Disease-Independent Skin Recruitment and Activation of Plasmacytoid Predendritic Cells Following Imiquimod Treatment

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Background: **Imiquimod, an immune response modifier that is used topically to treat different types of skin cancer, induces the production of proinflammatory cytokines that stimulate an antitumor immune response. We assessed characteristics of the imiquimod-induced immune activation in epithelial and lymphoproliferative neoplasias of human skin. We focused on plasmacytoid predendritic cells (PDCs), the primary producer of interferon α (IFN-α) after imiquimod activation in vitro.** ***Methods:*** **We used Affymetrix oligonucleotide arrays to compare gene expression profiles from tumors from 16 patients, 10 with superficial basal cell carcinomas (sBCCs), five with cutaneous T-cell lymphomas (CTCLs), and one with Bowen's disease, before and after topical imiquimod treatment. We used quantitative immunohistochemistry with PDC-specific antibodies against BDCA-2 and CD123 to characterize the PDC population before and after imiquimod treatment in these specimens. Activation status of PDCs from four sBCC patients was assessed by intracellular IFN-α staining and flow cytometry.** ***Results:*** **Expression of various IFN-α–inducible genes (e.g., CIG5, G1P2, OASL, IFIT1, STAT1, IFI35, OAS1, ISG20, MxA, and IRF7), the so-called IFN-α signature, was increased similarly in both sBCC and CTCL lesions after imiquimod treatment. PDCs were recruited and activated in both lesion types, and they produced IFN-α after imiquimod treatment in vivo (mean percentage of PDCs producing IFN-α = 14.5%, 95% confidence interval [CI] = 4.9% to 24%; range = 3.3%–27%, *n* = 4 lesions). Imiquimod induced similar immune activation patterns in all three diseases, and these patterns were associated with the number of PDCs recruited to the treatment site. Two imiquimod-treated sBCC patients who did not mount an inflammatory response to imiquimod and whose lesions lacked the IFN-α signature after treatment had fewer PDCs in treated lesions compared with other treated patients with such a response.** ***Conclusions:*** **Imiquimod induces immune activation patterns that relate to the number of the PDCs recruited to the treatment site, thus supporting the role of PDC in responsiveness to imiquimod in humans. [J Natl Cancer Inst 2005;97:1143–53]**

Dendritic cells are a heterogeneous group of antigen-presenting cells that link innate and adaptive immunity *(1,2)*. In humans, different subsets of dendritic cells have been described, some with a preference for specific organs *(1,3)*. Among these subsets, epidermal Langerhans cells and dermal dendritic cells are the resident dendritic cell populations in human skin. Plasmacytoid predendritic cells (PDCs) are a newly identified subset of dendritic cells that are found in blood and secondary lymphoid organs *(4)*. PDCs are characterized by a plasma cell–like morphology, a unique surface phenotype ($CD4^+$, $CD11c^-$, $CD123^+$, BDCA-2^+, and HLA-DR^+), and the lack of common lineage markers (such as CD3 on T cells, CD14 on monocytes, CD16 and CD56 on natural killer cells, and CD19 and CD20 on B cells) *(5)*. PDCs represent a key component in innate antiviral immunity because of their capacity to produce large amounts of IFN-α *(6,7)*. PDCs have also been described in lesions of inflammatory diseases of the skin (e.g., psoriasis vulgaris, contact dermatitis, and lupus erythematosus) but are essentially absent in normal skin *(8–11)*.

Imidazoquinolines are a group of low molecular weight synthetic compounds that are potent inducers of IFN-α in vivo. One imidazoquinoline, imiquimod, has potent antiviral and antitumor activity *(12)*. By using high-throughput gene expression profiling, we have recently described a group of IFN-α-inducible genes (e.g., IRF7, OAS1, OAS2, MxA, MxB, IFI44, IFIT1, IFI35, and G1P2), the so-called IFN-α signature, whose expression was increased after topical imiquimod treatment of skin cancer *(17)*. Imiquimod directly activates innate immune effectors through a Toll-like receptor (TLR) 7/MyD88-dependent pathway *(13)*. Through the induction of the surface expression of costimulatory molecules (such as CD80 and CD86) and through the production and release of specific cytokines (especially IFN-α, tumor necrosis factor α, and interleukin 12), imiquimod-treated dendritic cells induce T helper type 1 immunity *(13,14)*. In vitro, blood PDCs are the principal source of IFN-α produced in response to imiquimod *(15)*. Topical treatment with imiquimod induces several types of skin cancer to regress *(16)*. However, to date, PDCs have not been shown to play a direct role in the inflammatory response of imiquimod-treated human neoplastic skin lesions.

In this study, we compared gene expression profiles in lesions before and after topical imiquimod treatment of patients with a neoplastic epithelial disease, superficial basal cell carcinoma (sBCC) and Bowen's disease, or a lymphoproliferative disease of the skin, cutaneous T-cell lymphoma (CTCL). Because PDCs are considered the primary source of IFN-α after they are activated with imiquimod, we also assessed changes, before and after imiquimod treatment, in the PDC population in lesions from patients with these two diseases.

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See "Notes" following "References."

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Patients and Methods

Patient Samples

We used tissue samples from patients participating in two clinical open-label studies that investigated the immunohistology and gene expression profile of skin lesions treated with a 5% imiquimod cream (Aldara, 3M Pharmaceuticals, Saint Paul, MN): Study 1 *(17)* included five patients with sBCC and one patient with Bowen's disease. Study 2 included five patients with sBCC and five patients with CTCL. Both studies were approved by the institutional ethics committee. Before entering either study, patients provided written informed consent. At the screening visit that took place 2 weeks before treatment initiation, a biopsy specimen was taken for histologic confirmation of sBCC in study 1 or for confirmation of sBCC or CTCL in study 2. In study 2, a patient with CTCL was eligible if he or she had biopsy-proven stage Ib–IIa CTCL. In study 1, patients applied imiquimod to the target sBCC lesion once daily until the treated tumor showed signs of erosion (generally in 3–5 days), and in study 2, patients applied imiquimod to the target sBCC or CTCL lesions once daily for 5 days. In study 1, surgical excision of the treated sBCC was performed 3–5 days after treatment initiation, whereas in study 2 the treated lesion was either completely excised (sBCC) or subjected to biopsy examination (CTCL) 5 days after treatment initiation. Every pre- and posttreatment tissue sample was divided in two parts—one half was immediately snap frozen and stored at −80 °C, and the other half was fixed in formalin and subsequently embedded in paraffin. If the excised tumor was large enough, a piece of fresh tissue material was processed to obtain single-cell suspensions.

Gene Expression Profiling

Total RNA was isolated from tissues by use of the TRIzol reagent (Invitrogen AG, Basle, Switzerland). Double-stranded cDNA was generated with a cDNA synthesis kit (MessageAmp, Ambion Inc., Huntingdon, UK) and a $(dT)_{24}$ primer containing a T7 RNA polymerase promoter at the 3′ end. Labeled complementary RNA (cRNA) was prepared from double-stranded cDNA by in vitro transcription with a T7 polymerase in a reaction mixture containing biotin-11-cytidine triphosphate and biotin-16-uridine triphosphate (Enzo Diagnostics, Farmingdale, NY), and cRNA was purified with RNA clean-up columns according to the manufacturer's recommendations (MessageAmp Kit). Biotinylated cRNA (15 μg) was fragmented and hybridized to HG-U95Av2 GeneChip arrays (Affymetrix, Santa Clara, CA) containing probe sets representing approximately 12 000 genes. Chips were hybridized, washed, and stained according to protocols recommended by Affymetrix. The overall fluorescence intensity was scaled to a global intensity of 500 to enable the comparison between chips.

Raw data were imported in GeneSpring, version 7.2, microarray data analysis software (Silicon Genetics, Redwood City, CA). The following criteria were used to select differentially regulated genes in imiquimod-treated versus untreated specimens during microarray data analysis: 1) a paired Student's *t* test with Benjamini–Hochberg multiple-testing correction and a statistical significance level at $P<.05$; 2) more than a twofold change in either direction; 3) a detection "present" call that coincided with the regulation in question—e.g., if a candidate gene was called "up-regulated" in imiquimod-treated samples, at least one of the samples in the treated group had to be called "present"; and vice versa, if a gene was called "down-regulated," at least one of the samples in the untreated group had to be called "present." The selected differentially expressed genes were normalized to a mean of 0 and a standard deviation of 1, $\log_2$-transformed, and subjected to average-linkage hierarchical clustering by use of the uncentered Pearson similarity matrix. Clustering analysis was performed with the Gene Cluster program, and the display of the rearranged data was generated with the TreeView program *(18)* (both programs are available through http://rana.lbl.gov). The selected genes were annotated by use of the NetAffx Internet analysis system (Affymetrix) *(19)*.

Real-Time Polymerase Chain Reaction

Approximately 1 μg of total RNA was reverse transcribed with the 1st Strand cDNA Synthesis Kit for real-time polymerase chain reaction (PCR) (Roche Diagnostics AG, Rotkreutz, Switzerland) at 42 °C for 1 hour. PCR amplifications were carried out with the HotStart system (LightCycler–Faststart DNA Master SYBR Green I, Roche Diagnostics). The following primer sets were used: IRF7 (forward) = 5′-CAAGTGCAAGGTGTACTGG-3′ and IRF7 (reverse) = 5′-CAGGTAGATGGTATAGCGTGG-3′; MxA (forward) = 5′-ACTCTGTCCAGCCCCGTAGAC-3′ and MxA (reverse) = 5′-TCACAGCTTCCTGCTAAATCACC-3′; forward and reverse primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were from a GAPDH quantification kit (Search LC, GmbH, Dossenheim, Germany). To generate external standards for subsequent quantification, PCR products were purified and cloned into the pCRII-TOPO vector with the TOPO TA Cloning Kit (Invitrogen AG), according to the manufacturer's recommendations. Incorporation of the insert into the vector was confirmed by sequencing, and the insert's identity was confirmed with the BLAST online program (http://www.ncbi.nlm.nih.gov/BLAST).

Immunohistochemistry and Confocal Laser-Scanning Microscopy

Paraffin-embedded and acetone-fixed frozen tissue sections from the patients in our study were stained by use of the alkaline phosphatase–anti-alkaline phosphatase technique. Unspecific Fc receptor binding of antibodies used in this study was measured with isotype-matched control antibodies. The following antibodies were used only on frozen tissue sections: anti-BDCA-2 (clone AC144, Miltenyi Biotec, Bergisch Gladbach, Germany), anti-CD1c (L161, Immunotech, Marseilles, France), fluorescein isothiocyanate (FITC)-conjugated polyclonal rabbit anti-mouse immunoglobulins (DakoCytomation AG, Zug, Switzerland), and phycoerythrin-conjugated anti-CD123 and allophycocyanin-conjugated anti-CD4 antibodies (both from BD Biosciences, Basel, Switzerland). We also used anti-CD123 (7G3, BD Biosciences) and anti-MxA *(20)* (clone CL143) antibodies for paraffin-embedded tissue sections. Quantification of immunohistochemical staining was performed by two independent investigators who evaluated three random ×40 microscopic fields. Because imiquimod treatment results in an influx of inflammatory cells at the treatment site *(21,22)*, we evaluated the density of recruited inflammatory cells. The density of inflammatory infiltrated cells, if present, was scored by infiltrate density grade

as follows: grade 1 = mild, superficial, and subepidermal infiltrate without the tendency to confluence; grade 2 = moderate band-like infiltrate homogeneously distributed in the papillary dermis; and grade 3 = heavy infiltrate diffusely extending to reticular dermis or into the hypodermis.

Flow Cytometry Analysis of Ex Vivo PDCs

A portion of the skin biopsies obtained from study participants after imiquimod treatment was treated with dispase II (Roche Diagnostics) overnight at 4 °C to separate epidermis from dermis. Dermal sheets were then carefully separated from epidermal sheets, cut into small pieces of 1–5 mm, and enzymatically digested with a mixture containing collagenase and hyaluronidase type VII (each at 200 U/mL; both from Sigma-Aldrich Schweiz, Buchs, Switzerland), 0.01% DNase I (Roche Diagnostics), 1 m*M* sodium pyruvate, 1% antibiotic-antimycotic mixture (containing penicillin at 10 000 U/mL, streptomycin at 10 000 μg/mL, and amphotericin B at 25 μg/mL in a solution of 0.85% NaCl), and 10 m*M* HEPES buffer (all from Gibco Invitrogen AG, Basel, Switzerland) for 2 hours at 37 °C to obtain single-cell suspensions. Three-color staining for flow cytometry was performed by using FITC-conjugated anti-BDCA-2 and allophycocyanin-conjugated CD123 (both from Miltenyi Biotec) and phycoerythrin-conjugated antibodies against CD3, CD11c, CD14, CD20, CD56, CD80, CD86, or HLA-DR or their corresponding isotype controls (all from BD Biosciences). For intracellular IFN-α detection, cells were first surface stained with FITC-conjugated anti-BDCA-2 antibodies, permeabilized with 0.1% saponin in phosphate-buffered saline, and then stained with phycoerythrin-labeled anti-human IFN-α2 antibody (225.C, Chromaprobe Inc., Maryland Heights, MO) or IgG2b isotype control. Cells were then analyzed on a FACSCalibur flow cytometer, and data were processed with CellQuestPro software (both BD Biosciences).

Statistical Analysis

Quantitative immunohistochemistry data were expressed as the median number of cells per ×40 field. For the quantitative immunohistochemistry analysis, pairs missing respective pretreatment marker evaluation were excluded from matched statistical analysis (Wilcoxon test and Spearman's correlation). Statistical analysis was performed with the SPSS software package, version 12.0 (SPSS, Chicago, IL). *P* values of less than .05 were considered statistically significant. All statistical tests were two-sided.

Results

Characterization of the Imiquimod-Induced Changes in Gene Expression Profiles of Skin Tumors

To investigate the effects of topical imiquimod treatment on gene expression profiles of neoplastic epithelial and lymphoproliferative diseases of the skin, we generated gene expression profiles from specimens obtained before and after treatment with imiquimod from the following lesions: 11 sBCC lesions (from 10 patients, one of whom had two treated lesions), five CTCL lesions, and one Bowen's disease lesion. By using our filtering criteria, we found 709 genes that were differentially expressed in response to topical imiquimod treatment; expression of 366 genes was increased, and expression of 343 genes was decreased. We subjected this set of 709 differentially regulated genes to two-way hierarchical clustering analysis, which is a mathematical approach that essentially organizes the data by grouping genes with similar expression patterns. This analysis identified two major array clusters associated with imiquimod treatment—pretreatment and posttreatment clusters (Fig. 1, A).

To facilitate the analysis of genes that account for differences in overall gene expression, each gene was assigned to a defined functional category according to available annotations (i.e., a GO search on the NetAffx website, http://www.affymetrix.com/analysis/index.affx). We found that one third of the genes whose expression was stimulated by imiquimod were involved in various aspects of immune response (Fig. 2). A series of IFN-α–inducible genes including CIG5, G1P2, OASL, IFIT1, STAT1, IFI35, OAS1, ISG20, MxA, and IRF7, the so-called IFN-α signature, was the most prominently induced by imiquimod (Fig. 2). Imiquimod treatment stimulated the expression of these IFN-inducible genes in both sBCCs and CTCLs (Fig. 1, A). According to the clustering analysis, the expression of two IFN-α–inducible genes, IRF7 and MxA, was not increased in three imiquimod-treated samples (CTCL1, CTCL2, and CTCL3). To investigate the differences in IRF7 and MxA mRNA levels between samples before and after imiquimod treatment, we used real-time PCR to assess the expression of these two genes, which are highly specific for local IFN-α activity. When we compared the array data and the PCR data for the expression of IRF7 and MxA, we found good correlation (for IRF7 expression, Pearson's $r = .679$ and $P < .001$; for MxA expression Pearson's $r = .501$ and $P = .005$). Real-time PCR analysis confirmed the increased expression of IRF7 and MxA genes in all imiquimod-treated tumors, including tumors CTCL1, CTCL2, and CTCL3, compared with expression before treatment, irrespective of the disease type (Fig. 1, B). Immunohistochemistry also demonstrated consistently increased MxA protein expression in all imiquimod-treated tumors (data not shown), supporting the array data.

Recruitment of PDCs Into the Skin After Imiquimod Treatment

The distinct, disease-independent imiquimod-related expression of IFN-α–inducible genes raised the question of whether these results reflected the imiquimod-induced accumulation of PDCs in the treated lesions. First, microarray data indicated that PDCs may be present in BCC, CTCL, and Bowen's disease lesions because of the increased expression of several genes expressed by PDCs, including CD123 (IL3Rα) *(5)*, CD38 *(17)*, CD62L (L-selectin) *(7)*, ILT3 *(7)*, ILT7 *(23)*, and CD43 *(24)* after imiquimod treatment (Fig. 2). Second, we used an antibody specific for blood PDCs (i.e., anti-BDCA-2 antibody) *(25)* and an antibody against CD123 (IL3Rα), which is highly expressed on PDCs, to determine whether PDCs were present in lesions before and after imiquimod treatment. Eleven "baseline to posttreatment" matched samples (five BCCs, five CTCLs, and one Bowen's disease) were available for matched quantitative immunohistochemistry analysis of BDCA-2 and CD1c markers, and 16 matched pairs were available for quantitative immunohistochemistry analysis of CD123. We found that the number of BDCA-2^+ cells in skin lesions was statistically significantly higher after imiquimod treatment (from a median of 1.2 cells per ×40 field before imiquimod treatment to a median of 28.0 cells

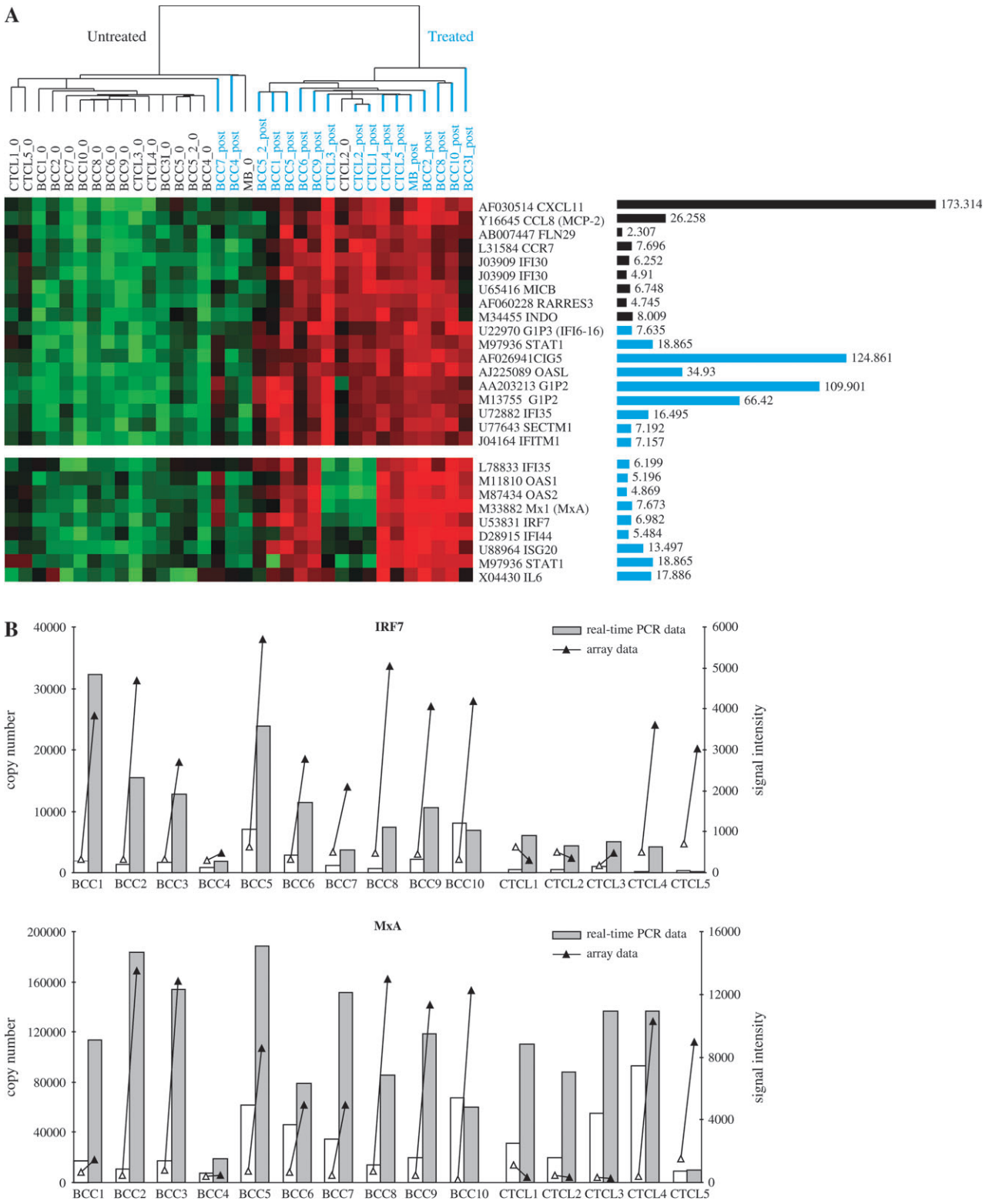

Fig. 1. Gene expression profiling of skin tumors treated with cream containing 5% imiquimod. **A**) Interferon (IFN) clusters obtained after hierarchical clustering analysis of 709 differentially expressed genes. Relationship between experimental samples is summarized in a dendrogram above the microarray, in which the pattern and the length of the branches reflect the relatedness of the samples. **Red** = high relative level of expression; **green** = low relative level of expression; **black** branches = samples before imiquimod treatment; **blue branches** = samples after imiquimod treatment. Genes are identified by their GenBank accession number and symbol. To the right of each gene symbol, changes in relative gene expression levels are represented by bars showing fold change increase in expression after treatment. **Blue bars** = IFN-α–inducible genes; **black bars** = IFN-γ–inducible genes. Names of pretreatment samples are followed by "_0"; names of posttreatment sample names are followed by "_post". **B**) Expression of MxA and IRF7 mRNA obtained by microarray analysis (**bars**) and by real-time polymerase chain reaction (PCR; LightCycler; **triangles**). Microarray gene expression data are expressed as signal intensity (*y* axis to the **right**). PCR results (the means of triplicate experiments are expressed as the copy number (i.e., as glyceraldehyde-3-phosphate dehydrogenase [GAPDH]–normalized ratios to compensate for variations in quantity and quality of starting mRNA) (*y* axis to the **left**). Low variability between triplicate experiments gave error bars (95% confidence intervals) that were too small to be visualized beyond the symbols. Pretreatment samples are shown as **open bars** (**triangles**), whereas posttreatment samples are shown as **shaded bars** (**black triangles**). BCC = basal cell carcinoma; CTCL = cutaneous T-cell lymphoma; MB = Bowen's disease.

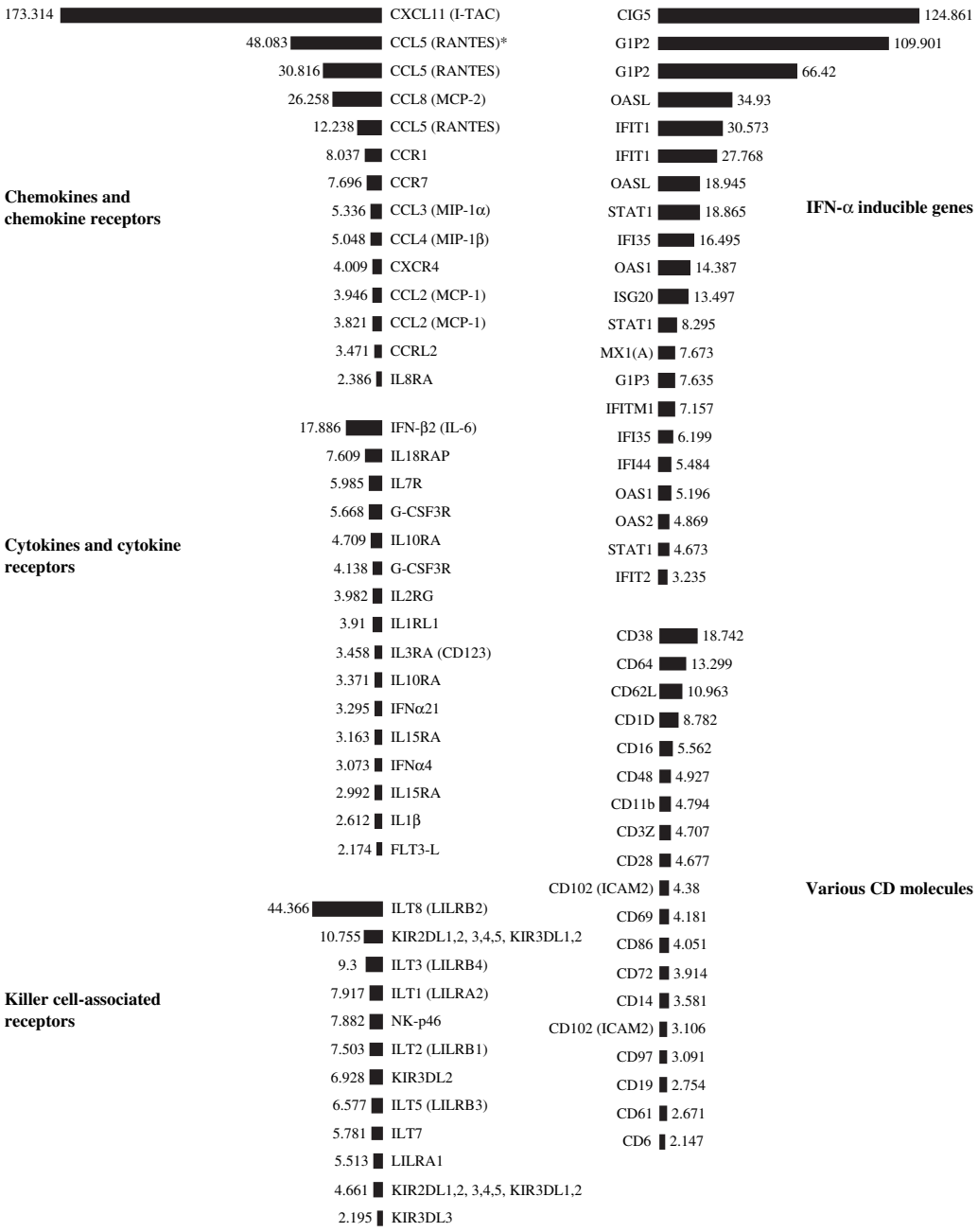

Fig. 2. Expression of various genes involved in the immune response after topical imiquimod treatment. **Bars** = the fold change in the mRNA level of the gene indicated after imiquimod treatment compared to mRNA levels before the treatment. These genes passed our filtering criteria. Genes are indicated by their gene symbol. * = repeated gene symbols represent gene expression levels obtained by use of different probes (i.e., oligonucleotide sets) on Affymetrix arrays. IFN = interferon.

per ×40 field after imiquimod treatment; the median difference among 11 matched samples of BDCA-2$^+$ cells = 26.8 cells per field; P = .003, Wilcoxon test; Figs. 3 and 4, A) than before treatment. Results were similar regardless of whether the lesion was from an epithelial disease group (sBCC and Bowen's disease) or a lymphoproliferative disease group (CTCL) (Fig. 3, B). The number of CD123-expressing cells increased statistically significantly also after imiquimod treatment (from a median of 2.3 cells per ×40 field before imiquimod treatment to a median of 39.3 cells per ×40 field after imiquimod treatment; the median difference among 16 matched samples of CD123$^+$ cells = 37 cells per field; P<.001, Wilcoxon test). The PDC phenotype was confirmed by costaining BDCA-2$^+$ cells with antibodies against CD123 and CD4, antigens that are coexpressed by PDCs, and viewing with confocal laser-scanning microscopy (Fig. 4, B and C). In addition, flow cytometry analysis of dermal cell suspensions revealed that BDCA-2$^+$,CD123^{++} cells were also HLA-DR$^+$,CD11c$^-$ but did not express the lineage markers CD3, CD14, CD20, and CD56; this phenotype corresponds to the PDC phenotype (Fig. 5, B). CD4 was not assessed by flow cytometry, because the treatment with dispase and collagenase disrupts CD4 cell surface expression so that it is undetectable *(26)*. According to flow cytometry analysis, PDCs accounted for up to 16.4% of the dermal mononuclear infiltrate (mean = 6.4%, 95% confidence interval [CI] = −0.2% to 13%; range = 2.2%–16.4%; n = 4 lesions).

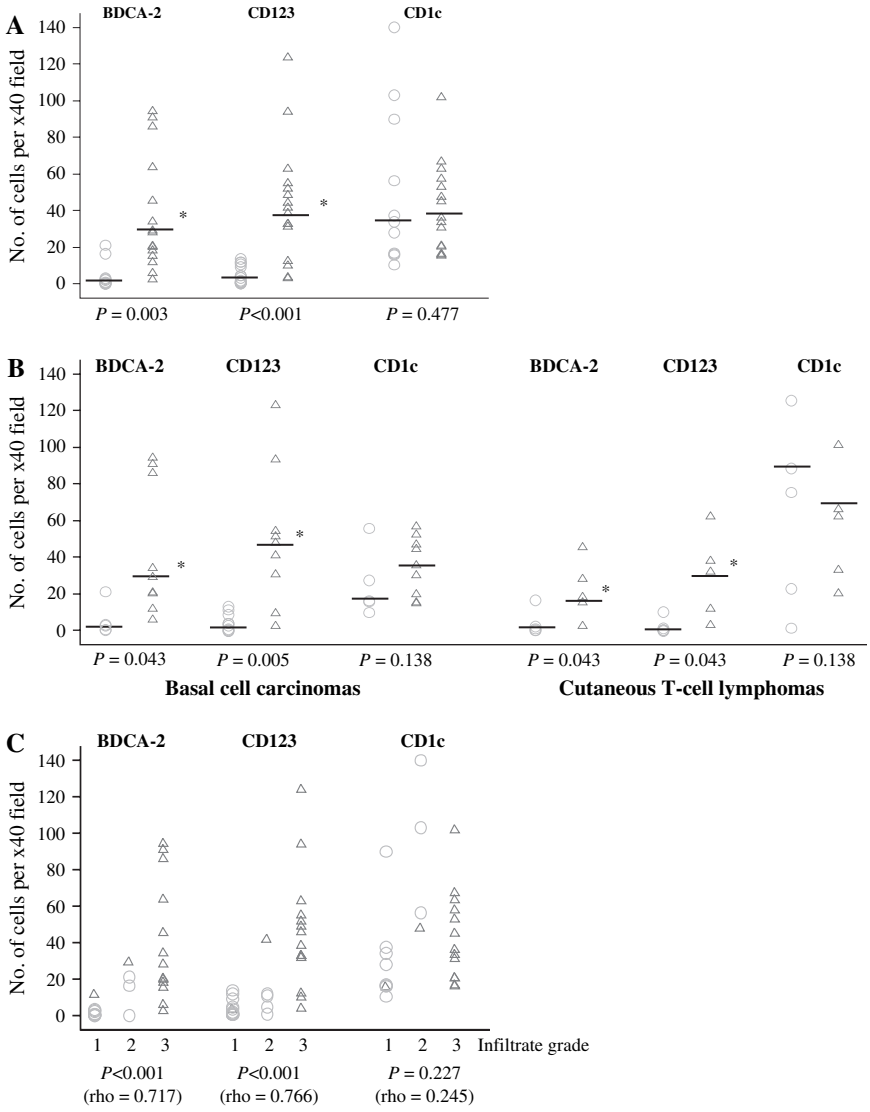

Fig. 3. Quantitative immunohistochemistry data. Data are expressed as the median number of cells expressing the respective marker per ×40 field. **A)** Expression of BDCA-2, CD123, and CD1c cell surface proteins in cells of skin tumors obtained before and after topical imiquimod treatment. **B)** Expression of BDCA-2, CD123, and CD1c cell surface proteins in skin tumors obtained before and after topical imiquimod treatment according to the disease histology. **C)** Expression of BDCA-2, CD123, and CD1c cell surface proteins in skin tumors obtained before and after topical imiquimod treatment according to infiltrate density grade. **A–C)** Horizontal lines = median cell count for each marker; **open circles** = before treatment; **open triangles** = after treatment. **A and B)** Statistical significances of differences between pretreatment and posttreatment marker expression are indicated with an asterisk (*); the corresponding *P* value shown below each graph (two-sided Wilcoxon signed rank test). **C)** Two-sided Spearman's ρ and corresponding *P* values are shown below each graph (Spearman's rank correlation).

Immunohistochemistry and confocal microscopy revealed that PDCs preferentially localized subepidermally, even though small groups of BDCA-2$^+$,CD123$^+$ cells could be observed in the vicinity of blood vessels or tumor islets (Fig. 4, B and C). To assess the alterations in dermal dendritic cells of myeloid origin (MDCs) in skin lesions before and after imiquimod treatment, we used quantitative immunohistochemistry for an MDC-specific marker, CD1c. Unlike PDCs, the number of CD1c$^+$ MDCs in the lesions was not changed by imiquimod treatment (i.e., a median of 33.5 cells per ×40 field before treatment to a median of 35.5 cells per ×40 field after treatment; the median difference among 11 matched samples of CD1c$^+$ cells = 2.0 cells per field; $P = .477$, Wilcoxon test; Fig. 3).

Furthermore, we found that the increase in the number of PDCs (BDCA-2$^+$ and CD123$^+$ cells) from baseline to posttreatment was accompanied by a statistically significant increase in infiltrate density grade (for BDCA-2 Spearman's ρ = .717 and $P<.001$; for CD123 Spearman's ρ = .766 and $P<.001$), thus indicating that PDCs along with other inflammatory cells are being actively recruited into the treated lesion (Fig. 3, C). The active recruitment of these cells into imiquimod-treated lesions is also supported by a 173-fold increase (range = 2.3-fold to 1232.9-fold increase) in expression in lesions of the CXCL11/ITAC gene, a chemokine that is involved in the recruitment of the PDC to inflamed skin *(27)*, compared with levels before treatment (Fig. 2). Changes in MDC (CD1c$^+$) cell numbers, however, did not correlate with the changes in infiltrated density grade, supporting their "resident" nature in the dermis (Spearman's ρ = .245 and $P = .227$).

Skin-Infiltrating PDCs and the Production of IFN-α After Imiquimod Treatment In Vivo

In vitro, PDCs produce large amounts of IFN-α in response to imiquimod treatment *(15)*. To determine whether skin-infiltrating PDCs also produce IFN-α after topical imiquimod treatment in vivo, we analyzed single-cell suspensions of dermal cells isolated from skin biopsy specimens of four sBCCs treated for 5 days with imiquimod. By intracellular staining with an antibody against IFN-α, up to 27% of BDCA2$^+$,CD123^{++} PDCs produced IFN-α (mean = 14.5%, 95% CI = 4.9% to 24.0%; range = 3.3%–27%, $n = 4$ lesions Fig. 5, C). In contrast, no other dermal cell types (BDCA-2$^-$,CD123$^-$ cell population) produced detectable amounts of IFN-α (<1%, $n = 4$ lesions), indicating that PDCs are the principal producers of IFN-α in imiquimod-treated lesions in vivo (Fig. 5, C).

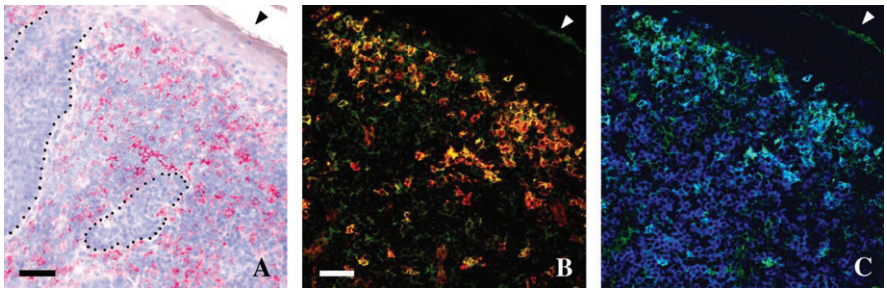

Fig. 4. In situ identification of plasmacytoid predendritic cells (PDCs) in the dermal infiltrate of a basal cell carcinoma treated with imiquimod. **A**) Immunohistochemical staining for the PDC-specific marker BDCA-2. Numerous BDCA-2$^+$ cells (**red**) recruited to the treatment site are shown (original magnification = ×20). For better discrimination of the infiltrate, tumor borders are indicated by **dotted lines. Arrowhead** = epidermis. **B** and **C**) Confocal laser-scanning microscopy of the co-expression of BDCA-2 (**green**), CD123 (**red**), and CD4 (**blue**) cell surface proteins on dermal PDCs. **B**) Co-staining for BDCA-2 and CD123 = **yellow** (magnification = ×40). **C**) Co-staining for BDCA-2 and CD4 = **turquoise** (original magnification = ×20). **White arrowheads** = epidermis. **Scale bar** = 50 μm.

Role of PDCs in the Clinical and Transcriptional Response to Imiquimod

To determine whether PDCs or other types of cells, such as MDCs, were shaping the gene expression profile of the skin lesions in response to imiquimod treatment, we next compared the number of PDCs (i.e., BDCA-2$^+$ cells) and MDCs (i.e., CD1c$^+$ cells) with the expression of various IFN-inducible genes detected by the Affymetrix arrays in skin lesions treated with imiquimod. The number of PDCs correlated (by the Spearman's correlation test) with the level of expression of known IFN-α–inducible genes (IRF7, Spearman's ρ = .542 and P = .004; MxA, ρ = .440 and P = .024; G1P2, ρ = .751 and P<.001; G1P3, ρ = .652 and P<.001; OASL, ρ = .728 and P<.001; IFI35, ρ = .720 and P<.001; ISG20, ρ = .718 and P<.001; IFIT1, ρ = .705 and P<.001; CIG5, ρ = .688 and P<.001; OAS1, ρ = .405 and P = .04; STAT1, ρ = .532 and P = .005; and IFI44, ρ = .608 and P = .001) and with the level of expression of genes for various chemokines, cytokines, and their receptors (CXCL11, ρ = .698 and P<.001; CCL8, ρ = 665 and P<.001; IL6, ρ = .646 and P<.001; CCL5, ρ = .539 and P = .005; CCL2, ρ = .550 and P = .004; CCL3, ρ = .622 and P = .001; CCL4, ρ = .419 and P = .033; IL18-RAP, ρ = .535 and P = .005; CXCR4, ρ = .523 and P = .006; and CD123/IL3RA, ρ = .625 and P = .001). In contrast, the number of CD1c$^+$ cells did not correlate with the expression of any of these genes.

We finally examined gene expression in the three tissue samples that were not assigned to the correct array group by the hierarchical clustering algorithm. Gene expression patterns of two samples treated with imiquimod (lesions BCC4 and BCC7) clustered with that of the pretreatment samples, and gene expression patterns of one untreated sample (lesion CTCL2) clustered with that of posttreatment samples. Lesions BCC4 and BCC7 had fewer PDCs after imiquimod treatment (Table 1). In addition, imiquimod treatment of lesion BCC4 did not result in an influx of inflammatory cells to the lesion (i.e., no increase of infiltrate density grade). Even though the infiltrate density

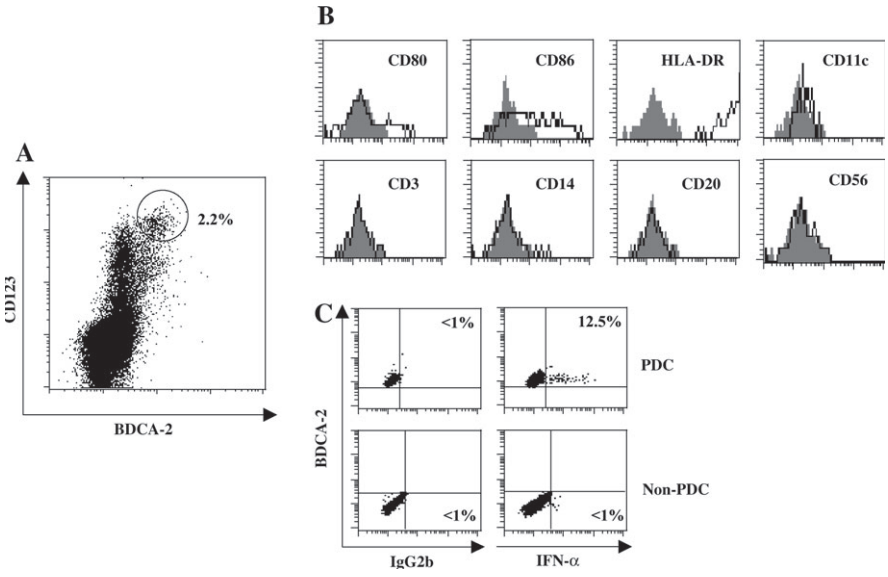

Fig. 5. Flow cytometry quantification of plasmacytoid predendritic cells (PDCs) in dermal cell suspensions. **A**) PDCs stained with anti-BDCA-2 and anti-CD123 antibodies in basal cell carcinoma (BCC) treated with imiquimod. The **round gate** shows the percentage of cells expressing BDCA-2 and CD123 surface markers. **B**) Marker expression in imiquimod-treated PDCs. After treatment with imiquimod, dermal PDCs from a BCC were stained with antibodies against HLA-DR, CD80, or CD86 or with antibodies against the lineage markers CD3, CD11c, CD14, CD20, or CD56. Isotype-matched control antibody = **shaded histogram**; BDCA-2$^+$, CD123$^+$ cells = **solid black line**. **C**) Flow cytometry analysis of intercellular interferon (IFN) α2 and BDCA-2 protein expression in PDCs in dermal cell suspensions. PDCs were stained with antibodies against IFN-α2 or with the isotype control (IgG2b). PDC = cells gated for BDCA-2 and CD123 expression shown in the **A**. Non-PDCs = all other nongated cells shown in **A**.

Table 1. Quantitative immunohistochemistry of BDCA-2, CD123, and CD1c expression in skin tumors before and after topical imiquimod treatment

	No. of positive cells per ×40 field							
	BDCA2		CD123		CD1c		Infiltrate grade	
Lesion*	Before	After	Before	After	Before	After	Before	After
BCC1	21	86	4	123	56	57	2	3
BCC2	—	94	3	51	—	52	1	3
BCC3	3	34	1	48	10	36	1	3
BCC4	1	12	0	3	17	15	1	1
BCC5	—	29	0	41	—	47	1	2
BCC6	—	—	0	31	—	—	1	3
BCC7	4	6	9	9	28	30	1	3
BCC8	0	91	4	93	16	45	1	3
BCC9	—	21	13	54	—	20	1	3
BCC10	—	20	12	45	—	15	2	3
CTCL1	3	28	2	62	37	20	1	3
CTCL2	17	45	10	12	140	101	2	3
CTCL3	0	18	0	38	16	33	1	3
CTCL4	1	15	0	32	90	62	1	3
CTCL5	0	2	0	3	103	66	2	3
MB	1	64	12	32	34	16	1	3

*BCC = basal cell carcinoma; CTCL = cutaneous T-cell lymphoma; MB = Bowen's disease; — = not done due to the lack of material.

grade increased in lesion BCC7, only single PDCs that were dispersed through the dermis were observed. Despite the allocation of the imiquimod-treated BCC7 to the pretreatment group, the expression of a series of IFN-α– but not IFN-γ–inducible genes was stimulated by imiquimod treatment (Fig. 1, A), suggesting the minimal activation of single PDCs present in the lesion (i.e., IFN-α–inducible genes) but no downstream activation of the T helper type 1 immune response (i.e., IFN-γ–inducible genes). In contrast, in lesion BCC4, the expression of neither group of IFN-inducible genes increased after imiquimod treatment (Fig. 1, A), indicating a complete lack of responsiveness to imiquimod treatment. This lack of response was also observed clinically; no inflammatory reaction (i.e., redness, edema, or scaling) was observed for lesions BCC4 and BCC7 during imiquimod treatment (Fig. 6). However, an inflammatory reaction was observed for lesion CTCL2, in which the gene expression profile of samples before and after imiquimod treatment clustered with the imiquimod-treated group. One explanation for this unusual array pattern may be in the fact that lesion CTCL2 had an increased number of $BDCA2^+$ cells and an unusually high number of $CD1c^+$ cells before treatment (Table 1).

Discussion

Our current study shows that, irrespective of whether the tumor was BCC, CTCL, or Bowen's disease, imiquimod induced a gene expression profile in these tumors that is reminiscent of activated PDCs and is related to the number of PDCs recruited to the treatment site. Our results also show that PDCs are the primary source of IFN-α in skin lesions in response to imiquimod treatment. In patients who did mount a clinical inflammatory reaction in response to imiquimod treatment, the IFN-α gene signature was not observed in treated lesions and fewer PDCs were detected in treated lesions than in other treated lesions that showed a clinical inflammatory reaction. Thus, PDCs appear to have a role in the clinical response to imiquimod.

Since Hemmi et al. *(13)* reported activation of the Toll-like receptor 7 (TLR7)/MyD88 signaling pathway and increased IFN-α production in mice treated with imiquimod, much effort has been directed at identifying the primary cellular target(s) for imiquimod and other imidazoquinolines in humans *(14,15,28,29)*. Of the 11 described members of the TLR family, human PDCs express only TLR7 and TLR9 *(30)*. After many reports on the induction of IFN-α production after imiquimod treatment *(14,15, 29,31–35)*, it was shown *(36,37)* that natural IFN-producing cells (also known as PDCs) express a TLR that is activated by imiquimod—a link that was missing for almost a decade was found.

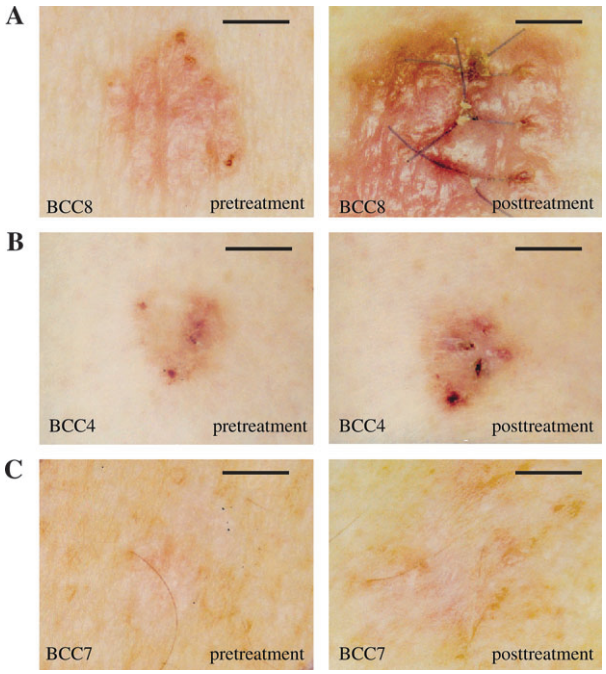

Fig. 6. Inflammatory reaction and gene expression before and after imiquimod treatment. **A**) Inflammatory reaction (redness, edema, and swelling) often observed clinically after 5 days of topical imiquimod treatment. A basal cell carcinoma (BCC) lesion, SCC8, is shown that was classified as a responder by array analysis. **B, C**) Lack of inflammatory reaction in two BCC lesions, BCC4 and BCC7, that were classified as nonresponders according to array analysis. **Scale bar** = 1 cm.

However, the question remained that, if PDCs are the primary target cells for imiquimod in the blood, are PDCs the primary target for imiquimod in the skin lesions? Recently Palamara et al. *(38)* observed the accumulation of PDC-like cells in the dermis of melanoma-bearing mice topically treated with imiquimod. We have demonstrated, to our knowledge for the first time, that PDCs (i.e., Lin^-, BDCA-2^+, $CD123^+$, $CD4^+$, and HLA-DR^+ cells) are recruited to cutaneous epithelial and lymphoproliferative tumors treated with imiquimod. PDC recruitment by imiquimod treatment was further supported by gene expression profiles showing that the expression of a set of genes that are known to be expressed by PDCs was consistently increased in lesions treated with imiquimod. PDCs accounted for up to 16% of dermal mononuclear cells in sBCC lesions treated with imiquimod, which is more than 10 times higher than the reported frequency (0.10%–0.30%) of PDCs among peripheral blood mononuclear cells *(6,7)*. This increase in the number of PDCs at the treatment site appeared to be independent of the underlying type of skin tumor evaluated in this study, supporting the specificity of the TLR response to a synthetic ligand, such as imiquimod *(3)*. Even though some tissue samples used in the microarray analysis were not available for the matched immunohistochemistry analysis (five of the total 16 samples; i.e., five of 10 sBCC), there was clear evidence of recruitment and accumulation of PDCs to the lesions after topical imiquimod treatment in the evaluated tumor groups. After the PDCs have migrated into the skin, they probably respond to either the imiquimod gradient at the entry point or the chemokine gradient induced by imiquimod, as reflected in their preferential grouping in the subepidermal zone of the area treated with imiquimod cream.

Furthermore, in contrast to other dermal cell populations, we found that PDCs recruited to the imiquimod-treated lesions were the principal producers of IFN-α. Each functional blood PDC is capable of producing 1–2 IU (3–10 pg) of IFN-α *(6,7,39)*. If we extrapolate this amount of IFN-α to that produced by at least 10 000 PDCs—the number of cells that we detected by flow cytometry in an excised imiquimod-treated BCC—then 1000 IU of IFN-α would be produced by PDCs in 2–3 cm^3 of skin. This level of IFN-α appears to specifically shape the gene expression signature in both epithelial and lymphomatous lesions treated with imiquimod. As we previously reported *(17)*, various IFN-α–responsive genes were induced in all treated samples. In three CTCL samples, we found almost no change in the expression of IRF7 and MxA genes before and after imiquimod treatment, as detected by microarrays. However, by using real-time PCR (a more sensitive method than arrays), we found that the expression of IRF7 and MxA genes in these three CTCL lesions and in other tumors was increased by imiquimod treatment. IRF7 is a transcription factor constitutively expressed in PDCs *(40)*. IRF7 appears to be the key regulator in TLR7-mediated IFN-α induction or production through formation of a complex with MyD88 and TRAF6 *(41)*. Induction of IRF7 gene expression by imiquimod treatment could, therefore, be reflected by an increase in the number of PDCs or the induction of IRF7 in other cell types in the lesion by IFN-α *(42,43)*.

Activation of TLR7 by imiquimod results in the production of IFN-α and other type I IFNs *(13,15)*, which in turn drives adaptive immunity toward a T helper type 1 immune response, either directly through the induction of IFN-γ in T cells or indirectly through the promotion of dendritic cell maturation [for review, see *(3,4)*]. The importance of IFN-α in initiating each of these downstream events is reflected in the lack of expression of many directly or indirectly IFN-α–inducible gene products in the two patients who did not respond to imiquimod treatment in our study and in other studies *(44–46)*. In addition to fewer PDCs, these two patients did not have a clinical inflammatory response, suggesting that their inability to respond to imiquimod (induction of IFN-α-responsive genes or a clinical response) may be the result of insufficient or impaired PDC recruitment to the treatment site.

Previous clinical studies have demonstrated a correlation between the regression of viral warts or high-grade intraepithelial neoplasia treated with imiquimod and the induction of IFN-α, IFN-γ, STAT1, and 2′–5′ oligoadenylate synthetase gene expression *(44–46)*. In patients with genital warts treated with imiquimod, erythema at the treatment site correlated with the expression of IFN-γ, CCL5/RANTES, and CCL2/MCP-1 genes *(44)*. Neither of the sBCC lesions studied that displayed no inflammatory reaction to imiquimod treatment showed increased expression of any of these genes (data not shown). However, we found good correlation between expression levels of IFN-α–inducible genes and the number of PDCs in all lesions, consistent with a role for PDCs in mediating the clinical response (generally painless inflammation) to imiquimod treatment. A recent study *(38)* in a melanoma mouse model also supports this result, by showing that the regression of the tumors treated with imiquimod correlated with the number of PDC-like cells recruited to the treated lesion.

When we further examined the expression of CXCL11, a chemokine that participates in PDC recruitment to inflamed skin *(27)*, we found that CXCL11 expression in these two nonresponders was not increased by imiquimod treatment. CXCL11/ITAC binds to CXCR3 (which is highly expressed on PDCs) and, in response to CXCL12/SDF-1, induces the recruitment of PDCs into the skin *(27)*. After imiquimod treatment, we also observed the increased expression of CXCR4, a cognate receptor for CXCL12/SDF-1 that is expressed at a high level on PDCs *(27,47)*. Even though the CXCL12/SDF-1 gene was expressed in samples before and after imiquimod treatment, its expression did not pass our filtering criteria, reflecting the fact that CXCL12/SDF-1 is constitutively expressed on dermal endothelial cells *(48)*. CXCR3 cell surface expression is rapidly lost after PDC stimulation because PDCs begin to secrete high levels of CXCR3 ligands and other chemokines *(49)*. Inhibition of the expression of CXCR3 by the autocrine secretion of the CXCR3 agonists (such as CXCL9, CXCL10, and CXCL11) is probably the reason that we did not observe the increased expression of CXCR3 in the array data. The genes CCL2/MCP1, CCL3/MIP-1α, CCL4/MIP-1β, CCL5/RANTES, and CCL8/MCP-2, whose expression was increased after imiquimod treatment, encode key chemokines produced in response to an active microbial infection of TLR triggering with a single goal to recruit inflammatory cells to the site of infection or for imiquimod treatment to the treatment site *(3,12,50)*. This massive production of various chemoattractants is particularly reflected in the rapid increase in the skin infiltrate density grade that we observed at the treatment site after 5 days after treatment was initiated. We found that the number of PDCs increased accordingly, suggesting that these cells and other inflammatory cells were being recruited. PDCs and MDCs appear to be capable of producing various chemokines, including CCL2/MCP1, CCL3/MIP-1α, CCL4/MIP-1β, and CCL5/RANTES, in response to bacterial or viral antigens and CpG motifs *(49,51,52)*.

Through the production of chemokines, PDCs may also be contributing to the early recruitment of other inflammatory cells to the treatment site.

The mechanism that triggers or induces the active recruitment of PDCs and other inflammatory cells to the skin in response to imiquimod remains elusive. Cells in the skin that are the primary target for imiquimod and thus a primary source of various PDC-chemoattracting substances remain to be defined. It is known that keratinocytes produce cytokines other than IFN-α in response to imiquimod treatment *(53)*, even though they do not express TLR7 *(54)*. Epidermal Langerhans cells do not express TLR7 either *(55)*. TLR7 expression on MDCs remains controversial, despite evidence of low TLR7 expression on MDCs *(30)* and evidence that MDCs produce interleukin 12 in response to imiquimod treatment *(14)*. It is conceivable that a high local concentration of imiquimod may be sufficient to activate MDCs in a TLR8-dependent manner, given the high structural similarity between TLR7 and TLR8 *(56,57)* and the fact that imiquimod and other similar TLR7 agonists are not necessarily TLR7-specific agonists. A recent report *(58)* describing the induction of TLR7 expression on MDCs by IFN-α suggests that in vivo MDCs and PDCs may coordinate their responses to imiquimod. Whether any of these cells or any other cells with yet undiscovered TLRs represent primary targets for imiquimod remains to be elucidated.

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Notes

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