentiated keratinocytes, we reasoned that Bid may be a key mediator in this process. It is known that UV-induced sunburn cell formation is dependent on the expression of tumor suppressor p53 (11, 12), and p53 up-regulates Bid gene transcription (13). Thus, Bid-dependent UV-induced responses may be up-regulated in cells that normally do not express high levels of Bid, such as normal primary basal cells. Indeed, Bid cleavage has been detected in human primary keratinocyte cultures in response to low doses of UV, which potentiates further cleavage in the presence of TRAIL (14). While Bid cleavage has been detected in epidermal cell lines and keratinocyte cultures after UV exposure in vitro (14, 15), the role of Bid as a critical mediator of cutaneous cell apoptosis remains undefined.

Interestingly, besides keratinocytes, epidermal Langerhans cells (LCs) are also important targets of UV-induced damage. It has been observed that within 24–48 h after UV exposure of epidermis, LC numbers and dendritic morphology diminish (16–18). Furthermore, the number of LCs able to reach the draining lymph nodes (LN) is also diminished. The loss of LCs may be due to apoptosis and/or inhibited migration to draining LNs (17, 19). It is also likely that LCs that do make it to the draining LNs have altered presentation function that promotes the development of regulatory T (Treg) cells (20–22). The altered LC function may be
due to direct DNA damage by UVB (which can be identified by Abs to cyclobutane pyrimidine dimers (CPD)) or to indirect immune modulation in response to keratinocyte-derived signals, such as soluble mediators like platelet-activating factor, cis-urocanic acid, IL-10, or expression of TNF-related activation-induced cytokine (TRANCE) (the ligand for receptor activator of NF-κB (RANK)) that is produced by UV-damaged keratinocytes (23–26). Direct DNA damage has been shown to correlate with UV immune suppression. This was shown in a series of experiments in which topical liposomal delivery of bacterial restriction enzymes induced IL-10 expression and inhibited contact hypersensitivity (CHS) responses (27, 28), while application of photolyase (which efficiently repairs pyrimidine dimers) inhibited local immunosuppression and blocked induction of Treg cells by UV-treated cutaneous APCs in vitro (29). However, these experiments do not directly assess the role of LC apoptosis in regulating UV-induced immune suppression.

In our previous studies, we showed that Bid KO mice could promote enhanced CHS to hapten, and isolated LCs were resistant to apoptosis and able to induce enhanced immunity when transferred to naive recipients (30). Because LC function figures prominently in the mechanism of UV-induced immune suppression, we investigated whether Bid KO mice demonstrated altered responses to UV-induced damage by keratinocytes and LCs. Our results indicate that epidermal keratinocytes and LCs depend on Bid to mediate UV-induced apoptosis. Furthermore, UV-induced immune suppression and tolerance are blocked in Bid KO mice, and this correlated with increased LC accumulation within draining LNs. Our data strongly support that Bid-dependent LC apoptosis is a critical response in the mechanisms of immune down-regulation following exposure to UV radiation (UVR).

Materials and Methods

Mice

Bid KO mice were bred on a C57BL/6 genetic background and were generously provided by Dr. Stanley J. Korsmeyer (Harvard Medical School, Boston MA). Age- and sex-matched (8–12-wk-old) C57BL/6 mice, termed wild type (WT), were purchased from The Jackson Laboratory. The institutional review board approved all animal protocols.

Chemicals and mAbs

Ammonium thiocyanate, 2,4-dinitro-1-fluorobenzene (DNFB), olive oil, BSA, acetone (reagent grade), sodium hydroxide, and sodium chloride were purchased from Sigma-Aldrich. Monoclonal anti-CPD or an isotype control Ab (lgG1) was purchased from Kamiya. mAbs specificities were as follows: I-A (2G9), CD11c (HL3), CD8a (53-6.7), and streptavidin-PerCP was from Dendritics (AbCys Biologie) or eBioscience (eBioRMUL.2). Peroxidase-conjugated steptavidin and a diaminobenzidine kit were from Vector Laboratories. The DeadEnd colorimetric TUNEL system was purchased from Promega. Dispase II and collagenase D were purchased from Roche Applied Science. Fluorescein-anti-G was purchased from Southern Biotech Associates, and attenu isofluran was supplied through the Animal Resource Program of the University of Alabama at Birmingham.

UVB irradiation

The dorsal sides of mice were shaved with electric clippers 1 day before UV exposure. Control groups of mock-treated mice were not exposed to UVB but were also shaved. UVB irradiation was done on anesthetized mice, and their eyes and ears were covered to protect from UBV exposure. A Daavlin UVA/UVB research irradiation unit, equipped with an electronic controller to regulate UV dosage, was used to emit URAV radiation from a band of four FS20 UVB lamps. The UV lamps emit UVA (280–320 nm; 80% of total energy) and UVA (320–375 nm; 20% of total energy), with very little UVC emission (<1%). Most of the resulting wavelengths of UV radiation are in the UBV range (290–320 nm), with peak emission at 314 nm. The UVB emission was also monitored with an IL-1700 research radiometer (International Light Technologies).

Histology

Midline dorsal skin or ears were excised promptly after euthanasia and placed immediately in 70% alcohol-buffered formalin. The tissues were fixed for 24 h in formalin and then embedded in paraffin. Sections of 6-μm thickness were cut and processed (UAB Skin Diseases Research Core Facility, the University of Alabama at Birmingham) for H&E staining to identify sunburn cells, TUNEL staining for apoptosis, or CPD staining (data not shown) on serial sections. Stained slide sections were examined by bright-field microscopy and images captured with an Olympus DP70 digital camera and further analyzed using ImagePro Plus software version 6.0 (Media Cybernetics).

Detection of apoptotic cells in epidermis

Paraffin-embedded tissue sections on the slides were immersed in the xylene (Sigma-Aldrich) twice for 5 min each to remove paraffin and then rinsed with 100% ethyl alcohol for 5 min. Tissue sections on slides were rehydrated through ethanol gradient in decreasing concentrations (100%, 95%, 85%, 70%, and 50%) and washed by 0.85% NaCl solution for 5 min. Tissues were fixed in 4% paraformaldehyde followed by two washes in PBS for 5 min each, treated for 20 min in proteinase K solution (Promega) with 20 μg/ml, 100 μtissue to permeabilize the tissue, and then permeabilized further in Triton-X buffer. Tissue sections were incubated in equilibration buffer for 10 min at room temperature, then labeled with rTdT enzyme and biotin-DUTP (Promega) reaction mix (100 μ/sample) during 60 min incubation at 37°C in the humidified chamber (negative control was treated with reaction mix except rTdT enzyme). After incubation, slides were immersed for 15 min in 2× SSC solution and washed with PBS 3 times for 5 min each. Endogenous H2O2 was blocked by immersing the slide in a 3% H2O2 (Sigma-Aldrich) solution for 5–10 min and then was washed with PBS twice for 5 min each. Tissue sections were incubated for 30 min in room temperature in 100 μ/sample of streptavidin-HRP (1/500) in PBS. Slides were rinsed with PBS 3 times for 5 min each and stained with 100 μ/sample diaminobenzidine freshly prepared solution for 3 min until a brown color developed. Sections were rinsed in deionized water three times. Tissue sections were mounted with Crystal/Mount (Biomed). Images were captured at ×20 and ×40 magnification, and 10 fields per section, three sections per animal, and three animals per treatment were analyzed in a blinded fashion for quantification of TUNEL− cells.

Staining of Langerhans cells in epidermis

Age-matched females of WT and BID KO were anesthetized and exposed to varying doses of UBV as indicated. After 24 h, ears were harvested from euthanized mice and separated into dorsal and ventral halves. Ear halves were frozen, dermis side down, on a 0.5% ammonium thiocyanate solution (Sigma-Aldrich) for 30 min at 37°C. Dermis was separated from epidermis, gently rinsed in PBS, and then fixed in cold acetone for 30 min on a rotator at 4°C. Acetone was replaced every 10 min following with three washes with cold PBS. Sheets were placed in a blocking Fluoro-Ab solution at a concentration of 4 μg/ml per sample (clone 2.4G2 hybridoma from American Type Culture Collection) for 30 min in 1% BSA at room temperature on a rotator. After the 30-min incubation, FITC-conjugated anti-I-A Abs or FITC-isotype control was added at 1 μg/sample (clone C9G9, BD Pharmingen) and incubated in the dark overnight on a rotator at 4°C. The next day sheets were washed three times in PBS and then mounted in Fluoromount-G. Fluorescent images were captured at ×20 or ×60 magnification and analyzed with MetaMorph software (Universal Imaging) provided by the UAB Imaging Core Facility or captured with a DP70 digital camera (Olympus) and further analyzed using ImagePro Plus software version 6.0 (Media Cybernetics). At least three sheets per treatment group were analyzed, and 10–20 fields per sheet were imaged for enumeration. The number of I-A−cells was enumerated after setting a standardized green intensity threshold to enhance contrast and reduce background staining.

Western blot analysis

Cytoplasmic fractions were obtained following the manufacturer’s instructions of a cytoplasmic extraction kit (no. 78833 from Pierce Biotechnology). This kit stringently clears supernatant of mitochondria. Blots of 10% SDS-PAGE gels loaded with 20 ± 5 μg protein were first blocked for 1 h with 5% nonfat milk TBST, then incubated further with Abs to β-actin (1/5000; mouse mAb no. A5316, Sigma-Aldrich) and cytochrome c (1/500; mouse mAb no. 13156, Santa Cruz Biotechnology) overnight at 4°C. HRP-conjugated goat anti-mouse antiserum (1/8000; no. 31400, Pierce) was incubated for 1 h at room temperature. ECL-treated membranes were wrapped in plastic and exposed to x-ray film for 45 s (actin) and 1.5 min (cytochrome c). The quantitative analysis was performed on a Macintosh
computer using the public domain National Institutes of Health Image program (developed at the U.S. National Institutes of Health and available at http://rsb.info.nih.gov/nih-image/).

**Cutaneous migratory dendritic cells (DCs) from split ear cultures**

Age-matched female mice (WT and Bid KO, n = 5/group) were anesthetized and exposed to UVB at 120 mJ/cm². Control mice had their ears tape-tripped 10 times on both dorsal and ventral sides to activate LC migration. After 4 h, mice were sacrificed and ears recovered and split into dorsal (UV-exposed) and ventral (nonexposed) sides to float in culture as described previously (30). After 3 days of culture, cells were counted in triplicate to determine cell recoveries and viability, then adjusted to 4 × 10⁶ cells/ml PBS. Samples of 100 μl were subjected to cytosin centrifugation at 1500 rpm for 5 min. Cytospin slides were fixed in cold acetone for 10 min and stored at −20°C until staining.

**Immunofluorescence assays for enumeration of CPD-positive cells in migratory DCs**

To detect CPD⁺ cells in migratory cells from the UVB-exposed ears, immunostaining was done as described previously (31), with a slight modification. Briefly, fixed cytosin slides were rehydrated in PBS for 10 min. DNA was denatured using 70 mM NaOH in 70% ethanol for 2 min, followed by neutralization with 100 mM Tris-HCl (pH 7.5) in 70% ethanol for 1 min. Slides were washed with PBS two times for 5 min each and then incubated for 30 min with 10% goat serum in PBS to block nonspecific binding. Slides were then incubated with anti-CPD mAb (0.5 μg/ml, Kamiya) or an isotype control (murine IgG1) for 1 h and washed with PBS three times for 5 min each. Then, slides were incubated with FITC-conjugated rat anti-mouse IgG for 30 min and washed with PBS three times for 5 min each, followed by incubation with fluorescence dye 4′,6′-diamidino-2-phenylindole (DAPI) for nuclear staining. A parallel set of cytosin slides was stained with anti-MHC II (I-A) Ab. Slides were incubated with an anti-CD16/CD32 Ab (2.4G2) and 3% BSA to block nonspecific binding and then stained with FITC-labeled anti-I-A (clone 2G9, BD Biosciences). Slides were counterstained with DAPI, washed, and then coverslipped over Gel Mount medium (BioMedica). Photomicrographs were taken with a ×20 objective. CPD⁺ or I-A⁺ cells were counted in 12 or 5 fields, respectively, for each group. The means and SDs were calculated and the difference between groups was analyzed statistically using Student’s t test.

**Local immune suppression of CHS**

Mice (WT or Bid KO) were exposed to UBV at 100 mJ/cm² daily for 4 consecutive days on the shaved dorsal skin. Ears were protected during UBV exposure. On day 5, mice were sensitized on UV- or mock-treated dorsal skin with 25 μl of either vehicle (4:1 acetonite and olive oil) or 0.5% DNFB emulsified in vehicle (S1). On day 5 post-sensitization, mice were challenged with DNFB Ag by painting ears with 10 μl of 0.3% DNFB on the dorsal and ventral side of each ear. CHS swelling responses were measured each day with an engineer’s micrometer (Mitutoyo). The averages of three readings per ear over the baseline measurement for each ear were recorded daily during the indicated period and expressed in micrometers. Groups of at least five mice were measured per treatment.

**UV-induced tolerance induction to hapten**

Mice (WT or Bid KO) were exposed to UBV at 100 mJ/cm² daily for 4 consecutive days on the shaved dorsal skin. Ears were protected during UBV exposure, unless otherwise noted. On day 5, mice were initially sensitized on UV- or mock-treated dorsal skin with 25 μl of either vehicle (4:1 acetonite and olive oil) or 0.5% DNFB emulsified in vehicle (S1). Two weeks postsensitization (day 19), mice were sensitized again (S2) on unexposed shaved ventral skin (abdomen) with 25 μl of 0.5% DNFB. After 5 days, (day 24) mice were challenged with 10 μl of 0.3% DNFB on the dorsal and ventral side of each ear. CHS swelling responses were measured each day with an engineer’s micrometer as above. Groups of at least five mice were measured per treatment, and the specific ear swelling responses are reported as the difference in ear thickness from baseline measurements obtained for each ear.

**Detection of CPD⁺ LCs in vivo**

UBV-induced DNA damage in LCs was determined using a procedure described previously (32). WT or Bid KO mice were shaved dorsally with an electric clipper on day −1. Mice had both backs and ears exposed to 100 mJ/cm² UV for 4 consecutive days. On day 5, backs and ears were painted with vehicle or 0.5% DNFB in vehicle as described. After 3 days, mice were sacrificed and superficial parotid (ear draining) and sublingual (often referred to as inguinal) LNIs (see Ref. 33 for nomenclature) were pooled per animal (n = 3/test group). LNIs were digested by collagenase D (2 mg/ml in HBSS containing HEPES buffer, Roche) for 30 min at 37°C. RPMI-10% FBS medium was added to neutralize collagenase D and filtered through 150-μm nylon mesh. Cell suspensions from each mouse were washed, counted, and then stained for flow cytometric analysis. Cells (5 × 10⁶/sample) were blocked with hamster and goat γ-globulin (Jackson ImmunoResearch Laboratories) (5 μg/sample) then stained with 0.5 μg PE-Cd11c or PE-hamster isotype. Washed cells were treated with Fix/Perm solution, then stained in Perm/Wash solution with 0.2 μg Alexa 647-Cd207 or 0.5 μg CPD mAb or isotype controls for 30 min on ice as per BD Biosciences intracellular staining protocol. FITC-goat anti-mouse (1/100 dilution, Jackson ImmunoResearch Laboratories) in Perm/Wash was washed an additional 2 times and washed and incubated another 30 min on ice. Cells were washed in PBS and fixed in 1% paraformaldehyde before flow cytometric analysis.

**FITC painting to track skin-derived cells in LN**

Mice (n = 3/group) were painted on the dorsal side of ears with 10 μl of 0.5% FITC in vehicle (acetone-dibutylphthalate, 1:1) or with vehicle alone. After 2 h, mice were exposed to daily doses of 0, 120, or 400 mJ/cm² UBV for 4 days. Parotid, ear draining LNs were harvested 3 days later and pooled for each individual mouse. Single-cell suspensions were counted and 5 × 10⁶ cells per sample were stained with PE-anti-Cd11c and CD8a. Multicolor flow cytometric analysis was performed on 10⁶ cells per sample. Student’s t test was applied to data for determining significant differences between groups.

**Statistical analysis**

Paired Student’s t test (two-tailed, unless noted otherwise) was performed using Prism 4.0 for Macintosh (GraphPad Software) or Excel (Microsoft) software to calculate significant differences. Significant values are reported in the text and figure legends.

**Results**

**UV-induced apoptosis is reduced in Bid KO epidermis**

UV radiation leads to morphological and functional alterations in epidermal cells, including apoptosis. To determine whether Bid KO mice sustained more or less UV damage than did WT mice, we compared levels of apoptosis in the epidermis from both strains 2 days after UV exposure. Damaged cells were identified as sunburn cells by H&E or apoptotic cells by TUNEL staining. Fig. 1a of H&E-stained epidermis sections revealed normal structural integrity of Bid KO epidermis, indicating that Bid is not required for normal keratinocyte differentiation. More sunburn and TUNEL⁺ cells (black arrows) were identified in UV-treated WT epidermis than in Bid KO epidermis. The numbers of TUNEL⁺ cells that developed in the epidermis after exposure to different doses of UV were compared (Fig. 1b). The mean numbers of TUNEL⁺ cells (per mm² ± SEM) for WT vs Bid KO epidermis were as follows: 36 ± 2 vs 22 ± 2 (n = 38) and 54 ± 5 vs 30 ± 2 (n = 65) for 100 and 200 mJ/cm² doses, respectively. Therefore, the level of UV-induced apoptosis in Bid KO epidermis was reduced by 38% and 44%, respectively. In other studies performed with cultures of primary skin keratinocytes, we observed that Bid KO cells maintained resistance to UV-induced apoptosis at in vitro doses up to 100 mJ/cm², as compared with WT cells, over longer times (e.g., 3 days in culture) (data not shown). These data suggest that Bid KO cells are not delayed in their apoptotic response. Bid KO cells also demonstrated reduced levels of cytochrome c release in the cytoplasmic fraction, as compared with WT derived samples, where 2-fold over control levels were measured 4 h after UVB exposure (Fig. 1, c and d). However, at higher doses of UVR (up to 200 mJ/cm²), necrotic cell death was observed at levels that were not significantly different between Bid-deficient and WT UV-exposed cells (data not shown). Collectively, these data indicate that Bid expression is not required for UV-induced necrosis, but that it is critical for mediating UV-induced apoptosis in epidermal keratinocytes.
Epidermal LC densities are retained in Bid KO mice after UV exposure

Since LC loss is an important marker of epidermal UV damage responses, we compared the number of remaining LCs in WT and Bid KO mice ear skin 2 and 7 days after a single exposure to various doses of UVB radiation (0–400 mJ/cm²). LC depletion increased in a UVB dose-dependent manner. However, the extent of depletion was significantly reduced in UV-exposed Bid KO epidermis as compared with WT epidermis at all UVB doses tested on day 2 (Fig. 2, left panel), and on all but the highest UVB dose (400 mJ/cm²) on day 7 (Fig. 2, right panel). Notably, the morphology of Bid KO LCs was enlarged as compared with WT LCs after exposure to low-dose UVB (50 mJ/cm²) and was easily observed on day 7, suggesting that Bid KO LCs were activated as opposed to undergoing apoptosis. The morphology of WT LCs differed strikingly from Bid KO LCs on day 7. LCs developed enlarged rounded cell bodies with blebbing (see WT at 100 mJ/cm² in Fig. 2a, inset), as well as diffuse smeary (WT 50 mJ/cm²) or blebbly dendritic processes (WT 200 mJ/cm²), providing morphological hallmarks of apoptosis in situ (indicated by the white arrow). We also noted that the tissue integrity of epidermis was maintained in Bid KO but became quite fragile in WT mice after larger UVB exposure doses. Continued depletion of LCs was observed after 7 days for both strains at the high UV doses (200–400 mJ/cm²), but at low UV doses (50 mJ/cm²) Bid KO LC numbers remained stable while WT LC numbers declined. These findings suggest that most of the UV-induced LC depletion (migration out of the epidermis or apoptosis) occurs within 2 days, and continuous apoptosis may occur thereafter. However, the Bid KO LCs remaining in epidermal layers 7 days following UV treatment displayed cell morphologies consistent with cell activation, indicating they were resistant to UV-induced apoptosis.

DNA-damaged DCs from WT and Bid KO mice exhibit identical capacities for migration from UV-exposed skin explant cultures

The different levels of LC depletion seen in the epidermis of UV-exposed WT and Bid KO mice suggested that we would observe correlative differences in the number of photodamaged cells that migrate from the skin of these two strains. Therefore, we examined migratory cells derived from ear skin harvested 4 h after a single dose of 120 mJ/cm² UVB radiation. Skin subjected to tape-stripping to activate LC migration served as a UV-negative control, as well as a positive control for LC migration. Ears were split and cultured in groups of dorsal skin (fully exposed to UVR and an indicator of direct UV damage) and ventral skin (a control for partial UV-exposed skin) for 3 days, then samples were pooled for each treatment group and assessed for total cell numbers, viability, CPD⁺ cells, and I-A⁺ cells. We observed that dorsal skin cultures accumulated similar total numbers of cells (Fig. 3) (7.9 × 10⁵ cells and 9.5 × 10⁵ cells per dorsal side, respectively, for WT and Bid KO samples), as well as numbers of CPD⁺ and I-A⁺ cells from both mouse strains. Interestingly, we observed a significant increase in the both the number of dead cells and CPD⁺ cells (Fig. 3b, bottom left and middle panels, respectively) recovered from WT cultures of ventral ear skin as compared with cells from parallel cultures of Bid KO ventral skin (Fig. 3b, top panels). Whether the increase in both dead and CPD⁺ subsets is related remains to be determined. All CPD⁺ staining was located in the nuclei of cells, consistent with direct photodamage. We failed to detect CPD staining in the cytoplasm of migratory cells, which might identify cells that were not directly damaged but had secondarily incorporated...
photodamaged cell DNA. Thus, our staining technique is limited to detecting cells that have sustained direct DNA photodamage. Collectively, data from UV-treated dorsal skin cultures indicate that WT cutaneous DCs are as viable and as active as Bid KO DCs during this 3-day timespan after a single-dose UVB treatment. This observation is consistent with our data presented in Fig. 2, showing that LC apoptosis is only detected in the epidermis late (5–7 days) after UVB treatment.

Bid KO mice accumulate greater numbers of LCs in draining LNs after 4 days of UV exposure

Because Bid KO LCs exhibited resistance to UV-induced apoptosis in situ, we investigated whether the numbers of LCs found in draining LNs might also differ between UV-treated WT and Bid KO animals. Mice were exposed to 100 mJ/cm² UV on 4 consecutive days, and then draining LNs were harvested for the following consecutive 3 days to enumerate LCs by flow cytometric analysis (Fig. 4). Consistent with our previous findings (30), steady-state LC numbers were the same in untreated mice from both strains (50,000 LCs/LN, Fig. 3b, bottom panel, day 0). A modest difference in the percentage of Langerin⁺ cells was observed (7.4% vs 5.3% in Bid KO vs WT, Fig. 4a) on day 0, but no consistent differences in the percentage of CD11c levels were observed at any time (Fig. 4b). However, opposite patterns of cellular composition were observed in LNs from these two mouse strains following UV exposure. One day after the final UV dose (day 1), increases in both the total numbers of LN cells (from 7.4 × 10⁶ to 11.3 × 10⁶ cells per LN) and the percentage of Langerin⁺ cells resulted in a 3-fold increase in LC numbers (in comparison to steady-state levels) within Bid KO LNs. In contrast, within WT LNs, total numbers of LN cells declined modestly and the percentage of Langerin⁺ cells dropped 2-fold, resulting in a 1.7-fold decrease in LC numbers. On the following day (day 2), the numbers of LCs from both strains dropped but at different rates, decreasing from day 1 levels by 50% and 70% for Bid KO and WT, respectively. On day 3, Bid KO LC numbers dropped 30% to control levels, while WT LC numbers rebounded 2-fold, achieving 50% of control levels. Mechanisms that may contribute to the increased numbers of Bid-deficient LCs in LNs include a greater capacity to 1) migrate to dLNs, 2) recruit Langerin⁺ cells from nonepidermal sources, and/or (3) resist mechanisms of DC attrition observed in LNs of UV-exposed WT mice.

Bid KO mice are resistant to UV-induced local immune suppression

To test if resistance to UV-induced apoptosis would alter UV-induced local immune suppression, we subjected Bid KO and WT...
mice to 100 mJ/cm² of UVB daily for 4 consecutive days on shaved backs, while ears were protected. The day following the last UV dose, dorsal skin was sensitized with hapten by painting with 0.5% DNFB or vehicle alone as a control. Five days post-sensitization, ears were treated with hapten by applying 10 μl 0.3% DNFB per side, and the elicitation of a swelling response due to CHS was measured each day following (Fig. 5). An enhanced CHS response was observed in Bid KO mice as compared with WT mice under normal conditions, as described previously (30). However, while UV exposure completely abrogated the CHS response in WT mice, only a modest reduction in CHS was observed in Bid KO mice.

**FIGURE 3.** Equivalent numbers of CPD⁺ and I-A⁺ cells migrate from UV-exposed dorsal ear skin in WT and Bid KO mice. Four hours after treatment with UVB 120 mJ/cm², mice were sacrificed and ear skin was harvested for ex vivo culture. Dorsal (UV-exposed) and ventral (variable UV exposure) split skin samples were cultured for 3 days. Pooled cells for each group were assessed for cell counts and viability before making cytospin slides. a. Fluorescence microphotographs are shown for sets of slides stained for I-A or CPD (green) and nuclei with DAPI (violet). Merged images demonstrate that CPD staining is detected only in the nuclei of cells. Views with higher than average % CPD⁺ nuclei are shown. I-A is detected only in the cytoplasm. Images were captured at ×20 magnification. Higher magnification images are shown to enhance detail; 50 μm bar is shown in yellow. b. The number of I-A⁺ and CPD⁺ cells in migratory populations from dorsal skin cultures are equivalent for UV-treated Bid KO and WT strains. Student’s t test p value designations are: *, p ≤ 0.05; **, p ≤ 0.01; ***, p ≤ 0.001; nd, not determined (due to low cell yields from WT).
Bid KO mice do not develop UV-induced tolerance to hapten-specific responses

We next tested whether the Bid deficiency might alter susceptibility to UV-induced tolerance. Following well-established protocols (32), the dorsal skin of UV-treated mice (UVB 100 mJ/cm² for 4 consecutive days) was sensitized with 0.5% DNFB (S1) or vehicle alone as control. Two weeks later the same mice were secondarily sensitized (S2) on the ventral side (UV untreated) with 0.5% DNFB or vehicle alone, as indicated (Fig. 6). UV-protected mouse ear skin was challenged 5 days later with 0.3% DNFB and CHS responses were measured by specific ear swelling responses on day 2. Striking differences in appearance of dorsal skin from UV-tolerized WT and Bid KO mice were observed. Bid KO mice that underwent the UV-tolerizing regime (group 3) developed inflamed lesions at sites of hapten sensitization, especially on UV-treated dorsal skin (indicated by the white arrows in Fig. 6a), whereas WT mice did not. This correlated with dramatic differences in ear swelling responses (Fig. 6, b and c). Increased ear swelling responses were observed by Bid KO mice as compared to WT mice for all treatment groups, outside of the control group. The ear thicknesses measured for WT vs Bid KO per treatment group are listed as follows (mean specific ear swelling response (µm) ± SEM values are shown. SEM values were usually smaller than the width of the circle. Results from one of two similar experiments are shown.

FIGURE 4. Enhanced accumulation of LCs detected in skin draining LNs of UV-exposed Bid KO mice compared with WT mice. Mice were irradiated with 100 mJ/cm² UVB (on shaved backs and ears) daily for 4 days. Mice were sacrificed on the days indicated following the last UV dose, and draining LNs (n = 6/mouse; two mice per group) were pooled and prepared for flow cytometric analysis. Data from untreated control mice are shown as day “0” samples. a, Contour plots of LN cells stained with PE-CD11c and Alexa 647-Langerin. Upper right quadrant number indicates percentage Langerin⁺ cells within the CD11c subset. b, Kinetics of LC cell accumulation differs markedly in UV-exposed Bid KO (black bars) vs WT mice (white bars). Total cell numbers, percentage CD11c, percentage Langerin, and number of Langerin⁺ cells (LCs) per node were determined. Means ± SD for two mice per group are shown. Values from duplicate animals showed significant differences between strains as shown (Student’s t test; *, p < 0.05). One of two similar experiments is shown.

FIGURE 5. Impaired local UV suppression of CHS responses in Bid KO mice. Mice were exposed to daily UVB for 4 days to induce local suppression to DNFB sensitization (as outlined in Materials and Methods, ears were shielded). After 5 days, ears were painted with DNFB (0.3%) and specific ear swelling responses (CHS) were measured as differences from baseline ear thickness in micrometers. CHS responses are shown for WT mice (a) and Bid KO mice (b). DNFB (0.5%) was applied on normal shaved backs (○), UVB-exposed shaved backs (●), or vehicle was applied to UVB-treated backs (○ with dotted lines) as a control. Four to five mice (8–10 ears) were measured per CHS group, while control groups had two mice (4 ears) per group. The mean and SEM values are shown. SEM values were usually smaller than the width of the circle. Results from one of two similar experiments are shown.
FIGURE 6. Bid KO mice are resistant to UV-induced tolerance induction. Mice were subjected to UVB for 4 consecutive days (with ears shielded, as outlined in Materials and Methods) on shaved backs (dorsal) or mocked treated (shaved, no UV). The first sensitization (S1) with 0.5% DNFB was applied to dorsal skin on day 5, and a second sensitization with 0.5% DNFB on non-UV-treated ventral skin (abdomen) was performed 2 wk later. After 5 days, ears were painted with 0.3% DNFB and ear swelling responses measured. a. Sensitized dorsal skin shows evidence of inflammation and ulceration (white arrows) in UVB-treated Bid KO but not in UVB-treated WT mice. Numbers indicate the treatment group (outlined in the text and in c). b, H&E-stained ear skin sections provide corroborative evidence of ear swelling response measurements. Numbers indicate the treatment group. c. Scatter plot of specific ear swelling responses from WT (○) and Bid KO (●) mice (n = 10 ears) are shown above descriptions of treatment group designation. Student’s two-tailed t test p values designations are as follows: *, p < 0.05; ***, p < 0.001. This experiment was repeated and provided identical results.

FIGURE 7. High numbers of LCs with undetectable CPD-staining accumulate in UV-treated Bid KO LN. Superficial parotid LN cells were analyzed for expression of CD11c, Langerin, and CPD from mice exposed once with UVB (100 mJ/cm²) and/or 0.5% DNFB 3 days prior. a. Contour plots of CD11c-gated cells show percentage of CPD+ cells within the Langerin+ population in the upper right quadrant. b, Summary and statistics of the phenotype and LN cell numbers (total, LCs, and CPD+ cells per node) are shown. Mean and SEM (n = 3) are displayed for each treatment of Bid KO (filled bars) and WT (open bars) mice. Student’s one-tailed t test p values indicate where significant differences between strains were evident: *, p < 0.05; **, p < 0.001. Results from one of two similar experiments are shown.
been shown to correlate with UV-induced suppression and tolerance induction (32). Therefore, we examined the levels of CPD $^+$ cells that accumulate in LNs of Bid KO and WT mice after 4 consecutive days of UV exposure (on ears and back) and subsequently sensitized with DNFB or vehicle as a control. Three days after the last UV dose, cells from parotid and subiliac LNs were pooled per individual mouse and double stained for CD11c, Langerin, and CPD DNA adducts and examined by flow cytometry (Fig. 7a). Consistent with our previous experiments (shown in Fig. 3), fewer LN cells were recovered from WT mice and more cells were recovered from Bid KO mice treated with UV, but not DNFB (Fig. 7b, top panels). Similarly, LC numbers from Bid KO mice were at control levels in UV-treated groups but significantly higher levels after treatment with DNFB alone, while in WT mice diminished numbers of LCs were recovered for all treatment groups. Of the few remaining LCs recovered from WT mice, up to 26% were CPD $^+$ in UV-treated groups, while only 3% were CPD $^+$ in DNFB-treated groups, demonstrating a correlation with UV and CPD levels (Fig. 7b, bottom panel). Surprisingly, only 1% of the Bid KO LCs were CPD $^+$ in UV-treated groups, which dropped to $\sim$0.2% in control groups. These results show that LCs from UV-treated WT mice have high levels of CPD DNA adducts and undergo rapid loss (presumably due to Bid-dependent apoptosis) and that this phenomenon correlates with suppressive function. In contrast, Bid KO LCs initially accumulate to high levels and then drop down to control levels over 3 days, where only 1% of LCs are CPD $^+$. Our results demonstrate that it is the presence of high numbers of CPD $^-$ LCs, not the absolute number of CPD $^+$ LCs (which was equivalent to WT; Fig. 7b, bottom panel), that correlates with resistance to mechanisms of UV-induced tolerance.

Bid KO cutaneous DCs exhibit resistance to turnover in LNs of UV-treated mice

To address whether the increased numbers of LCs found in draining LNs of UV-treated Bid KO mice were due to increased turnover and recruitment of hematopoietic DCs or whether skin-derived DCs demonstrated longer lifespans, UV-treated and untreated mice were painted with the hapten FITC on ear skin after the last UV exposure. LN cells were examined 3 days later to provide time for FITC $^+$ cells to migrate and to mimic conditions used for tolerance induction. We observed that a daily UV dose of 400 mJ/cm$^2$ over 4 days induced substantial necrosis in mouse skin of both Bid KO and WT strains. Therefore, we used this dose to compare with a standard UV dose of 120 mJ/cm$^2$ and untreated mice to test the effect of necrotic epidermal environment on the turnover rate of skin-derived DCs. Our data presented in Fig. 8 demonstrate that Bid DCs maintain a profound resistance to apoptosis, even when skin is exposed to high doses of UV.
necrotic epidermal environment per se does not significantly affect the turnover rate of Bid KO DCs, in contrast to the depletion seen by WT DCs.

To begin to address if differences in DC subsets might account for differences in Bid KO and WT phenotypes, samples were costained for CD8α expression, and the frequency of DC subsets was determined. In mice treated with 120 mJ/cm² UV, WT CD11c⁺ DCs were depleted in LNs by 23%, while in Bid KO mice a 9% increase in CD11c⁺ DCs was observed. Moreover, the depletion rate of cells was greatly enhanced in FITC⁺ DC subsets from LNs of WT, but not Bid KO, mice. FITC⁺ DCs were depleted by 63% and FITC⁺ lymphoid DC subsets (CD8α⁺CD11c⁺) were depleted by 49% in WT mice, whereas Bid KO FITC⁺ DCs were depleted by only 22%, and FITC⁺CD8α⁺CD11c⁺ DCs remained at control levels. The enhanced depletion seen within the total FITC⁺ fraction suggests that these cells were more susceptible to UV-induced apoptosis.

Interestingly, in response to high-dose irradiation treatment, elevated numbers of CD11c⁺ DCs were present in draining LNs of WT mice (WT, 127% increase vs Bid KO, 109% of control numbers; Fig. 8c, top panel), consistent with the increase in total cells recovered (Fig. 8b, top panel). Increased numbers of total cells were observed for both mouse strains in response to high-dose UV radiation, and this was evident for Bid KO at the lower dose as well. However, for Bid KO, the increased cellularity did not contribute to an increase in CD11c cells (Fig. 8b) and was consistent with our previous studies (Fig. 4b).

We noted that the median fluorescence level of FITC staining of CD8α⁺ DCs tended to be lower than CD8α⁻ DCs, consistent with the model that CD8α⁺ DCs are resident LN cells that specialize in endocytosing apoptotic debris (e.g., dying FITC-stained migratory cells) for crosspresentation to T cells. The increased FITC levels within CD8α⁺ DC subset in LNs of WT mice observed in response to 400 mJ/cm² of UV radiation provide further evidence for this (Fig. 8c, upper panel set). The FITC fluorescence intensity measured for both DC subsets of Bid KO mice remained unchanged, regardless of UV radiation dose.

Discussion

Our study reveals that the Bid molecule plays a prominent role in UV-induced apoptosis in vivo, and that this Bid-dependent process is strongly linked to immune suppression and tolerance induction. We found that Bid deficiency resulted in marked resistance to apoptosis in both keratinocytes and LCs following UV exposure. Our data also indicate that Bid activation is required to initiate the mitochondrial signaling pathway. Additionally, Bid deficiency abrogates UVB-induced local immune suppression of hapten sensitization and systemic tolerance induction. These studies provide the first evidence for the importance of Bid-dependent pathways in cutaneous immune regulation.

The link between apoptosis and UV-induced immune suppression have been examined in genetically engineered mice that either overexpress Bcl-2 (34) or are deficient for the CD95 (Fas) (10, 35). The immune suppression was shown to be significantly inhibited in these animals. The involvement of both intrinsic (Bcl-2) and extrinsic (CD95) apoptosis pathways supports our data demonstrating that Bid is an important mediator of this response. UV-induced death receptor signal initiation can occur through ligand-dependent and independent (receptor aggregation) mechanisms, but to what extent the pathway depends on Bid was not known. Our findings provide evidence for a critical role of Bid in mediating epidermal cell apoptosis in response to UVR. Further investigations are required to understand the molecular mechanism underlying the role of Bid. Bid may act chiefly as a primary target of initiator caspase 8, unequivocally defining keratinocytes as “type II cells,” or as a target of JNK-induced mechanisms of activation (reviewed in Ref. 36), or as a downstream target of effector caspases that cannot efficiently induce apoptosis without Bid activation to recruit mitochondrial mechanisms.

Our studies demonstrate that both local suppression and systemic tolerance induction are regulated by Bid-dependent apoptosis. These data contrast, in part, with studies in CD95 (Fas)-deficient mice, in which inhibition of systemic tolerance but not local UV immune suppression was seen (37). Taken together, these data suggest that other death receptors may be critical in triggering local immune suppression mechanisms and are consistent with studies demonstrating that low doses of UVR sensitize human basal cell cultures to TRAIL-mediated apoptosis but not to agonist anti-Fas Ab treatment (14).

A hallmark photodamage response is the depletion of LCs in the epidermis and a reduction of LC numbers recovered from draining LNs. The mechanism of this depletion is not fully understood. Migration out of the epidermis of these cells likely accounts for most of the epidermal depletion, but other mechanisms such as apoptosis, inhibition of migration from dermis to lymphatics (17), and increased susceptibility to apoptosis within the LN due to UV-induced cytokine production or T cell activation (38) have been proposed to contribute to low LC recovery. UV-induced apoptosis has been detected after in vitro irradiation of purified LCs after relatively long culture periods (48–72 h) (19) but has been difficult to detect in vivo (39). However, most in vivo studies examine epidermal LCs within 24–48 h after UV treatment, which may be too early to detect apoptosis. In accordance with previous findings in WT mice, we found that most UV-induced LC depletion from the epidermal layer occurred within 48 h and was UV dose-dependent. However, significantly more epidermal LCs were retained in Bid KO mice. At this early time point, morphological evidence of LC apoptosis in situ was not observed (data not shown). However, when we examined UV-treated skin a week later, LC apoptotic morphology was prominently observed in all UV-treated samples from WT but not Bid KO mice. In striking contrast to the apoptotic blebs seen for WT LCs, the remaining epidermal Bid KO LCs exhibited enlarged cell bodies with thickened dendritic processes, consistent with an activated morphology. These observations support the notion that in WT mice, UV-induced LC apoptosis is delayed such that LCs can reach the LNs before undergoing cell death.

The capacity of cells from WT or Bid KO mice to migrate from UV-exposed skin was not impeded and comparable to control samples (where tape-stripping was used to activate migration). Furthermore, the capacity of CPD⁺ cells to migrate from dorsal ear skin and remain viable after 3 days in culture was the same for both mouse strains. However, we did observe more dead cells in WT cultures of ventral ear skin as compared with Bid KO cultures, suggesting that a subset of cells was more susceptible to apoptosis. Taken together, the data support the notion that UV-induced LC apoptosis is delayed, and that resistance to apoptosis by Bid KO LCs can account for the increased numbers remaining in UV-treated epidermis. Furthermore, photodamaged LCs maintain their capacity to migrate from the epidermis to enter the lymphatics over the course of 3 days, since identical absolute numbers of CPD⁺ cells migrating from skin cultures and reaching LNs were detected in samples from both strains.

The magnitude of CHS responses has been shown to correlate with epidermal LC density (18), so reduced LC numbers in the epidermis at the time of hapten application may play an important role in the mechanism of local immune suppression. However, while significant differences in LC density were observed between
Bid KO and WT strains, we think that this does not completely account for the dramatic differences observed in their ability to sensitize UV-treated mice to hapten. Many reports indicate that UVR changes the quality of LC function such that Ag (hapten)-specific suppression is developed (22). In one study, purified epidermal LCs that were UV treated and haptenated in vitro were shown to induce CHS suppression and long-lasting tolerance when injected into untreated mice (40). The nature of the relevant qualitative changes are under investigation by several laboratories, and a role for apoptosis has been implicated (37). Therefore, we tested whether Bid KO mice differed from WT mice in their susceptibility to UV-induced local immune suppression and systemic tolerance induction. We found that Bid KO mice were highly resistant to UVB-induced immune suppression and tolerance induction, demonstrating that the Bid-dependent apoptosis plays an essential role in these processes.

Several studies have shown that UV-induced DNA damage, in particular the generation of pyrimidine dimers present in migrating cutaneous DCs, correlates with immune suppression (29, 41). Therefore, we investigated the level of DNA damage found in LCs derived from UV-treated skin draining LNs from WT and Bid KO mice using flow cytometric methods (32, 42). Our analysis revealed three important differences between these strains: 1) in contrast to WT LCs, which contained >25% CPD- cells, the Bid KO LC population exhibited very low levels of DNA damage; 2) greater numbers (2- to 3-fold) of Bid KO LCs were recovered from draining LNs of UV-treated Bid KO mice as compared with UV-treated WT mice; and 3) the lifespan of migratory cutaneous DCs was significantly extended in LNs of Bid KO mice. We addressed whether fewer CPD- cells reached draining LNs in Bid KO mice. Reduced CPD staining could be a secondary consequence of reduced epidermal cell apoptosis limiting a source of apoptotic CPD- debris for LC uptake. However, we were unable to detect cytoplasmic CPD- staining with our technique. Furthermore, we determined that the number of cells with CPD- nuclei were not significantly different in populations of migratory cells from UV-treated skin explants derived from either strain. Thus, both strains are equally susceptible to DNA damage by UVR, and such damaged cells are fully capable of migration activity. Another consideration may be related to differences in DNA repair capacities between WT and Bid KO LCs. For example, IL-12 has been shown to be a key cytokine for the activation of DNA repair in response to UVR (43). It is possible that Bid KO mice produce enhanced levels of IL-12 in response to UVR, as compared with WT mice.

We favor the hypothesis that reduced levels of DNA damage observed by Bid KO LCs is likely due to an enhanced recruitment of nondamaged Langerin+ cells, as well as to profound resistance to UV-induced apoptosis exhibited by Bid KO cutaneous DCs. The longer lifespan of such cells was shown convincingly by demonstrating that Bid KO LCs supported the maintenance of skin-derived FITC+ DCs at all UV doses tested. These findings are consistent with our previous report demonstrating that Bid KO LCs exhibited resistance to apoptosis induced by Ag-specific T cell activation (30). Additionally, increased numbers of LCs observed in LNs from UV-treated Bid KO mice likely did not originate from the epidermis, and this may account for the low percentage of CPD- cells observed. This interpretation is consistent with recent findings by three independent groups of investigators, demonstrating that a novel epidermal LC-distinct, blood-derived Langerin+ subset traffic through the dermis in the steady-state and contributes to the majority of Langerin+ cells found in skin draining LNs (44-46). The contribution of dermal LCs may explain why FITC-labeled cells from skin exposed to toxic levels of UV (dep-
References


