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Immunomodulation by imiquimod in patients with high-risk primary melanoma

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Abstract

Imiquimod is a synthetic Toll-like receptor 7 (TLR7) agonist approved for the topical treatment of actinic keratoses, superficial basal cell carcinoma, and genital warts. Imiquimod leads to an 80–100% cure rate of lentigo maligna, but studies of invasive melanoma are lacking. We conducted a pilot study to characterize the local, regional, and systemic immune responses induced by imiquimod in patients with high-risk melanoma. After treatment of the primary melanoma biopsy site with placebo or imiquimod cream, we measured immune responses in the treated skin, sentinel lymph nodes (SLN), and peripheral blood. Treatment of primary melanomas with 5% imiquimod cream was associated with an increase in both CD4+ and CD8+ T cells in the skin, and CD4+ T cells in the SLN. Most of the CD8+ T cells in the skin were CD25 negative. We could not detect any increases in CD8+ T cells specifically recognizing HLA-A*0201-restricted melanoma epitopes in the peripheral blood. The findings from this small pilot study demonstrate that topical imiquimod treatment results in enhanced local and regional T cell numbers in both the skin and

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CONFLICT OF INTEREST

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SLN. Further research into TLR7 immunomodulating pathways as a basis for effective immunotherapy against melanoma in conjunction with surgery is warranted.

INTRODUCTION

Invasive melanoma accounts for 4% of skin cancers, but causes approximately 71% of skin cancer deaths, largely due to aggressive metastatic disease. Primary melanomas can induce immune suppression in the sentinel draining lymph node (SLN) (Lee *et al.*, 2005;Shu *et al.*, 2006). Specifically, antigen presentation by dendritic cells (DCs) can be negatively impacted in the sentinel nodes, leading to deficient tumor-specific T cell induction and inefficient control of metastatic spread (Pinzon-Charry *et al.*, 2005). Identification of immunomodulating agents to enhance immune responses may result in better systemic immunity and improved control of metastases. After a biopsy demonstrates an invasive melanoma with a Breslow thickness greater than 1 mm, the current standard of care is to immediately excise the melanoma with wide surgical margins and to biopsy the SLN. However, the use of immune stimulating agents before surgical removal of the primary melanoma and SLN biopsy may provide additional immunoprotective benefit to patients with thicker high-risk melanoma (Erickson and Miller, 2010).

Imiquimod is a topical immunomodulator that is FDA-approved for use in external genital warts, superficial basal cell carcinoma, and actinic keratosis (Gaspari *et al.*, 2009). It belongs to the imidazoquinoline small molecule family of Toll-like receptor (TLR) agonists. TLRss are conserved receptors that are important in pathogen recognition as well as linking innate and acquired immunity (Takeda and Akira, 2004). Imiquimod modulates immune responses through activation of TLR7 (Hemmi *et al.*, 2002) located on endosomal membranes of antigen presenting cells (APCs), including myeloid and plasmacytoid dendritic cells (pDCs), monocytes, and macrophages. TLR7 activation induces secretion of proinflammatory cytokines, predominantly IFN-α, IL-12, and TNFα (Stanley, 2002), and facilitates the maturation and migratory capabilities of DCs (Prins *et al.*, 2006). These responses help to bridge innate and acquired immune responses, leading to CD4+ T helper type 1 cell induction, as well as cytotoxic CD8+ anti-tumor T cell responses. Persistent TLR signaling may also be required to bypass regulatory T cell-induced tolerance (Yang *et al.*, 2004). Previous studies have demonstrated that other TLR-stimulating agents, such as CpG oligodeoxynucleotide (TLR9 agonist), enhance DC responses in the SLNs of cancer patients (Molenkamp *et al.*, 2007).

There have been few clinical studies to systematically evaluate neoadjuvant immunomodulators in primary melanoma patients undergoing surgical excision. Here we describe a pilot study of a two-week topical course of imiquimod cream or placebo applied to the primary melanoma site prior to surgical excision of tumor and SLN biopsy. Cellular immunological parameters in the skin and lymph nodes were evaluated following treatment. We hypothesized that treatment of primary cutaneous melanoma with imiquimod prior to surgery would lead to a program of inflammation at the tumor site and SLN that might enhance systemic anti-melanoma immunity. Because enhanced DC responses might lead to increased tumor antigen presentation and naïve T cell induction, we included in the study

measurements of melanoma antigen-specific T cell responses in the peripheral blood before and after therapy.

RESULTS

Patient selection and treatment

Fourteen patients with primary melanoma tumors measuring >1.0 mm Breslow thickness were enrolled in the study. Patients were randomly assigned to pre-operative topical administration of either 5% imiquimod cream or vehicle (placebo) cream to their tumor areas, every day for two weeks prior to surgical removal of tumor and sentinel lymph nodes. The final day of treatment was the day before surgery (day -1). Blood samples were obtained from each patient before and after treatment (day -14 and day 0). Tumor sites were examined and photographed on these days for comparison. One patient from each group did not complete the SLN resection for clinical reasons unrelated to this study, and these patients were not included in the SLN analysis.

No severe local or systemic adverse reactions were observed in any of the patients after two weeks of treatment. Signs of local inflammation, including erythema and edema, were expected to occur in some patients in the imiquimod-treated group. Four out of the six analyzable imiquimod-treated patients had clinical evidence of a pharmacologic response, i.e., obvious signs of local inflammation, including erythema, crusting, or scaling in the area of treatment. Two imiquimod-treated patients had no clinical evidence of a pharmacologic response, and displayed no visible differences in the skin pre- and post-treatment. The patient with the most overt, marked inflammatory response (Patient 2) had an obviously enlarged SLN (greater than 1 cm). However, despite having obvious visible tumor prior to treatment on day - 14 (Figure 1), melanoma cells could not be detected in the excised tumor area or SLN on day 0. In the placebo-treated group, only one patient developed mild erythema, while five others displayed no clinical differences between pre- and post-treatment. On day 0, tumors and SLNs were surgically removed from all patients.

Local cutaneous effects of TLR7 activation

Based on prior reports, we hypothesized that topically applied imiquimod would lead to localized skin inflammation. After applying cream topically for two weeks, we evaluated patients' primary tumor sites for the infiltration of immune cells following imiquimod or placebo treatment. Paraffin-embedded serial sections were stained with antibodies to identify lymphocytes and monocyte/macrophage/dendritic lineage cells, and assessed by immunohistochemistry (IHC). Significantly higher levels of CD3+ T cells, both CD4+ and CD8+, were observed in the local skin regions of the primary tumor site following treatment with imiquimod, compared to placebo (Figure 2a). Subtype analysis of these cells revealed that most CD8+ cells were CD25- (separately submitted manuscript). During analysis of non-lymphocyte markers, conversely, we were unable to detect significant differences between imiquimod- and placebo-treated groups in levels of myeloid DCs (CD1a+ and CD11c+), macrophages (CD14+ and CD68+), antigen presenting cells (HLA-DR+, S100+), plasmacytoid DCs (CD123+), or Langerhans cells (Langerin+) (Figure 2b). Within the imiquimod-treated group, patients displaying the highest levels of most immune markers in

the skin by IHC corresponded with the clinically visible responders. However, the study was not powered to permit further statistical analysis based on this clinical response classification within the imiquimod group.

Immunological effects of TLR7 activation in sentinel lymph node

Fresh SLN sections were obtained prior to histopathological analysis, and single-cell suspensions were prepared for flow cytometric quantitation of immune cell numbers. Antibody staining of lymph node cells was performed using a similar array of biomarkers as in the skin, but also included CD209 and CD83 for a subset of patients. As in the skin, imiquimod treatment induced increased percentages of CD3+ lymphocytes compared with placebo. The percentage of CD4+ cells was significantly higher and the percentage of CD8+ cells trended higher in the imiquimod group as well (Figure 3a).

We used both IHC and flow cytometry to quantitate the levels of monocyte/macrophage/dendritic cell infiltration of the lymph nodes. Single stains with the antibodies used in Figure 2 revealed no significant differences between placebo-treated and imiquimod-treated groups, as shown by IHC (Figure 3b). A subset of patients was assessed for additional monocyte lineage surface markers by flow cytometry. We were unable to detect significant differences in CD123+, CD123+CD11c− (defining a plasmacytoid DC subset), and CD209+ populations between placebo- and imiquimod-treated groups (Figure 3c). Double staining with CD83 allowed assessment of the activation status of these populations. These measurements also failed to detect differences in the numbers of activated CD1a+, CD11c+, and CD209+ populations between the two groups of patients (Figure 3d).

Assessment of melanoma-specific peripheral T cells before and after imiquimod treatment

Lymphocytes were purified from PBMCs harvested from patients before and after imiquimod (or placebo) treatment, and potential tumor specificity was assessed in the subset of HLA-A*0201-positive patients using tetramer staining assays. We chose defined HLA-A*0201-binding epitopes derived from MART-1, tyrosinase, and gp100, as these represent commonly found T cell receptor specificities in melanoma patients (Comin-Anduix *et al.*, 2008). Following staining and flow cytometric analysis, CD8+ cells that stained positive with these tetramers were assessed. Slight increases in the percentage of tetramer+ cells were seen in some individuals following the two-week treatment period (Figure 4). However, in terms of overall number of responses observed, there were no significant differences between placebo- and imiquimod-treated groups for any of the three tumor-antigen-specificities analyzed.

DISCUSSION

Imiquimod has shown clinical efficacy in a number of different skin neoplasms, including superficial basal cell carcinoma and actinic keratoses, but its exact mechanism of action is largely undefined. Imiquimod has been widely used off-label in cases of lentigo maligna (LM) and cutaneous metastatic melanoma (Naylor *et al.*, 2003;Cotter *et al.*, 2008;Buettiker *et al.*, 2008;Powell *et al.*, 2009). Additionally, others and we have demonstrated the ability of imiquimod to enhance vaccine-associated protection against melanoma in mice (Ma *et*

al., 2010;Craft *et al.*, 2005;Prins *et al.*, 2006). Although surgical excision is generally the main treatment modality for both LM and invasive melanoma, imiquimod has been used in patients with confounding co-morbidities which limit their surgical candidacy, and in patients with tumors in anatomic areas that would lead to aesthetic or functional impairments. Currently, only case studies have been reported on imiquimod as a monotherapy for cutaneous metastases of melanoma, although there has been a Phase I/II study of topical imiquimod combined with intralesional IL-2 that demonstrated some clinical responses in cutaneous metastases (Green *et al.*, 2008). Topical imiquimod treatment can lead to 75% cure rates of melanoma *in situ* without surgery (Cotter *et al.*, 2008). However, overall data remains inconclusive, due to small study sizes and short follow-up periods (Rajpar and Marsden, 2006;Erickson and Miller, 2010). We hypothesized that topical imiquimod treatment of high-risk invasive primary melanoma in humans would lead to increased anti-tumor immune responses in the skin and SLN. In this pilot study, we identified, quantified, and compared local tumor site and draining lymph node immune responses in imiquimod- and placebo-treated primary malignant melanoma patients. Clinical outcomes associated with treatment were not the focus of this study.

Our data demonstrates that topical imiquimod treatment significantly elevates levels of CD4+ and CD8+ lymphocytes, both locally in the skin, and in the draining sentinel lymph nodes, as compared to patients treated with placebo. We were unable to detect, however, significant differences in the levels of DCs, macrophages, Langerhans cells, or other antigen presenting cells. The fact that this was a pilot study with small numbers of patients allowed only large differences between groups to be detected. Previous studies have shown evidence that topical imiquimod treatment is associated with local infiltration of both myeloid and lymphoid immune cells (DeGiorgi *et al.*, 2009;Torres *et al.*, 2007). Infiltration of T lymphocytes and DCs into superficial basal cell carcinomas (Barnetson *et al.*, 2004) and actinic keratoses (Ooi *et al.*, 2006) were associated with imiquimod treatment. We initially hypothesized that imiquimod-treated patients would have either increased numbers of monocyte lineage cells, or higher activation levels. Although we examined the activation marker CD83 and could not detect increased levels in the small number of imiquimod-treated patients in this study, we did not examine potential functional differences, such as cytokine or chemokine expression, that might influence lymphocyte infiltration.

The predominant cell type that responds to TLR7 stimulation in humans is the plasmacytoid dendritic cell (pDC) (Gibson *et al.*, 2002), identified in this study by CD123+CD11c− staining, although conventional myeloid DCs, identified as CD11c+, have also been shown to respond to TLR7 agonists (Ito *et al.*, 2002). One major response of activated pDCs is the expression of type I IFNs (IFNαand IFNβ) via signaling through NFκB. Indeed, a microarray analysis of imiquimod treatment of superficial basal cell carcinomas and cutaneous T cell lymphomas revealed increased expression of IFNα-induced genes that correlated with higher numbers of tumor-infiltrating activated pDCs (Urosevic *et al.*, 2005). Our data did not reveal significant differences in pDC populations in the SLN or the excised tumor; however, the previous study was performed only 5 days following initiation of imiquimod treatment, compared to 2 weeks in our study. In addition to pDCs, other cell types expressing TLR7 may also be involved in the inflammation observed in our study, and

in the clinical effect observed in the treatment of skin tumors here and in prior reports. These cells include myeloid DCs, T cells, B cells, and monocyte/macrophages. Although statistical significance was not reached due to the limited sample size, cells expressing the monocyte/macrophage marker CD68 were detected in higher numbers in imiquimod-treated tumor sites than in skin treated with placebo cream. Cytokine release may also lead to indirect effects on other cell subpopulations, reflecting the complex inter-relationships among immune cells during imiquimod treatment.

Signs of localized visible inflammation are strong predictors of therapeutic benefit in LM patients treated with imiquimod (Powell *et al.*, 2009). Among the imiquimod-treated patients in this study, varying degrees of clinical inflammation were evident following the two-week treatment course. Comparing post-treatment to pre-treatment, 4 out of 6 patients treated with imiquimod showed clinical inflammation—erythema, crusting, or scaling—while two of six had no obvious visible changes after 14 days of treatment. Thus, because 5% topical imiquimod was not always associated with obvious local changes, we characterized subsets within the treatment group as pharmacologic responders and non-responders based on visible local inflammation of the skin. It is possible that genetically distinct subsets of the population respond differently to immunomodulation by imiquimod. Single nucleotide polymorphisms (SNPs) have been described for TLR7 and TLR8, including alleles that are associated with disease outcomes for HCV (Schott *et al.*, 2008) and HIV infection (Oh *et al.*, 2008), suggesting that genetic variation might potentially contribute to varied clinical responses to TLR ligand stimulation. Whatever the cause of variability in TLR ligand responses, larger future studies could be designed to account for individual patients having varying degrees of pharmacologic responses to imiquimod.

Recent evidence has implicated CD8+/CD25−/granzymeB+/perforin+ T cells as a population that is capable of responding immediately to immune stimulation (separately submitted manuscript). Activation and proliferation of T cells through the TCR normally leads to CD25 upregulation. The increased numbers of CD8+ T cells that we observed in treated skin and SLN sections of imiquimod-treated patients were found by co-staining to be CD25 negative (data in a separate manuscript under review). This observation suggests that these cells are likely infiltrating and proliferating in response to TLR7 stimulation in the absence of antigen.

In patient PBMCs, we were unable to detect any T cells that recognize epitopes from three common melanoma targets, MART-1, tyrosinase, and gp-100. Because imiquimod was applied to tumor sites before surgical excision, the tumor itself might serve as an antigen source for endogenous DCs that are activated by imiquimod. Any antigen-loaded DC could then traffic to the draining lymph nodes to activate tumor-specific T cells. The fact that no melanoma antigen-specific T cells could be detected in PBMCs from these melanoma patients does not rule out their existence, since the epitopes examined do not represent a complete array of potential targets that might be recognized by T cells. Additionally, any potentially generated antigen-specific T cells might be predicted to leave the circulation and home to the tumor, thus precluding their detection in the blood. Ultimately, larger studies with longer follow-up times are needed to determine the importance of CD8+/CD25− T

cells, or if imiquimod-induced infiltration of T cells into the skin and SLN leads to increased circulating melanoma antigen-specific T cells or clinical survival benefit.

Many clinicians and scientists hypothesize that immune-based therapies delivered before the SLNs are excised from patients with melanoma could result in improved systemic immunosurveillance for micrometastases. Because the risk of topical imiquimod use is exceedingly low, the results from this pilot study suggest a large-scale randomized, placebo controlled clinical trial may be warranted. The proposed trial would use imiquimod or other TLR agonists as topically-applied, pre-surgical neoadjuvants to determine if long-term, disease-free survival or overall survival is improved. Further characterization of the effector cells induced by imiquimod and the mechanisms involved in tumor clearance are also warranted.

MATERIALS AND METHODS

Study design, patients, and treatment schedule

This prospective pilot study was designed to determine the clinical variability of responses to topical imiquimod in patients with high risk primary melanoma, and was modeled after a previous sentinel lymph node analysis performed in melanoma patients (Vuylsteke *et al.*, 2004). Thus, although the study was not powered to detect differences in clinical outcomes, it was still designed as a randomized, double-blind, placebo-controlled study to model potential future applications. For this pilot, the target enrollment was n=6 in each group. Inclusion criteria were: 18 years of age and older, and diagnosis of primary melanoma with a pathological Breslow thickness greater than 1.0 millimeter. Exclusion criteria included: primary biopsy of the wide excisional type with depth-appropriate margins, location of the tumor on the head and neck, prior treatment with immunotherapy or chemotherapy, ongoing immunotherapy, prior diagnosis with autoimmune disorder, presence of co-morbid conditions precluding lymph node biopsy, evidence of any clinically evident metastases, and pregnancy and/or breastfeeding. All patients received the standard of care for high-risk melanoma including pre-surgical staging work-up, wide local excision of tumor, lymphoscintigraphy and SLN biopsy, and post-surgical care. Fourteen patients enrolled in the study after giving written, informed consents. Patients were randomized in a 1:1 ratio to receive imiquimod 5% cream (3M Pharmaceuticals, Saint Paul, MN and later Graceway Pharmaceuticals) or vehicle cream (3M Pharmaceuticals, Saint Paul, MN and later Graceway Pharmaceuticals). On day -14 before surgical excision, primary lesions were photographed and 100 mLs of blood were collected for pre-therapy immune monitoring studies and HLA typing. Starting on day -14, patients were instructed to apply the cream (250 mg) once daily over the affected area for 14 days. Patients were not advised to occlude the area. Patients were evaluated on day -7 and a second photograph of the tumor site was taken. On the day of surgery (day 0), a post-treatment photograph was taken and another 100 mLs of blood were obtained for post-therapy immunoassays. Patients then underwent wide excision of the tumor and SLN biopsy using standard surgical techniques, and samples were processed for IHC, flow cytometry, and/or immunoassays as described below. This study was approved by the Institutional Review Board at the Los Angeles Biomedical Research Institute and the University of California Los Angeles (UCLA), and was conducted under

Good Clinical Practices guidelines at the Harbor-UCLA Medical Center and the UCLA Medical Center, as well as adherence to the declaration of Helsinki Guidelines.

Immunohistochemistry of Paraffin-embedded Tumor

Sections of excised tumors were placed in 10% formalin and processed as typically performed by the Pathology department. Serial sections from the paraffin-embedded blocks were subsequently obtained and evaluated for infiltration of immune cells using standard IHC procedures. Antibodies against CD3 (BD Biosciences), CD4 (US Biological), CD8 (Ancell), CD14 (Novocastra), CD1a (US Biological), S100 (US Biological), Langerin (US Biological), CD68 (US Biological), CD83 (US Biological), CD86 (Abcam), CD11c (Abcam), CD123 (US Biological), and HLA-DR (US Biological) were used for staining. Image analysis of stained slides was performed by an observer blinded to the treatment assignment using the Image-Pro Plus software (Media Cybernetics, Silver Spring, MD). A compiled image of the region containing the tumor was taken for each slide (10×), and stained areas within the tumor were measured using the color segmentation function. For each interpretable slide, the observer measured percentages of positively stained area as a function of the total area of the epidermis or dermis containing the tumor.

Isolation of SLN cells

Sentinel lymph nodes were collected immediately after dissection and placed in sterile, ice-cold medium. Nodes were bisected and viable cells were collected by removing a thin, cross-sectional slice of the interior of the node in a sterile manner. Tissue was incubated in complete medium [IMDM (Invitrogen), 10% human AB Serum (Cellgro), 50 IU/ml penicillin-streptomycin (Invitrogen), and 0.05 mmol/2-mercaptoethanol (Invitrogen)] containing 0.01% DNase I (Sigma) and 0.14% collagenase A (Roche) for 45 minutes, and subsequently macerated between frosted glass slides to obtain a single cell suspension. Cells were then washed with complete medium, counted, and processed for flow cytometry or IHC.

Flow Cytometry of SLN Cells

Isolated SLN cells were resuspended in IMDM supplemented with 10% human AB serum to block FcγIII/IIR (CD16/CD32). Cells were stained for the following markers obtained from BD Pharmingen (San Diego, CA): CD1a-FITC, CD3-FITC, CD4-PE, CD8-PE, CD11c-PECy5, CD14-FITC, CD83-PE, CD86-PECy5, CD123-PE, and CD209-FITC. Matched isotype monoclonal antibodies served as negative controls. Subsequently, cells were analyzed by flow cytometry (FACSCalibur, BD Biosiences, Mountain View, CA) at 100,000 events per measurement, and positive gates for each biomarker were defined.

Immunohistochemistry of SLN

Immunohistochemical analysis of immune cells in the SLN was performed as described above. IHC results are reported for each SLN as the percentage of the total nodal area positively stained for the specified marker. All slides were evaluated without knowledge of the SLN tumor status or treatment group.

Blood Collection and cryopreservation

Patients underwent two peripheral blood collections of 100 mL whole blood: once prior to treatment initiation on study day -14, and once following treatment, on day 0. Blood samples from day -14 were sent to UCLA Genetics Laboratory for HLA-A*0201 typing. Peripheral blood mononuclear cells (PBMC) were obtained by Ficoll-Hypaque (GE Healthcare) centrifugation of whole blood. PBMCs were cryopreserved at a concentration of 1×10^7/mL in RPMI (Invitrogen) supplemented with 20% (v/v) heat-inactivated human AB serum (Cellgro) and 10% DMSO (Sigma) at −80°C, to allow for large batch processing on a single day.

Peptides and Tetramer Analysis

Tetramer analysis was performed on HLA-A*0201 positive patients, using the following HLA-A*0201-binding peptide epitopes: $MART1_{26–35}$ (ELAGIGILTV), $tyrosinase_{368–376}$ (YMDGTMSQV) and $gp100_{209–217}$ (ITDQVPFSV), representing three immunodominant tumor antigens, as well as negative control [$(AFP)_{325–332}$ (GLSPNLNRFL)] and positive control [(CMV)$pp65_{495–503}$ (NLVPMVATV)] epitopes. All tetramers were purchased from Beckman Coulter Inc., San Diego, CA, as peptide preloaded reagents, and the assay was performed as previously described (Comin-Anduix *et al.*, 2006). Briefly, each patients' PBMCs were thawed simultaneously and treated with DNase for one hour at 37°C. Then, $1{\times}10^6$ cells were stained at room temperature for 30 minutes with each MHC tetramer, plus CD8-FITC (Caltag) and a CD4/CD13/CD19 antibody cocktail (Caltag) for gating. Cells were subsequently washed and analyzed immediately using a FACScan flow cytometer (Becton Dickinson). Lymphocytes were gated based on forward and side scatter, and those positive for CD4/CD13/CD19 were gated out. The percentages of $CD8^+$ cells that stained with tetramer before and after treatment were measured and compared using a standardized gating algorithm. Results were analyzed based on the criteria previously defined (Comin-Anduix *et al.*, 2006).

Statistical Analysis

The stained area percentages and the flow cytometric data in the primary tumor and SLN studies were presumed to have normal distributions based on previous measurements. The mean and difference in the percentage of area (IHC) or cell number (flow cytometry) stained in the melanoma and lymph node tissue samples for imiquimod-treated versus placebo groups were compared using an unpaired two-tailed t-test. Pre- and post- treatment values of tetramer staining were analyzed using the Wilcoxon signed-rank test. P-values of <0.05 were considered to be significant, and multiple comparison adjustments were not applied given the number and nature of the cell markers being analyzed. Changes in cell counts were summarized in 6 imiquimod-treated and 6 placebo patients with means and standard deviations, and with the group difference 95% confidence interval for the estimated treatment effect.

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Abbreviations used

APC	antigen presenting cell
DC	dendritic cell
IHC	immunohistochemistry
LM	lentigo maligna
MM	metastatic melanoma
pDC	plasmacytoid dendritic cell
SLN	sentinel lymph node
TLR	Toll-like receptor

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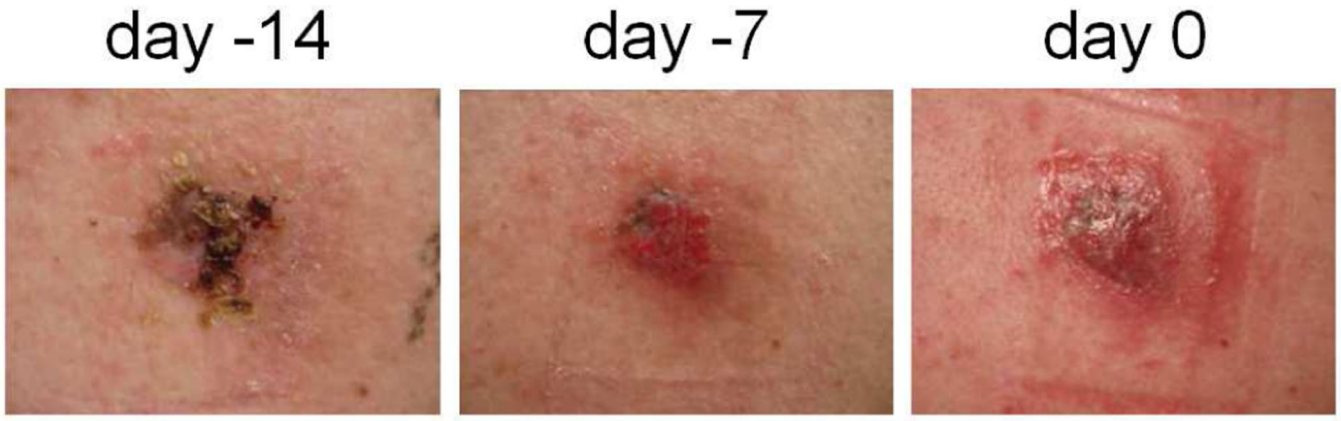

Figure 1. Melanoma site of "responder", pre- and post-treatment with imiquimod

Patient applied topical imiquimod to tumor site starting on day -14, and ending on day -1, before surgery on day 0.

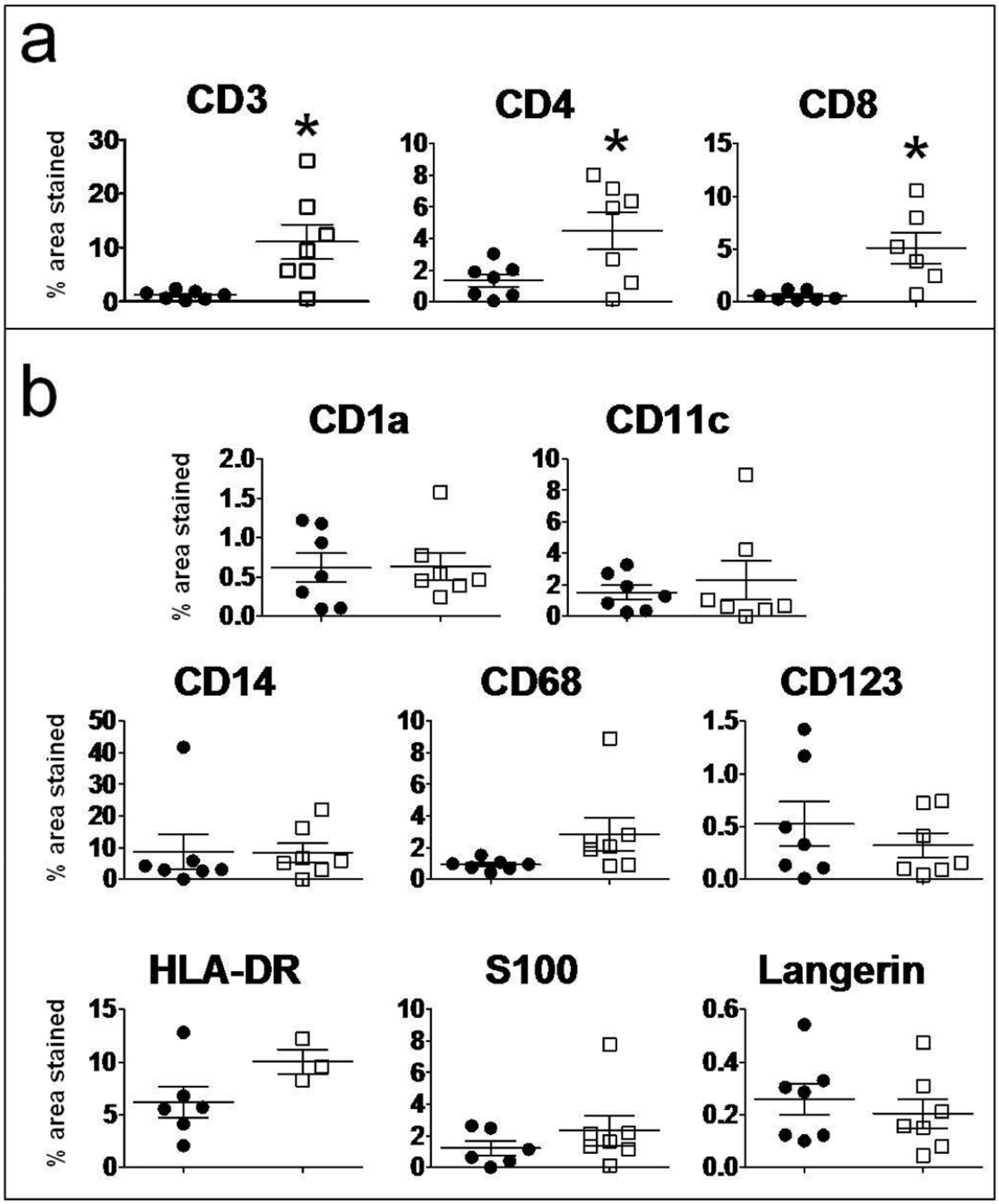

Figure 2. Immune biomarkers in local tumor sites

Skin sections from placebo-treated (closed circles) and imiquimod-treated (open squares) patients were assessed by IHC. Percent areas staining positive for lymphocyte markers (a) and monocyte/macrophage/dendritic cell lineage markers (b) were quantitated using Image Pro software. Means +/− SEM are shown, N=7 for all groups except CD8-imiquimod (6), HLA-DR-placebo (6), and HLA-DR-imiquimod (3). P values for CD3=0.01, CD4=0.03, CD8=0.01.

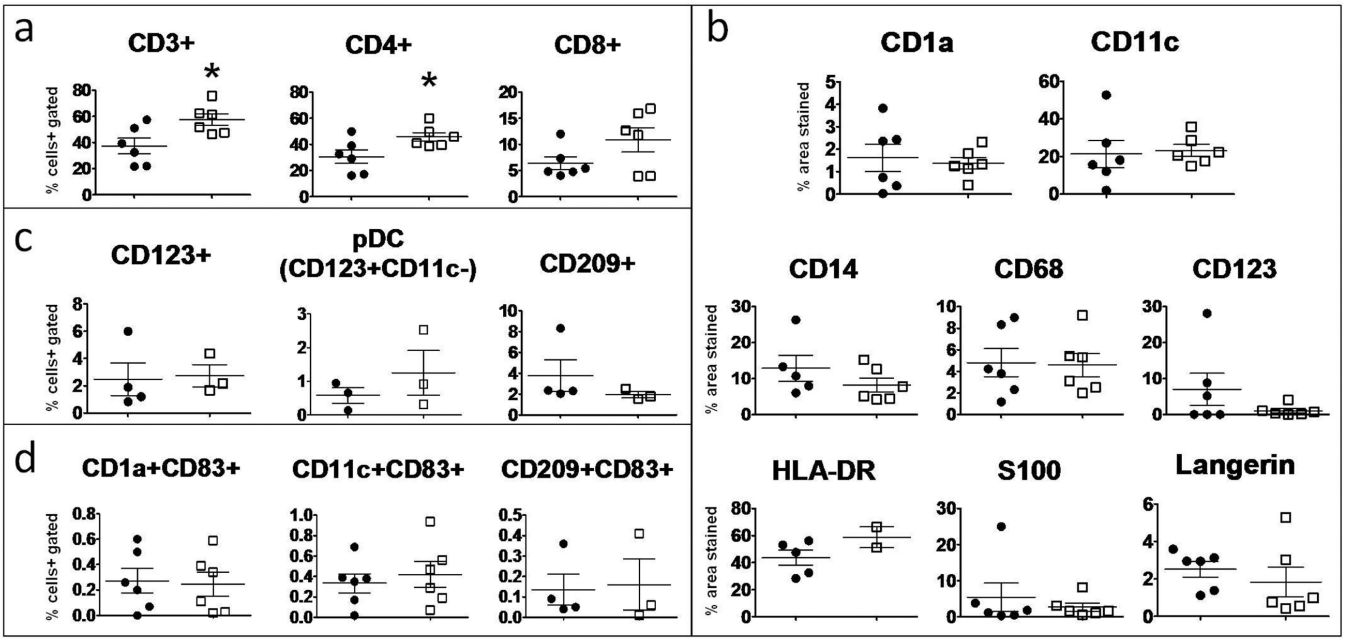

Figure 3. Immune biomarkers in primary sentinel lymph nodes, by flow cytometry or IHC

Lymph node sections and single cell suspensions from placebo-treated (closed circles) and imiquimod-treated (open squares) patients were assessed by IHC and flow cytometry, respectively. Lymphocytes were quantitated by flow cytometry (a), monocyte/macrophage/DC lineage cells were quantitated by IHC (b) or flow cytometry (c), and activation markers on monocyte/DC cells were quantitated by flow cytometry (d). Means +/− SEM are shown, N=6 for all groups except in: (b) CD14-placebo (5), HLA-DR-placebo (5), and HLA-DR-imiquimod (2); (c) all subgroups were 4 or 3; (d) CD209/CD83 was 4 and 3. P values in (a) for CD3=0.02, CD4=0.04, CD8=0.12.

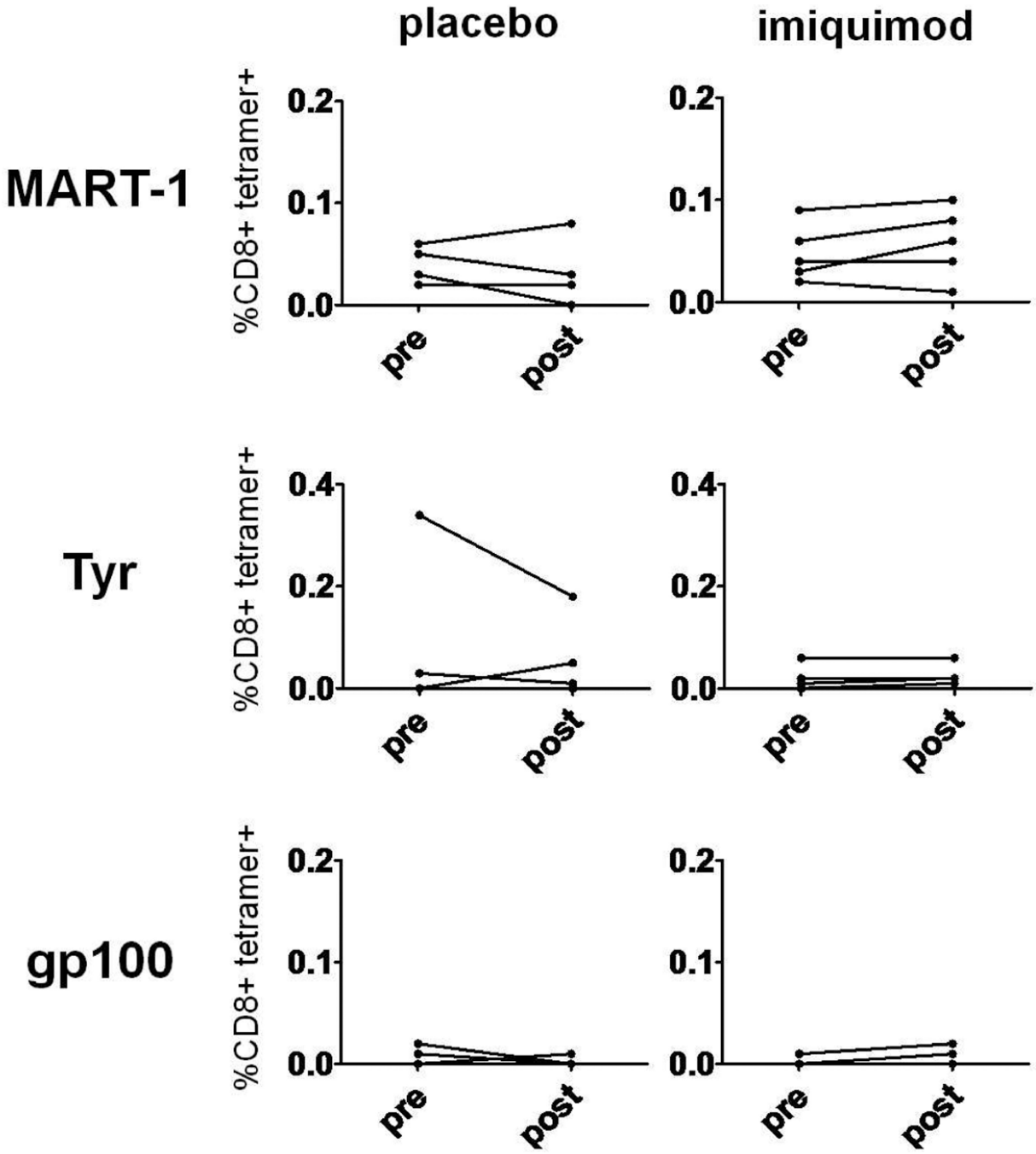

Figure 4. Tetramer staining of PBMC CD8+ lymphocytes following imiquimod or placebo treatment

Lymphocyte-enriched PBMC fractions from HLA-A*0201-positive patients (4 placebo-treated, 5 imiquimod-treated) were assessed for recognition of HLA-A*0201-binding melanoma epitopes derived from MART-1, tyrosinase, or gp100, by tetramer staining. Differences between pre- and post-treatment were assessed per individual, and placebo- and imiquimod-treated group differences compared using the Wilcoxon signed-rank test.