JDA Japanese Dermatological Association
THE JOURNAL OF DERMATOLOGY
doi: 10.1111/1346-8138.13821
Journal of Dermatology 2017; ●●: 1–6

ORIGINAL ARTICLE

Increased number of mast cells in the dermis in actinic keratosis lesions effectively treated with imiquimod

Satomi OYAMA,[1] Yoko FUNASAKA,[1] Shin-ichi TSUCHIYA,[2] Seiji KAWANA,[1] Hidehisa SAEKI[1]

Departments of [1]Dermatology, [2]Pathology, Nippon Medical School, Tokyo, Japan

ABSTRACT

Actinic keratosis (AK) is a cutaneous cancer *in situ* which develops as a result of excessive exposure to ultraviolet (UV). Toll-like receptor (TLR)7 agonist imiquimod is a topical immune response modifier and is effective for the treatment of non-melanoma skin cancers. Recently, the diagnostic role of the dermatoscope has been reported in the course of treatment of AK. In addition, mast cells are now considered to contribute to both the innate and adaptive immune systems in topical imiquimod therapy. We assessed the effect of imiquimod treatment by dermatoscopic and immunohistochemical findings in 14 patients with a total of 21 AK lesions. With the dermatoscope, though the mean erythema score was not significantly different between the cured lesions and the unresponsive lesions, the erythema/red pseudo-network ("strawberry") pattern was decreased significantly in the cured lesions. By immunohistochemistry, the number of Ki-67-positive proliferative cells in the epidermis was decreased and that of CD117-positive mast cells in the dermis was increased in the responding lesions. To the best of our knowledge, this is the first study demonstrating that the number of mast cells in the dermis was increased in AK lesions effectively treated with imiquimod. Our present result suggests that mast cells may contribute an antitumor effect in human skin treated with topical imiquimod.

Key words: actinic keratosis, dermatoscope, imiquimod, mast cell, plasmacytoid dendritic cell.

INTRODUCTION

Actinic keratosis (AK) is a skin cancer *in situ* commonly observed on sun-exposed areas.[1] A fair skin type, age and chronic ultraviolet (UV) exposure are considered to be risk factors for developing AK.[2] The rate of progression of AK to squamous cell carcinoma is estimated to range 0.025–16%/year.[3] For this reason, AK should be treated appropriately at an early stage. Ishihara *et al.*[4] reported that the number of AK patients seen at major medical institutions in Japan has tended to increase over the past 15 years. While a variety of effective treatments are available for AK, topical application of imiquimod was approved for its treatment in November 2011 in Japan.[5] Recently, the diagnostic role of the dermatoscope has been reported in the course of treatment of AK.[6] Additionally, mast cells are now considered to contribute to both the innate and adaptive immune systems in topical imiquimod therapy.[7] In this study, we assessed the effect of imiquimod treatment not only by dermatoscopic findings such as erythema/red pseudo-network,[6] but also by immunohistochemical findings such as the numbers of Ki-67-positive proliferative cells in the epidermis and CD117-positive mast cells in the dermis.

METHODS

Patients

From January to December 2012, the patients who had clinically typical non-hyperkeratotic AK located on the face or the front of the head, confirmed by the histopathological diagnosis with two or three basal layers of nuclear atypia (mild or moderate AK), and who chose imiquimod therapy but not operation at the Department of Dermatology, Nippon Medical School Hospital, were enrolled in this study. In detail, the size of each clinical lesion was more than 1 cm^2 and less than 4 cm^2 and the lesion showed slight palpable erythema, Olsen grade 1,[8] and histopathologically Bowenoid type (severe AK) was excluded. Each patient was instructed to apply imiquimod 5% cream (Beselna® cream 5%; Mochida Pharmaceutical, Tokyo, Japan) to the AK lesion(s) three times per week for 4 weeks consecutively, and to attend follow-up visits on weeks 1, 2 and 4 during the treatment period. After the first course of 4-week

Correspondence: Satomi Oyama, M.D., Department of Dermatology, Nippon Medical School, 1-1-5 Sendagi, Bunkyo-ku, Tokyo 113-8603, Japan.
Email: syama@nms.ac.jp
Received 5 April 2016; accepted 1 February 2017.

treatment, a 4-week interval without treatment was taken, then a second course of another 4-week treatment was performed if the residual AK lesions were suspected.

Assessment of clinical and dermoscopic images

At each follow-up visit, the AK lesion(s) were examined using a dermatoscope by two educated dermatologists to evaluate the erythema/red pseudo-network ("strawberry") pattern, which is typified by telangiectatic vessels and keratotic plugs in the hair follicles surrounded by whitish halos.[9] Local skin reaction 10 was graded 0–4 based on the classification of erythematous reaction (grade 0, no erythematous reaction; grade 1, mild; grade 2, moderate; grade 3 severe; grade 4, very severe).

Histopathological analysis

A skin biopsy was obtained from treated lesions 4 weeks after the end of treatment. All biopsy specimens were stained with hematoxylin–eosin (HE) for histopathological examination. When no atypical cells were identified in the HE biopsy specimens, the lesion was defined as cured.

In addition, immunohistochemistry study using antibodies against Ki-67 (Dako, Glostrup, Denmark) and CD117 (Dako) was performed on all biopsy specimens, and the ultraView DAB universal kit (Roche, Kaiseraugst, Switzerland) was used as the detection system. To detect mast cells, toluidine blue staining was also performed. Immunohistochemistry was assessed by counting positive cells from two random fields of view at ×200 magnification and then taking the average.

Statistical analysis

All statistical analyses were performed using IBM SPSS Statistics (version 21; IBM, Armonk, NY, USA). Data were analyzed by Mann–Whitney *U*-test, Wilcoxon signed-rank test or McNemar's test. $P < 0.05$ was considered statistically significant. All statistical tests were two-sided.

RESULTS

Clinical status

Fourteen patients, six women (43%) and eight men (57%), with a total of 21 AK lesions completed the study and were included in the analysis. The median age of the patients was 72 years (range, 67–90). In all patients, AK were located on the face or the front of the head. As the AK lesions did not show complete clearance clinically by one course of 4-week treatment, all the lesions were treated with two courses of 4-week treatment. After two courses of treatment, the cure rate was 57% (histopathologically cured lesion, 12/21; unresponsive lesion, 9/21).

Dermatoscopy findings

The erythema scores were compared between the cured ($n = 12$) and the unresponsive lesions ($n = 9$). There was no significant difference between the two groups in initial erythema score before treatment (Fig. 1; Mann–Whitney *U*-test, $P = 0.545$). The mean erythema scores after treatment were significantly increased in both groups (Wilcoxon signed-rank test: cured group, $P = 0.007$; unresponsive group, $P = 0.027$). Although there was a tendency for the erythema score in cured lesions to be slightly higher than that in unresponsive lesions, the mean erythema score was not significantly different between the two lesions (Fig. 1; Mann–Whitney *U*-test, $P = 0.157$).

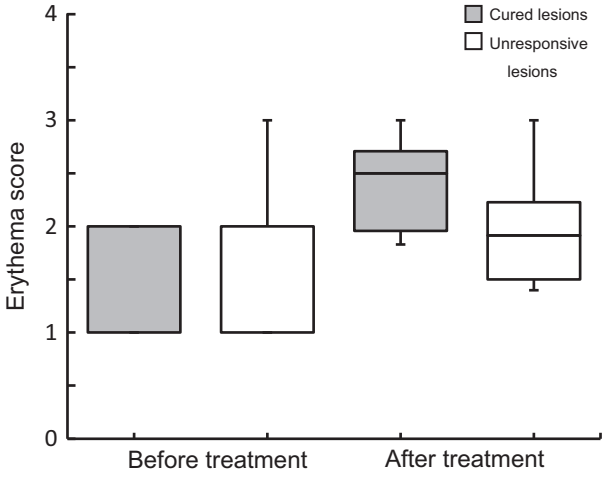


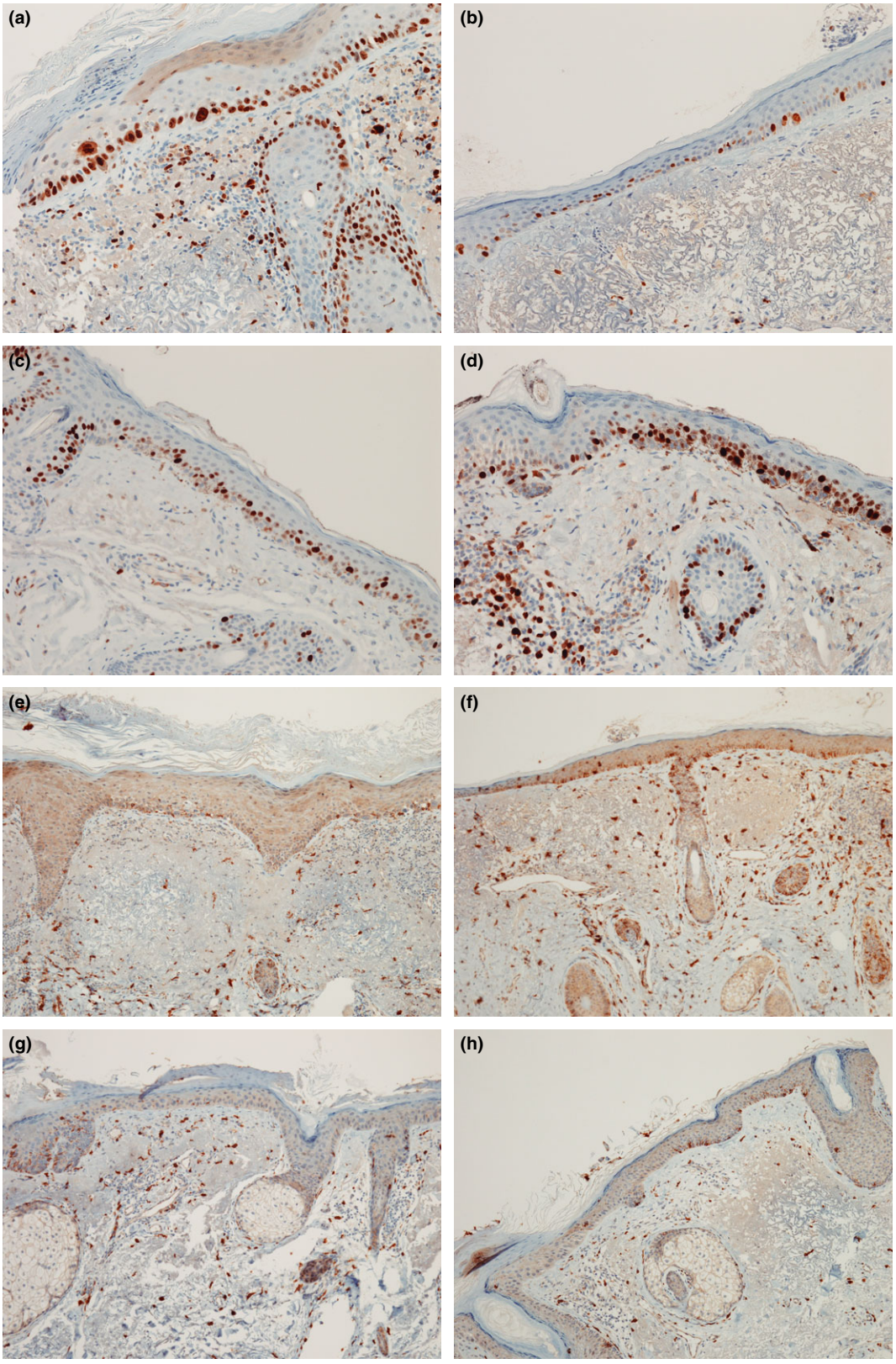
Figure 1. Box plots showing that erythema score of cured (gray boxes) and unresponsive lesions (white boxes) before and after treatment. There was no significant difference between the groups (both Mann–Whitney *U*-test).

The dermatoscope before treatment revealed erythema/red pseudo-network (strawberry) patterns in 16 of 21 lesions (76%). After the course of treatment, this had decreased to six of 21 lesions (29%). In the histopathologically cured group, a pseudo-network was observed in nine of 12 (75%) lesions before treatment, and this had decreased to two of 12 lesions (17%) after treatment. On the other hand, in the unresponsive group, a pseudo-network was present in seven of nine lesions (78%) before treatment, but this had decreased to four of nine lesions (44%) after treatment. Although these pseudo-networks had decreased in each group, the change was significant in the cured group between before and after treatment, and not significant in the unresponsive group (McNemar's test: cured group, $P = 0.016$; unresponsive group, $P = 0.250$).

Immunohistochemical analysis

The mean number of Ki-67-positive cells in the epidermis of cured lesion had decreased significantly after treatment (Figs 2a,b,3a; Wilcoxon signed-rank test, $P = 0.023$). In contrast, there was no significant change in those of unresponsive

Figure 2. Immunohistochemical staining for (a–d) Ki-67 and (e–h) CD117 before and after treatment (original magnification ×100). (a,b,e,f) Cured lesions. (c,d,g,h) Unresponsive lesions. (a,c,e,g) Before treatment. (b,d,f,h) After treatment. In a cured lesion, (a,b) Ki-67-positive cells in the epidermis were decreased and (e,f) CD117-positive cells in the dermis were increased after treatment.



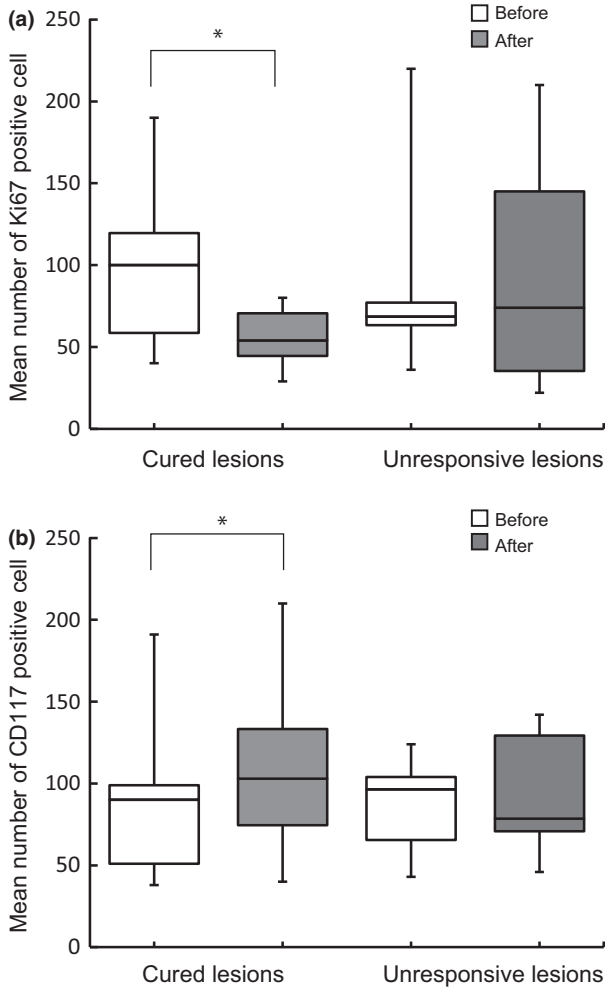


Figure 3. Box plots of the mean number of (a) Ki-67-positive cells in the epidermis and (b) CD117-positive cells in the dermis from two random fields. In a cured lesion, Ki-67-positive cells in the epidermis had significantly decreased after treatment (*P = 0.023, Wilcoxon signed-rank test). The number of CD117-positive cells in the dermis after treatment was significantly increased in cured lesions (*P = 0.041, Wilcoxon signed-rank test).

lesions before or after treatment (Figs 2c,d,3a; Wilcoxon signed-rank test, P = 0.515). Furthermore, the number of CD117-positive cells in the dermis after treatment was significantly increased in cured lesions compared with that before treatment (Figs 2e,f,3b; Wilcoxon signed-rank test, P = 0.041). There was no significant difference in CD117 expression in unresponsive lesions before and after treatment (Figs 2g,h,3b; Wilcoxon signed-rank test, P = 0.521). Neither was any significant difference noted between the groups of cured and unresponsive lesions before topical imiquimod treatment (Mann–Whitney U-test: Ki-67, P = 0.651; CD117, P = 0.702). Similar results were obtained by toluidine blue staining for detection of mast cells (Figs S1,S2).

DISCUSSION

Imiquimod, a synthetic Toll-like receptor (TLR)7 agonist with a molecular weight of 240.3 Da,[10] is recently well used to treat multiple AK lesions. TLR are a type of pattern recognition receptor. TLR1–10 have been confirmed in humans,[11] and are believed to recognize lipopolysaccharides, peptidoglycans and other pathogen-specific molecules (pathogen-associated molecular patterns), and thereby to activate the innate immune system via activation of the TLR/MyD88/nuclear factor-κB signaling pathway.[12] TLR7, which is present in the endosomal membranes of dendritic cells, monocytes, macrophages[13] and mast cells,[14] recognizes imiquimod and virus-derived ssRNA.[15,16]

Strawberry patterns of the skin were reduced after treatment in both responding and non-responding lesions though there was significant difference only in the former. In addition, although it has been reported that the complete clearance rate of AK tended to increase as the intensity of erythema increased,[17] no significant difference in erythema response score was observed between the two groups in our results. Strawberry pattern is typified by linear vessels surrounding the hair follicles, as previously mentioned.[6] The erythema score is considered to reflect the degree of local skin inflammation. Imiquimod, activating the innate immune system, inhibits tumor angiogenesis through promoting the production of interferon (IFN)-γ.[18] Although the strawberry patterns in responding lesions were attenuated significantly, the erythema score and therapeutic effects did not correlate, perhaps because the erythema had become less noticeable by angiogenesis inhibition.

In this study, lesions in which topical imiquimod was effective had significant post-treatment reductions in Ki-67-positive cells in the epidermis. Ki-67, a cell proliferation marker that localizes in the nucleus, is expressed in the G1 phase as well as between the S and M phases of the cell growth cycle.[19,20] Its expression is thought to positively correlate with malignancy grade,[19] suggesting that topical imiquimod treatment lowers the AK malignancy grade.

Several theories regarding the mechanism by which imiquimod exerts an antitumor effect have been proposed, including the induction of type I IFN secretion via TLR7 stimulation of plasmacytoid dendritic cells (pDC) in the dermis,[21] inhibition of angiogenesis[22] and induction of tumor cell apoptosis.[23] Recently, topical imiquimod was reported to have activity towards mast cells. In a melanoma mouse model, local recruitment of pDC was shown to be promoted by the CC chemokine ligand 2, which is produced from mast cells via TLR7 stimulation, and mature pDC were shown to exert a direct antitumor effect.[24] Mast cells activated by TLR7 stimulation are also reportedly involved in the migration of Langerhans cells and intensity of the cytotoxic T-cell response.[7] Hence, mast cells have been implicated in both the innate and adaptive immune systems in topical imiquimod therapy.[7]

Interestingly, lesions in which imiquimod treatment was effective in this study had a significant increase in CD117-positive cells in the dermis compared with those before treatment. CD117 is expressed in melanocytes and mast cells in the normal skin.[25] It is consequently considered that the

CD117-positive cells with granules observed in the dermis were mast cells. To the best of our knowledge, this is the first study to demonstrate that the number of mast cells in the dermis was increased in AK lesions effectively treated by imiquimod. Lately, mast cells have been reported to play a critical role in the initial inflammatory response induced by topical imiquimod. TLR7 stimulation provoked a local initial inflammatory response that was dependent on tumor necrosis factor-α produced from mast cells.[26] Moreover, in a contact hypersensitivity model using hapten, mast cells and dendritic cells were reported to activate one another and play key roles in establishing sensitization.[27] Therefore, an association between pDC and mast cells would possibly be observed in skin treated with topical imiquimod. Local skin reaction such as erythema and edema are well-known side-effects of imiquimod; therefore, mast cell induction could also be related to the side-effects of imiquimod.

Recent report revealed that the erythema score, which reflects the clinical inflammation induced by imiquimod, was significantly correlated to the duration for AK clearance, implying that inflammation elicited by topical imiquimod accelerates AK clearance.[28] We evaluated erythema score after two courses of topical therapy with imiquimod, and there was no significant difference between cured and non-responsive lesions at this time. It is possible that tumor cells had already been removed in the cured group which resulted in less inflammation. Our finding that prolonged mast cell induction was observed in cured lesions may contribute to the prolonged clearance of AK lesions, as imiquimod is known to keep tumor-free states even after the cessation of the treatment.

Sun-exposed skin, where AK occurs most frequently, has a high probability of possessing p53 genetic aberration.[29] In addition, UV exposure lowers local immunity,[30] such that skin surrounding AK is believed to be in a state of field cancerization, a condition in which tumors are more likely to occur.[31] Imiquimod has been reported to curb tumor recurrence[32] and rejuvenate photo-aged skin at sites where it was topically applied.[33] Thus, when considering the skin as a source of tumor generation, we propose that topical imiquimod is a suitable method for treating AK.

In summary, we assessed the effect of imiquimod treatment for AK by dermatoscopic and immunohistochemical findings. Strawberry patterns of dermatoscopy were significantly reduced after treatment in responding lesions. By immunohistochemistry, the number of Ki-67-positive proliferative cells in the epidermis was decreased and that of CD117-positive mast cells in the dermis was increased in responding lesions. These findings are useful in assessing the effectiveness of imiquimod treatment for AK.

CONFLICT OF INTEREST: None declared.

REFERENCES

1 Spencer J, Rosen T, Lebwohl MG. Prevalence and awareness of actinic keratosis: barriers and opportunities. *J Am Acad Dermatol* 2013; **68**: S2–S9.

2 Harvey I, Frankel S, Marks R, Shalom D, Nolan-Farrell M. Non-melanoma skin cancer and solar keratoses II analytical results of the South Wales Skin Cancer Study. *Br J Cancer* 1996; **74**: 1308–1312.

3 Glogau RG. The risk of progression to invasive disease. *J Am Acad Dermatol* 2000; **42**: S23–S24.

4 Ishihara K, Saida T, Otsuka F, Yamazaki N. Statistical profiles of malignant melanoma and other skin cancers in Japan: 2007 update. *Int J Clin Oncol* 2008; **13**: 33–41.

5 Idezuki T. Field cancerization and field therapy - the therapy of actinic keratosis by imiquimod. *Gan To Kagaku Ryoho* 2013; **40**: 1–5 (in Japanese).

6 Kaçar N, Sanli B, Zalaudek I, Yildiz N, Ergin S. Dermatoscopy for monitoring treatment of actinic keratosis with imiquimod. *Clin Exp Dermatol* 2012; **37**: 567–569.

7 Heib V, Becker M, Taube C, Stassen M. Advances in the understanding of mast cell function. *Br J Haematol* 2008; **142**: 683–694.

8 Olsen EA, Abernethy ML, Kulp-Shorten C *et al.* A double-blind, vehicle-controlled study evaluating masoprocol cream in the treatment of actinic keratoses on the head and neck. *J Am Acad Dermatol* 1991; **24**: 738–743.

9 Zalaudek I, Giacomel J, Schmid K *et al.* Dermatoscopy of facial actinic keratosis, intraepidermal carcinoma, and invasive squamous cell carcinoma: a progression model. *J Am Acad Dermatol* 2012; **66**: 589–597.

10 Vidal D. Topical imiquimod: mechanism of action and clinical applications. *Mini Rev Med Chem* 2006; **6**: 499–503.

11 Lee CC, Avalos AM, Ploegh HL. Accessory molecules for Toll-like receptors and their function. *Nat Rev Immunol* 2012; **12**: 168–179.

12 Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. *Cell* 2006; **124**: 783–801.

13 Hennessy EJ, Parker AE, O'Neill LAJ. Targeting Toll-like receptors: emerging therapeutics? *Nat Rev Drug Discov* 2010; **9**: 293–307.

14 Kulka M, Metcalfe DD. TLR3 activation inhibits human mast cell attachment to fibronectin and vitronectin. *Mol Immunol* 2006; **43**: 1579–1586.

15 Sandig H, Bulfone-Paus S. TLR signaling in mast cells: common and unique features. *Front Immunol* 2012; **3**: 185.

16 Diebold SS, Kaisho T, Hemmi H, Akira S, Reis e Sousa C. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. *Science* 2004; **303**: 1529–1531.

17 Korman N, Moy R, Ling M *et al.* Dosing with 5% imiquimod cream 3 times per week for the treatment of actinic keratosis: results of two phase 3, randomized, double-blind, parallel-group, vehicle-controlled trials. *Arch Dermatol* 2005; **141**: 467–473.

18 Majewski S, Marczak M, Mlynarczyk B, Benninghoff B, Jablonska S. Imiquimod is a strong inhibitor of tumor cell-induced angiogenesis. *Int J Dermatol* 2005; **44**: 14–19.

19 Gerdes J. Ki-67 and other proliferation markers useful for immunohistological diagnostic and prognostic evaluations in human malignancies. *Semin Cancer Biol* 1990; **1**: 199–206.

20 Scholzen T, Gerdes J. The Ki-67 protein: from the known and the unknown. *J Cell Physiol* 2000; **182**: 311–322.

21 Batinac T, Zamolo G, Jonjic N, Gruber F, Petrovecki M. p53 protein expression and cell proliferation in non-neoplastic and neoplastic proliferative skin diseases. *Tumori* 2004; **90**: 120–127.

22 Gibson SJ, Lindh JM, Riter TR *et al.* Plasmacytoid dendritic cells produce cytokines and mature in response to the TLR7 agonists, imiquimod and resiquimod. *Cell Immunol* 2002; **218**: 74–86.

23 Schön MP, Schön M. Imiquimod: mode of action. *Br J Dermatol* 2007; **157**: 8–13.

24 Drobits B, Holcmann M, Amberg N *et al.* Imiquimod clears tumors in mice independent of adaptive immunity by converting pDCs into tumor-killing effector cells. *J Clin Invest* 2012; **122**: 575–585.

25 Lammie A, Drobnjak M, Gerald W, Saad A, Cote R, Cordon-Cardo C. Expression of c-kit and kit ligand proteins in normal human tissues. *J Histochem Cytochem* 1994; **42**: 1417–1425.

26 Heib V, Becker M. Mast cells are crucial for early inflammation, migration of Langerhans cells, and CTL responses following topical application of TLR7 ligand in mice. *Blood* 2007; **110**: 946–953.

27 Honda T, Egawa G, Grabbe S, Kabashima K. Update of immune events in the murine contact hypersensitivity model: toward the understanding of allergic contact dermatitis. *J Invest Dermatol* 2013; **133**: 303–315.
28 Kopera D, Kerl H. Visualization and treatment of subclinical actinic keratoses with topical imiquimod 5% cream: an observational study. *Biomed Res Int* 2014; **2014**: 135916.
29 Ouhtit A, Ueda M, Nakazawa H *et al*. Quantitative detection of ultraviolet-specific p53 mutations in normal skin from Japanese patients. *Cancer Epidemiol Biomarkers Prev* 1997; **6**: 433–438.
30 Kripke ML. Ultraviolet radiation and immunology: something new under the sun-presidential address. *Cancer Res* 1994; **54**: 6102–6105.
31 Stockfleth E. The paradigm shift in treating actinic keratosis: a comprehensive strategy. *J Drugs Dermatol* 2012; **11**: 1462–1467.
32 Sidky YA, Borden EC, Weeks CE, Reiter MJ, Hatcher JF, Bryan GT. Inhibition of Murine Tumor Growth by an Interferon-inducing Imidazoquinolinamine. *Cancer Res* 1992; **52**: 3528–3533.
33 Smith K, Hamza S, Germain M, Skelton H. Does imiquimod histologically rejuvenate ultraviolet radiation-damaged skin? *Dermatol Surg* 2007; **33**: 1419–1428; discussion 1428–9.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. (a–d) Toluidine blue staining before and after treatment (original magnification ×100).

Figure S2. Box plots of the mean number of mast cells in the dermis from two random fields.