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Imiquimod-Induced TLR7 Signaling Enhances Repair of DNA Damage Induced by Ultraviolet Light in Bone Marrow-Derived Cells

Rita Fishelevich,* Yuming Zhao,* Papapit Tuchinda,* Hannah Liu,* Ayako Nakazono,* Antonella Tammaro,* Tzu-Ching Meng,† Jim Lee,‡ and Anthony A. Gaspari* * ,‡

Imiquimod is a TLR7/8 agonist that has anticancer therapeutic efficacy in the treatment of precancerous skin lesions and certain nonmelanoma skin cancers. To test our hypothesis that imiquimod enhances DNA repair as a mechanism for its anticancer activity, the nucleotide excision repair genes were studied in bone marrow-derived cells. Imiquimod enhanced the expression of xeroderma pigmentosum (XP) A and other DNA repair genes (quantitative real-time PCR analysis) and resulted in an increased nuclear localization of the DNA repair enzyme XPA. This was dependent on MyD88, as bone marrow-derived cells from MyD88−/− mice did not increase XPA gene expression and did not enhance the survival of MyD88−/−-derived bone marrow-derived cells after UVB exposure as was observed in bone marrow-derived cells from MyD88+/− mice. Imiquimod also enhanced DNA repair of UV light (UVL)-irradiated gene expression constructs and accelerated the resolution of cyclobutane pyrimidine dimers after UVL exposures in P388 and XS52. Lastly, topical treatment of mouse skin with 5% imiquimod cream prior to UVL irradiation resulted in a decrease in the number of cyclobutane pyrimidine dimer-positive APC that were found in local lymph nodes 24 h after UVL irradiation in both wild-type and IL-12 gene-targeted mice. In total, these data support the idea that TLR7 agonists such as imiquimod enhance DNA repair in bone marrow-derived cells. This property is likely to be an important mechanism for its anticancer effects because it protects cutaneous APC from the deleterious effects of UVL.

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UV-light (UVL) is a well-characterized carcinogen that causes genetic lesions in epidermal keratinocytes, which over time accumulate and play a critical role in the development of skin cancers (1, 2). Equally important in the development of skin cancer is the immunosuppressive cutaneous microenvironment that develops as a result of chronic UVL exposures. Immunosuppressive cytokines such as IL-10 are induced after UVL exposure (3, 4). Additionally, there is impaired Ag presentation by skin-derived dendritic cells such as Langerhans cells (LC), and immune suppressive macrophages migrate into skin after UV exposures (5, 6). As a result of UVL impairment of cutaneous APC, there is impairment in the development of Th1 immunity and loss of sensitization to topically applied haptenes (7, 8). Instead of sensitization, there is hapten-specific immune tolerance that can be adoptively transferred to naive hosts by regulatory T cells (9). This loss of contact hypersensitivity is thought to be a sentinel event that correlates well with the loss of immunologic reactivity against tumor Ags that develop in parallel as a result of chronic UVL exposures. Thus, instead of preventing the development and progression of UVL-induced skin tumors, there is immune tolerance and a permissive environment for skin cancer development.

The fundamental lesion for both skin cancer development as well as photoinmunosuppression is DNA damage. Within 24 h of UVL exposures, dendritic APC with cyclobutane pyrimidine dimers (CBPD) have been found in the paracortical area of draining lymph nodes of UVL-irradiated mice (10). Such APC that bear DNA damage are in part responsible for the loss of protective cutaneous immunity against skin cancer development (11). A biomarker for the loss of cutaneous immunity is the loss of contact sensitization. Local exposure of the skin to UVL results in both local and systemic effects. In model systems to protect mice from cutaneous immunosuppression after UVL exposures, a variety of treatments have been applied in an attempt to enhance DNA repair by APC, potentially restoring immune competence after UVL exposures. DNA repair-promoting agents include bacterial DNA repair enzymes (T4 endonuclease encapsulated into liposomes) (12), green tea polyphenols (13), and cytokines such as IL-12 and IL-23, platelet-activating factor, and serotonin receptor antagonists (14–16).

In recent studies, the effect of topically applied imiquimod was studied in a mouse model of UVL-induced loss of hapten sensitization. This treatment prevented the loss of contact hypersensitivity as well as the development of hapten-specific tolerance (17). Even though imiquimod preserved contact hypersensitivity in vivo, it did not protect from UVL-induced depletion of LC from the epidermis of UVL-irradiated skin (17). In vitro, imiquimod activation of TLR7 prior to UVL exposure induced resistant APC maturation, preserving Th1 lymphocyte-derived cytokines. This is in marked contrast to UVL-irradiated APC that were not activated...
with imiquimod. These data lead us to hypothesize that imiquimod, via TLR7 activation, renders cutaneous APC resistant to UVL-induced immunosuppression because it enhances DNA repair. In this study, we describe the effects of imiquimod on APC DNA repair of UVL damage. We found that imiquimod induces DNA repair gene expression that is dependent on MyD88 as well as an intact NF-κB signaling pathway, which are early and late components of TLR signaling pathway. As well, imiquimod-activated APC were resistant to UVL, and ionizing radiation induced loss of viability. Additionally, imiquimod-activated APC exhibited enhanced functional DNA repair compared with resting APC. Lastly, topical application of imiquimod prior to UVL exposure resulted in a marked reduction of CBPΔ-positive APC and a reduction in suppressive cytokine gene expression in local lymph nodes of such treated mice when compared with vehicle or untreated, irradiated mice. These data lead us to conclude imiquimod-mediated, TLR7 signaling enhances repair of UVL damage to genomic DNA and may play a therapeutic role in the preservation of an intact cutaneous immune response after exposure to UVL.

Materials and Methods

Experimental mice
C57BL/6 female mice, 8 wk old, were purchased from The Jackson Laboratory (Bar Harbor, ME). These animals were chosen because they have been demonstrated to exhibit a phenotype of susceptibility to UV-induced suppression of contact hypersensitivity (18, 19). IL-12p40 gene-targeted female mice (B6.129S1IL2btm1Jm/J, stock number 002693) in the C57BL/6 background were purchased from The Jackson Laboratory (20). Animals were kept in standard housing conditions, fed mouse chow ad libitum, and exposed to a 12-h light/dark cycle. To study the appearance of APC in local lymph nodes, experimental mice (either wild-type or IL-12 gene-targeted mice) were unirradiated or irradiated with a single dose of UV B (UVB) at a dose of 150 mJ/cm². Some of the UVB-irradiated mice received topical applications of 5% imiquimod cream or its vehicle to the dorsal aspect of the pinna of the ear (once daily for 2 d); 24 h later, the animals were then exposed to UVB. For the UVB exposure, the animals were anesthetized with a single dose of avertin (i.p. injection), then the animals’ ears were taped to a restraining chamber and the dorsum exposed to the indicated dose of UVB. Twenty-four hours after this, the animals were sacrificed, the cervical lymph nodes were dissected, and DNA extracted using standard methods. All experimental procedures were reviewed and approved by the University of Maryland Institutional Animal Care and Use Committee.

Cell lines
A20 was purchased from American Type Culture Collection. P388 cell line was a generous gift from Dr. David Scott (University of Maryland Baltimore). XS52 was a generous gift from Dr. Akira Takashima (University of Toledo) and was cultured using standard conditions. Wild-type macrophages and MyD88 knockout cell lines were a generous gift from Dr. Doug Gelb, University of Massachusetts. Xeroderma pigmentosum (XP) A20 was purchased from American Type Culture Collection. P388 cell line and MyD88 knockout cell lines were a generous gift from Dr. Doug Golomb (University of Massachusetts). Xeroderma pigmentosum (XP) A and MyD88 knockout cell lines were a generous gift from Dr. David Scott (University of Maryland Baltimore). P388 cell line was a generous gift from Graceway Pharmaceuticals (Exton, PA). For the experiments, imiquimod powder was used (also provided by Graceway Pharmaceuticals). It was prepared as a stock solution in sterile water at 1 mg/mL and diluted to the appropriate working concentration in complete medium. BAY 11-7085 (Calbiochem) was dissolved in DMSO and added to the culture medium at 0 h and exposed to imiquimod treatment at the indicated concentrations to block NF-κB (24).

Cytotoxicity assay
A thiazoyl blue tetrazolium bromide (MTT) assay was used to measure cell viability 24 h after UVL or ionizing radiation exposures (22). Either adherent cells (P388) or nonadherent lymphoblasts (EBV-immortalized cells) were cultured at a cell density of ~5000 cells/well of a 96-well plate. For adherent P388 cells, the cells remained adherent to the microtiter wells; for the nonadherent lymphoblasts (XP or control lymphoblasts), the microtiter plates were centrifuged (1200 rpm for 10 min) prior to any washes or medium changes. Using standard methods (22), MTT solution (20 μL of 5 mg/mL MTT stock solution) was added to cell cultures, then incubated for 4 h at 37°C. The culture medium was then removed, and 150 μL MTT solution (4 mg/mL MTT in HCl 0.1% Nonidet P-40 in isopropanol) was added, and the plates were covered with tin foil and placed on an orbital shaker for 15 min. The OD was read at 950 nm with a reference filter of 620 nm. Background OD for MTT was assayed in wells that contained no cells.

UVL source
Groups of mice were irradiated with a panel of 48° Q-Sun light bank (Q-Panel Laboratory Products, Cleveland, OH) (equipped with a UVC WG320 filter) at a distance of 12 inches from the light source to their shaved abdominal skin. The spectral emission profile of this light source closely mimics that of natural sunlight with the predominant UVA. A UVB radiometer (National Biologic Corporation, Twinsburg, OH) was used to determine UVB output and calculate the time necessary to deliver the desired doses of UVB. Plasmid DNA, cell monolayers, or experimental mice received UVL at the doses indicated in each of the individual experiments.

Ionizing radiation
A [137]Cs radiation source was used to deliver ionizing radiation to either control or XPA lymphoblasts. Exposure time of the irradiated cells was calculated to deliver the desired dose of radiation (see Results) using calibration curves.

Transient transfection by electroporation and luciferase assays
For the gene reactivation assay, control (medium alone) or imiquimod-treated cells (at the indicated doses for 24 h prior to electroporation) were transfected with the following constructs: pGL3, an empty vector that contains no luciferase gene as a negative control; and pGL4.1[hLuc], which encodes for firefly luciferase as a positive control; these negative and positive controls were cotransfected with pGL4.70[hRluc] (Renilla luciferase), which serves as a control for transfection efficiency. In parallel, control or imiquimod-treated cells were transfected with UVL-irradiated pGL4.10 [hLuc] constructs, with UVL radiation doses ranging from 1000–5000 mJ/cm². The UVL-irradiated constructs were produced by placing a 5-μl volume of the plasmid DNA on a glass slide and exposing to the indicated doses of UVL at a 12-inch distance from the UVL source. After UVL irradiation, these constructs were cotransfected with unirradiated pGL4.70 [hRluc], which served as a control for transfection efficiency.

The lucinomancy data represent a normalized ratio of firefly luciferase to Renilla luciferase. These normalized data were then normalized to the negative (pGL3/pGL4.7) and positive control values (pGL4.1/pGL4.7). The normalized expression of the UVL-irradiated pGL4.10[UVL/pGL4.7] was then compared with its respective positive and negative controls. Data are reported as percent of control because imiquimod treatment of host cells had significant effects on transfection efficiency (expression of positive control construct, data not shown). Thus, the formula for calculating gene reactivation was as follows: [(pGL4.1[UVL]/pGL4.7) − (pGL3/pGL4.7)]/(pGL4.1[UVL]/pGL4.7) × 100 = percent gene reactivation compared with control. We used methods as previously described for the luminometry assays (23).

Cell lines (P388, XS52, macrophage cell line) used in the gene reactivation assay were transfected at ~70% confluence using the Amaxa nucleofector kit (Amaxa) according to the manufacturer’s protocol. Briefly, cells were transfected with 2 μg supercoiled plasmid DNA per well in transfection of six-well plates. A positive control plasmid for luciferase (pGL4.10 [hLuc]) encodes a synthetic firefly luciferase gene. An internal control plasmid, pGL4.70 [hRluc], which expresses the Renilla luciferase gene, was also used for correcting variations in the transfection efficiency. After 15 min, 1 ml growth medium was added and incubated for 48 h. Lysates were harvested, and extracts were prepared using freeze-thaw lysis. Lysates were monitored for luciferase activities using a commercially available Dual Luciferase (firefly and Renilla) Reagent kit (Promega) in a Turner 20/20 luminometer (Turner Biosystems, Sunnyvale, CA). Firefly luciferase activity was normalized to the Renilla luciferase activity. Briefly, trypsinized 1 million cells were electroporated with 3 μg DNA and incubated for 48 h prior to harvesting the cellular lysates. All gene reporter assays were repeated at least three times.

Chemicals
Imiquimod (1-[2-methylpropyl]-1H-imidazo[4,5-c]quinolin-4-amine, R-837) 5% cream and its vehicle cream were used for topical applications and were provided by Graceway Pharmaceuticals (Exton, PA). For the in vitro studies, imiquimod powder was used (also provided by Graceway Pharmaceuticals). It was prepared as a stock solution in sterile water at 1 mg/ml and diluted to the appropriate working concentration in complete medium. BAY 11-7085 (Calbiochem) was dissolved in DMSO and added to the culture medium at 0 h and exposed to imiquimod treatment at the indicated concentrations to block NF-κB (24).

Western blot
Western blotting was performed as previously described (24, 25). Equal quantities of total protein from different samples were separated on a 10%
Bis-Tris NuPage denaturing gel (Invitrogen Life Technologies, Carlsbad, CA). Gels were transferred to nitrocellulose membranes, probed with appropriate first Abs, and detected using a Western Breeze Chemiluminescent detection kit (Invitrogen Life Technologies). The bands were detected using a Chemi-Doc densitometer and Quantity One software (Bio-Rad, Hercules, CA). β-actin signals from each sample were used for normalizing the intensity of specific bands of interest. A mouse anti-XPA Ab (clone B1; Santa Cruz Biotechnology, Santa Cruz, CA) was used to Western blot XPA in control or imiquimod-treated cells. This clone was raised against human aa 1–123 derived from full-length human XPA. This mAb reacts with a polypeptide of ∼38–40 kDa.

Cell fractionation

Nuclei from cultured cells were isolated using the Active Motif Nuclear Extraction Kit (Active Motif North America, Carlsbad, CA). Nuclear extracts were prepared using the manufacturer’s instructions.

RNA extraction, cDNA synthesis, and quantitative real-time PCR

Standard methods were used to extract total cellular RNA and cDNA synthesis. Detection of XPA, TNF-α, and TLR7 mRNA was performed using quantitative real-time PCR (qPCR) according to previously published methods (26). The annealing temperature was 55˚C, followed by 35 cycles of amplification using a PCR Sprint (Hybaid). Primers to amplify mouse DNA repair genes were as follows: XPA (reference number 592) and XPC (catalog number 04375e), XPG (catalog number 04636a), XP Filip1 (catalog number 05392a), Rpa3 (catalog number 03481a), Xab2 (catalog number 0491a), Rad23b (catalog number 04360a), and Ddb1 (catalog number 04381a). Primers to amplify other genes included: TLR7 (reference number 1136), TNF-α (reference number 879), Foxp3, IL-4, IL-10 (catalog #074), TGF-β (catalog number 035), and 18S (reference number X03205). All of these primers were purchased from SuperArray Biosciences (Qiagen, Valencia, CA). Real-time PCR (LightCycler; Roche, Indianapolis, IN) was used to confirm differences in the levels of expression of selected mRNA. The primers, PCR protocol, and product quantification for 18S rRNA were exactly as reported previously (SYBR Green detection) (26). Amplification (40–50 cycles) of a single PCR product was confirmed by gel electrophoresis and melting curve analyses. The cDNAs were assayed in duplicate, and mean values are depicted in the graphics for the quantitative PCR.

Statistical analyses

GraphPad Instat and Prism software programs (GraphPad, San Diego, CA) were used to compare quantitative data from experimental groups for statistical significance. For comparisons between multiple groups, ANOVA was used; p < 0.05 was considered to be statistically significant.

Results

Imiquimod induces DNA repair gene expression in a variety of bone marrow-derived cells

To determine whether the TLR7 agonist imiquimod increased DNA repair gene expression in bone marrow-derived cells, we cultured a variety of cell lines with this agent and studied DNA repair gene expression. Although there are >30 genes involved in the repair of DNA damage induced by UVL, we focused on XPA gene expression because it is involved in the recognition of UV damage to DNA, an early event in the DNA repair process (6). The importance of this gene in DNA repair is further confirmed by the clinical correlation that defects in this DNA repair gene can result in a severe XP phenotype (27). As demonstrated in Fig. 1, P388 cell line (monocyte lineage) exhibited enhanced XPA gene expression as a result of the addition of imiquimod to the cell culture medium (Fig. 1A). Twenty-four hours after imiquimod treatment, XPA protein (as detected by Western blotting, see Materials and Methods) was assayed in duplicate, and mean values are depicted in the graphics for the quantitative PCR.

**FIGURE 1.** Imiquimod enhances gene expression and nuclear localization of the DNA repair enzyme XPA in bone marrow-derived cells. A, The P388 cell line was incubated with medium alone or medium containing the indicated doses of imiquimod for 6 h, and then gene expression was assayed using qPCR analysis (single representative experiment, see Materials and Methods). B, Western blotting of nuclear XPA (∼38 kDa) 24 h after P388 was incubated with medium alone or medium containing the indicated doses of imiquimod. C, Densitometry scanning of normalized XPA/β-actin indicated that there was an ∼2-fold increase in nuclear XPA protein in imiquimod-treated cells. D, Fold-change expression of multiple DNA repair genes in P388 cells cultured with 10 μg/ml imiquimod relative to cells cultured in medium alone (not shown, but depicted as horizontal line). The data in this experiment represent the mean and SD of three different gene expression experiments. **p < 0.01, ***p < 0.001, ANOVA.
Methods) is increased in the nucleus of imiquimod-stimulated P388 compared with control (unstimulated) P388 (Fig. 1B); densitometry scanning of the Western blot autoradiogram was then normalized (XPA/β-actin ratio) and is presented as a histogram in Fig. 1C, with ~2-fold increase in nuclear XPA protein at 24 h after 10 μg/ml imiquimod exposure and a smaller increase induced by imiquimod at 20 μg/ml. Because DNA repair of UVL damage is a complex process that involves a family of DNA damage recognition and repair, we screened the P388 cell line for changes in other repair genes at the 10 μg/ml dose of imiquimod and found that the transcription of the entire family of genes was modestly increased (range of increase between 1.5- and 3.5-fold above control cells (Fig. 1D). We found that the optimal dose for the increasing XPA gene expression, as well as the amplitude of the increase, varied among these three cell lines. The optimal dose of imiquimod for increasing XPA gene expression varied between 10 and 30 μg/ml, which is slightly higher than the optimal dose (5–10 μg/ml) for triggering cytokine secretion by TLR7-expressing mouse APC (28).

**Imiquimod-enhanced DNA repair gene expression and survival after UV exposure is dependent on MyD88**

All of the TLR receptors, including TLR7 and -8, use MyD88 as an adaptor protein to initiate early events that eventually result in NF-κB signaling in the nucleus, which reprograms cells to express inflammatory cytokine genes (29–31). Bone marrow-derived cells from MyD88 gene-targeted mice exhibit impaired inflammatory responses after exposure to TLR ligands (3). Thus, if imiquimod induction of the DNA repair gene XPA is indeed dependent on TLR7 signaling, then cells deficient in MyD88 would exhibit impaired ability to upregulate XPA gene expression after stimulation by imiquimod. Macrophage cell lines from wild-type mice (MyD88+/+) and MyD88 knockout mice (MyD88−/−) were exposed to medium alone or medium containing imiquimod for 4 h and XPA gene expression was assayed. Whereas macrophages from wild-type mice (MyD88+/+) responded by increasing XPA gene expression at both 5 and 10 μg/ml, the macrophages from MyD88−/− knockout mice exhibited a blunted response to 5 μg/ml and an absent response to the 10 μg/ml dose, which induced optimal gene expression in macrophages from MyD88+/+ mice (Fig. 2A).

![Graph showing relative change in gene expression](image)

**FIGURE 2.** MyD88 expression is necessary for the enhanced XPA gene expression and survival after exposure to UVL in bone marrow-derived cells. A, Macrophage cell lines derived from MyD88+/+ (wild-type) mice or MyD88−/− (gene-targeted) mice were incubated with the indicated doses of imiquimod, and XPA gene expression was assayed 6 h later. B, Bone marrow-derived cells from MyD88+/+ (wild-type) mice were incubated with medium alone or medium containing the indicated doses of imiquimod, and XPA gene expression was assayed 6 h later. C, Bone marrow-derived cells from MyD88−/− (gene-targeted) mice were incubated with the indicated doses of imiquimod for 24 h and then exposed to increasing doses of UVL, and survival was assayed after another 24 h using an MTT assay (see Materials and Methods). Data depicted represent the mean ± SD of six replicates from a single representative experiment that was repeated three times. *p < 0.05, **p < 0.01, ***p < 0.001, ANOVA.

After exposure to UVL, an irradiated cell will sense DNA damage and can go on to repair the DNA damage and survive. Alternatively, if the DNA damage is too severe, an irradiated cell will then undergo apoptosis (32, 33). We hypothesized that if DNA repair induced by TLR7 was functionally significant, there would be enhanced survival after exposures to increasing doses of UVL. As shown in Fig. 2B, treatment of MyD88+/+ macrophages enhanced the survival of these cells (MTT assay) compared with control (medium alone) cells. In contrast, MyD88−/− macrophages did not respond to imiquimod with enhanced survival to UVL (MTT assay) (Fig. 2C). Other cell lines such as P388 also exhibited imiquimod-induced enhanced survival after UVB exposure (data not shown).

A late event in the TLR signaling pathway is translocation of the transcription factor NF-κB from the cytoplasm to the nucleus of activated cells (28–30). Under steady-state conditions, NF-κB proteins are sequestered in the cytoplasm by members of the IκB family. Upon cellular stimulation by TLR agonists, activation of the IκB kinase phosphorylates IκB, which dissociates the NF-κB–IκB complexes. Free NF-κB heterodimers then translocate into the nucleus, where they bind to enhancer elements of target genes (24). The chemical compound BAY 11-7085 inhibits the NF-κB signaling pathway by preventing the degradation of IκB (24) and hence prevents the translocation of NF-κB from the cytoplasm to the nucleus, thus blocking the activation of its target genes. If the induction of a DNA repair gene expression is dependent on classical TLR signaling, we hypothesized that Bayer compound would block the upregulation of XPA gene expression after TLR7 stimulation by imiquimod. Consistent with our hypothesis, BAY 11-7085 blocked the increase in XPA gene expression in the P388 cell line (Fig. 3A). Similarly, BAY 11-7085 blocked the imiquimod-induced...
enhanced nuclear localization of NF-κB and XPA protein (Western blotting of nuclear extracts, Fig. 3B). Additionally, BAY 11-7085 blocked the ability of imiquimod to enhance survival of P388 after exposure to UVL (Fig. 3C). Thus, the upregulation of XPA gene expression is dependent upon both early (MyD88-dependent, Fig. 2A) and late events (nuclear activation of NF-κB, Fig. 3A, 3B).

Imiquimod stimulation of bone marrow-derived cells enhances functional DNA repair in a gene reactivation assay and the resolution of CBPD

Next, to determine whether imiquimod stimulation of bone marrow-derived cells enhanced DNA repair, we used a functional assay (known as the gene reactivation assay) to study this phenomenon (34, 35). In this assay, a firefly luciferase-encoding
plasmid is UVL irradiated and then cotransfected (along with an unirradiated Renilla luciferase-encoding plasmid to control for transfection efficiency) into a host cell. Visible light emission (luciferase functional activity) was studied over time (luminometer, see Materials and Methods). The P388 cell line was pretreated with medium alone or medium containing imiquimod 5 or 10 μg/ml for 24 h, then transfected with control (unirradiated) or UVL-irradiated luciferase constructions. Forty-eight hours after transfection, a lysate was prepared, and visible light was measured. As is depicted in Fig. 4A, 10 μg/ml imiquimod significantly enhanced the repair of the irradiated constructs. Higher doses of imiquimod did not further enhance repair (data not shown). Although the lower dose of imiquimod enhanced the repair relative to the untreated host cells, this was not statistically significant. We studied two other cell lines (XS52 and macrophage cell line) for responsiveness to imiquimod in the gene reactivation assay and found that both responded similarly to 10 μg/ml TLR7 agonist (Fig. 4B, 4C). We conclude that pretreatment of bone marrow-derived cells with imiquimod enhances the repair (and hence gene expression) above that of background levels of exogenously irradiated plasmid DNA that is introduced into three different host cells using electroporation method.

In another functional assay of DNA repair that is more relevant to in vivo exposures, we first treated bone marrow-derived cells with medium alone or medium containing imiquimod at the indicated doses and then exposed the bone marrow-derived cells to UVL at doses of 10 or 25 mJ/cm². The resolution of DNA damage was followed over time using a DNA dot blot in which genomic DNA was extracted from cells, blotted onto nylon membranes, and then probed with an mAb that reacts with CBPD (14). P388 cells stimulated with 10 μg/ml imiquimod had noticeably less DNA damage 7 h after a single exposure to 10 mJ/cm² UVB compared with P388 treated with medium alone or lower doses of imiquimod (Fig. 5A, 5B). With higher doses of UVB (25 mJ/cm²), there was a delay in the accelerated resolution of damage to 24 h that occurred in P388 cells stimulated with imiquimod 10 μg/ml when compared with either the control P388 cells or P388 cells stimulated with lower doses of imiquimod (5 μg/ml) (Fig. 5C, 5D). XS52 cells were also treated with imiquimod prior to 10 mJ/cm² UVB and then studied for the resolution of CBPD over time. Similar to P388, XS52 more rapidly resolved CBPD (by 7 h) compared with control cells (Fig. 5E, 5F).

*Imiquimod-induced resistance to ionizing or UV radiation is dependent on intact DNA repair activity*

To determine whether TLR7 activation by imiquimod enhanced the survival of human cells after ionizing radiation, we exposed EBV-immortalized lymphoblasts (from a healthy individual or an XP patient [XPA gene defect]) to 4000 rad from a [137Cs] irradiator. Treatment of control EBV lymphoblasts with imiquimod (5 or 10 μg/ml) prior to exposure to ionizing radiation significantly (p < 0.05, ANOVA) enhanced cell survival 24 h later (MTT assay, see Materials and Methods) (Fig. 6A). In contrast, exposure of XP lymphoblasts to imiquimod did not enhance cell survival 24 h after exposure to the same amount of ionizing radiation (4000 rad) (Fig. 6B). Identical results were obtained with these two cell lines after exposure to a single dose of 35 mJ/cm² UVB. Whereas exposure of control lymphoblasts to either 5 or 10 μg/ml imiquimod enhanced survival 24 h after UVB (Fig. 6C), XPA lymphoblasts did not respond to these same doses of imiquimod (Fig. 6D).

**FIGURE 5.** Imiquimod enhances functional DNA repair activity in bone marrow-derived cells as measured in a DNA dot blot assay. P388 cells (A–D) or XS52 cells (E, F) were pretreated with medium or medium containing imiquimod at the indicated doses and then exposed the bone marrow-derived cells to UVL at doses of 10 or 25 mJ/cm². The resolution of DNA damage was followed over time using a DNA dot blot in which genomic DNA was extracted from cells, blotted onto nylon membranes, and then probed with an mAb that reacts with CBPD (14).
These data suggest that enhanced survival after ionizing or UVB irradiation induced by TLR7 signaling is dependent on intact DNA repair mechanisms. This is consistent with our hypothesis that TLR7 signaling enhances DNA repair.

**Imiquimod decreases UVL-induced DNA damage in an in vivo model**

After UVB irradiation of mouse skin, MHC class II-positive APC that bear CBPD can be detected within the local lymph nodes within hours after UVB exposure and persist for days after a single UVB exposure (10, 36). These damaged APC are thought to be responsible for systemic immune suppression that occurs after UVB irradiation (11). Topical applications of imiquimod decreased the amount of CBPD-bearing APC in local lymph nodes 24 h after UVB exposure, as seen in a DNA dot blot derived from draining lymph nodes of UVB-irradiated mice (Fig. 7A), as well as reversed the presence of immunosuppressive cytokines (IL-10, TGF-β) and regulatory T cell-associated transcription factor FoxP3 (9) (Fig. 7C).

**Discussion**

TLR are a major class of innate immune signaling receptors (37). These receptors, after sensing certain microbial products or damage-associated molecular patterns, trigger an inflammatory response that is orchestrated by a family of cytokines such as IL-1, TNF-α, and IL-6. In total, these inflammatory cytokines are pleiotropic and recruit inflammatory cells, enhance vascular permeability, regulate cell death, and induce the production of acute-phase reactants (29, 38).

TLR7, -8, and -9 are distinct members of the larger TLR family and are clustered in a group of intracellular receptors that recognize intracellular viral and bacterial infections (31). Imiquimod is a small, synthetic adenosine analog that activates TLR7 in mice and is known to elicit immune responses that suppress both the ability of immune cells to cause the clearance of precancerous skin lesions (actinic keratoses) or fully developed nonmelanoma skin cancer (superficial basal cell carcinoma). First, because of its adjuvant effects on TLR7/8 APC, this agent induces the maturation of such APC and the production of a number of cytokines that favor the development of Th1 lymphocytes during Ag presentation (17), which play an important role in antitumor responses. This has been confirmed at the tissue level, as histologic analysis of imiquimod-treated nonmelanoma skin cancers develop lymphocyte-rich (CD4, CD8 T lymphocytes, and NK cells) infiltrates that are thought to be antitumor effector cells (41). Second, imiquimod induces a strong local inflammatory response in treated cancerous tissue (42) and cytokine production (such as TNF-α), which may be cytotoxic to tumor cells. Third, imiquimod has been demonstrated to promote the development of cytotoxic killer dendritic cells, which may also attack tumors (43). Lastly, imiquimod can induce mitochondrial-mediated cell apoptosis of proliferating tumor cells (i.e., transformed epithelial cells) directly through its interaction with nucleoside transition molecules (44).

The dominant focus of research on TLR signaling is related to its properties of generating danger signals to the immune system (i.e., an inflammatory response) (29, 31, 37, 38), as well as the ability of TLR-mediated signaling to shape adaptive immunity (i.e., T lymphocyte memory). However, there is evidence that TLR signaling can play a role in tissue repair. MyD88-deficient mice were found to be more susceptible to radiation and dextran sulfate sodium-induced intestinal and colonic epithelial injury (45). Additionally, TLR9 agonists enhance T lymphocyte survival after exposure to ionizing radiation, which was dependent on MyD88 expression, involved ATM/ATR kinases, and the phosphorylation of the DNA repair enzymes Chk1/2 (46). There was also evidence that enhanced DNA repair of ionizing radiation-induced DNA damage, as detected by a comet assay. Thus, there is precedent that other TLR agonists enhance DNA repair.

Our previous studies with topical imiquimod in a mouse model of UVL-induced immune cutaneous immune suppression indicated that this TLR7 agonist protected mice from the loss of contact hypersensitivity, as well as the associated hapten-specific immune response. Imiquimod decreases UVL-induced DNA damage in an in vivo model. This is supported by the observation that imiquimod-treated nonmelanoma skin cancers develop lymphocyte-rich (CD4, CD8 T lymphocytes, and NK cells) infiltrates that are thought to be antitumor effector cells (41). Treatment with imiquimod also reverses the presence of immunosuppressive cytokines (IL-10, TGF-β) and regulatory T cell-associated transcription factor FoxP3 (9). These data suggest that enhanced survival after ionizing or UVB irradiation induced by TLR7 signaling is dependent on intact DNA repair mechanisms. This is consistent with our hypothesis that TLR7 signaling enhances DNA repair.

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After UVB irradiation of mouse skin, MHC class II-positive APC that bear CBPD can be detected within the local lymph nodes within hours after UVB exposure and persist for days after a single UVB exposure (10, 36). These damaged APC are thought to be responsible for systemic immune suppression that occurs after UVB irradiation (11). Topical applications of imiquimod decreased the amount of CBPD-bearing APC in local lymph nodes 24 h after UVB exposure, as seen in a DNA dot blot derived from draining lymph nodes of UVB-irradiated mice (Fig. 7A), as well as reversed the presence of immunosuppressive cytokines (IL-10, TGF-β) and regulatory T cell-associated transcription factor FoxP3 (9) (Fig. 7C).

**Discussion**

TLR are a major class of innate immune signaling receptors (37). These receptors, after sensing certain microbial products or damage-associated molecular patterns, trigger an inflammatory response that is orchestrated by a family of cytokines such as IL-1, TNF-α, and IL-6. In total, these inflammatory cytokines are pleiotropic and recruit inflammatory cells, enhance vascular permeability, regulate cell death, and induce the production of acute-phase reactants (29, 38).

TLR7, -8, and -9 are distinct members of the larger TLR family and are clustered in a group of intracellular receptors that recognize intracellular viral and bacterial infections (31). Imiquimod is a small, synthetic adenosine analog that activates TLR7 in mice and is known to elicit immune responses that suppress both the ability of immune cells to cause the clearance of precancerous skin lesions (actinic keratoses) or fully developed nonmelanoma skin cancer (superficial basal cell carcinoma). First, because of its adjuvant effects on TLR7/8 APC, this agent induces the maturation of such APC and the production of a number of cytokines that favor the development of Th1 lymphocytes during Ag presentation (17), which play an important role in antitumor responses. This has been confirmed at the tissue level, as histologic analysis of imiquimod-treated nonmelanoma skin cancers develop lymphocyte-rich (CD4, CD8 T lymphocytes, and NK cells) infiltrates that are thought to be antitumor effector cells (41). Second, imiquimod induces a strong local inflammatory response in treated cancerous tissue (42) and cytokine production (such as TNF-α), which may be cytotoxic to tumor cells. Third, imiquimod has been demonstrated to promote the development of cytotoxic killer dendritic cells, which may also attack tumors (43). Lastly, imiquimod can induce mitochondrial-mediated cell apoptosis of proliferating tumor cells (i.e., transformed epithelial cells) directly through its interaction with nucleoside transition molecules (44).

The dominant focus of research on TLR signaling is related to its properties of generating danger signals to the immune system (i.e., an inflammatory response) (29, 31, 37, 38), as well as the ability of TLR-mediated signaling to shape adaptive immunity (i.e., T lymphocyte memory). However, there is evidence that TLR signaling can play a role in tissue repair. MyD88-deficient mice were found to be more susceptible to radiation and dextran sulfate sodium-induced intestinal and colonic epithelial injury (45). Additionally, TLR9 agonists enhance T lymphocyte survival after exposure to ionizing radiation, which was dependent on MyD88 expression, involved ATM/ATR kinases, and the phosphorylation of the DNA repair enzymes Chk1/2 (46). There was also evidence that enhanced DNA repair of ionizing radiation-induced DNA damage, as detected by a comet assay. Thus, there is precedent that other TLR agonists enhance DNA repair.

Our previous studies with topical imiquimod in a mouse model of UVL-induced immune cutaneous immune suppression indicated that this TLR7 agonist protected mice from the loss of contact hypersensitivity, as well as the associated hapten-specific immune response.

**Imiquimod decreases UVL-induced DNA damage in an in vivo model**

After UVB irradiation of mouse skin, MHC class II-positive APC that bear CBPD can be detected within the local lymph nodes within hours after UVB exposure and persist for days after a single UVB exposure (10, 36). These damaged APC are thought to be responsible for systemic immune suppression that occurs after UVB irradiation (11). Topical applications of imiquimod decreased the amount of CBPD-bearing APC in local lymph nodes 24 h after UVB exposure, as seen in a DNA dot blot derived from draining lymph nodes of UVB-irradiated mice (Fig. 7A), as well as reversed the presence of immunosuppressive cytokines (IL-10, TGF-β) and regulatory T cell-associated transcription factor FoxP3 (9) (Fig. 7C).

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Our studies using the XS52 cell line (an LC-like APC line) suggested the TLR7 agonist imiquimod induced UVL-resistant XS52 maturation during T cell interactions, as measured by the ability of this cell line to stimulate IFN-γ secretion during Ag presentation, even after UVL exposure. One possible explanation for this resistance to UVL immune suppression is the enhanced repair of UVL-induced DNA damage, subsequent survival, and preservation of immune competence. We demonstrate that imiquimod has direct effects on APC on XPA gene expression, as well as nuclear localization of XPA protein (Fig. 1). This effect occurred rapidly (within 4–6 h after exposure to imiquimod), suggesting that it is a primary effect rather than a late secondary effect of cytokines triggered by imiquimod such as IL-12 (39). Our studies of important components of the TLR signaling pathway indicated that MyD88 was necessary for both the enhancement of the expression of the DNA repair gene XPA (and possibly other DNA repair genes as well) (Fig. 2A) as well as the enhanced cell survival after UVL exposure (Fig. 2B).

All TLR receptors activate NF-κB through a canonical pathway involving MyD88, TRAF6, TAK1, and Ikk complex (10, 29, 37, 28). Our studies using the pharmacologic compound BAY 11-7085 (inhibitor of the degradation of Ikk) indicates that this late component of the TLR pathway also directly regulates both XPA gene expression and the enhanced survival of UVL-irradiated cells (Fig. 3A–C). NF-κB is a central regulator of immune responses and inflammation, but it also targets genes related to cell survival and proliferation, such as cyclin D1, cyclin D2, c-Myc, c Myb, cycloxygenase 2, BcL-2, and BcL-XL (47–49). Thus, the observation that imiquimod enhanced cell survival after exposure to either UVB or ionizing radiation (Figs. 2, 3, 6) may in part be secondary to its broader effects of NF-κB signaling on cell survival gene expression. However, against this idea is the observation that imiquimod does not enhance the survival of lymphoblasts derived from an XPA patient after exposures to ionizing radiation or UVB. These data indicate that intact DNA repair mechanisms are necessary for imiquimod’s effects on survival to agents that
damage genomic DNA (in this case, ionizing radiation or UVB) (Fig. 6).

Our data indicate that TLR7 signaling can enhance the XPA gene expression and nuclear localization. XPA is involved in recognition of UVB damage and is thought to be an important early component of nucleotide excision repair of UV-induced CBPD (27). Although nucleotide excision repair genes are constitutively expressed, there is evidence that they are inducible (49, 50). Cytokines such as IL-12 and IL-18, natural products such as green tea polyphenols, and DNA oligonucleotides all enhance nucleotide excision repair of UV-damaged DNA (12–15, 51–54). Imiquimod also induces the nucleotide excision repair gene XPA (and presumably other nucleotide excision repair genes) as well as promoting functional DNA repair (gene reactivation and DNA dot blot assays, Figs. 4, 5).

Our studies of the local (cervical) lymph nodes after UVB irradiation of the dorsal surface of the pinna of the ear demonstrate that imiquimod treatment prevents the appearance of APC-bearing CBPD (Fig. 7A, 7B) and prevents the expression of immunosuppressive genes (IL-4, IL-10, TGF-β, and Foxp3) (Fig. 7C) in the skin-associated lymphoid tissue, preventing systemic immune suppression and the loss of contact sensitization, as we had previously demonstrated (16). Although imiquimod induced less efficient resolution of UV-induced CBPD in the local lymph nodes of IL-12 knockout mice (Figs. 7D, 7E) when compared with wild-type mice (Fig. 7A, 7B), there remained significant DNA repair in these gene-targeted mice. These data indicate that there is TLR7-inducible DNA repair even in the absence of IL-12, suggesting that TLR7 signaling has direct effects on DNA repair.

In summary, the TLR7 agonist imiquimod enhances DNA excision repair of UVB-induced CBPD. Thus, in addition to triggering inflammation and acting as an adjuvant, this topically applied anticancer agent also serves to protect the immune system against the deleterious effects of UVB by promoting the repair and subsequent survival of UVB damage by APC. These data also imply that imiquimod and its related analogs can be used as a therapy to prevent skin cancer by accelerating the repair of UV-induced DNA damage in cutaneous APC, thus preserving immune competence.

Disclosures
T.-C.M. and J.L. are employees of Graceway Pharmaceuticals. The other authors have no financial conflicts of interest.

References