Nonmelanoma skin cancers (NMSC) are the most common cancers in the Caucasian population and include squamous cell carcinoma (SCC) and basal cell carcinoma (BCC). According to WHO, 2 to 3 million new cases of NMSC occur globally each year (http://www.who.int/uv/faq/skincancer/en/index1.html). NMSC presents a low metastatic potential; however, the tumor can cause substantial local damage if not treated early. The standard surgical care of SCC and BCC often causes severe pain and disfiguration (recently reviewed in ref. 1).

The risk to develop NMSC significantly increases up to 250-fold in immunosuppressed patients, such as organ transplant recipients (OTR). In addition, aggressive and metastatic variants of SCC develop in OTR, which represents a critical health burden (2, 3). The ratio BCC/SCC is 4:1 in immunocompetent patients, but 1:10 or higher in OTR, suggesting a critical role for immunosurveillance in SCC but not in BCC. However, studies explicitly addressing this issue are lacking.

Although NMSCs are frequently infiltrated by immune cells (for review, see ref. 4), the immune system often seems incapable of eradicating the tumor. Downregulation of E-selectin and recruitment of regulatory T cells (5) as well as malfunctioning intratumoral myeloid dendritic cells (6) have been proposed as mechanisms that compromise local tumor-specific immunity in SCC. In the case of BCC, the absence or downregulation of MHC-I (7, 8) and the presence of regulatory T cells (9) were observed.

Immunotherapy is an alternative treatment approach, especially for cancers that cannot be surgically removed due to their site or to multiple metastases. Usually, patients are immunized with tumor-associated antigens to induce or boost tumor-specific immunity, and this approach has shown objective clinical responses in some patients. Many of those vaccines contain tumor-specific differentiation antigens (Melan-A/MART-1, tyrosinase,
I (MHC-I) expression and infiltration by CD8+ T cells, we when comparing BCC and SCC with respect to MHC class antigen expression in SCC and BCC in immunocompetent of CT-antigens in BCC and SCC are available. no other data about the expression and immunogenicity spontaneous SCC on an immunohistochemical level (13), but expression of the CT-antigen MAGE-A4 was identified in cu-
taneous SCC, which is accompanied by infiltrating CD8+ cells. Also, we present the novel finding that imiquimod upregulates MHC class I expression in basal cell carci-
noma, which is accompanied by infiltrating CD8+ T cells. Taken together, these data can lead to new immuno-
otherapy approaches especially to unresectable NMSC and open new application ranges for the immu-
response modifier imiquimod, which can possibly be translated to other malignancies.

In this article, we present the first wide screening for CT-
antigen expression in SCC and BCC in immunocompetent and immunosuppressed (OTR) patients. Furthermore, when comparing BCC and SCC with respect to MHC class I (MHC-I) expression and infiltration by CD8+ T cells, we found that both immunologically relevant parameters were significantly lower in BCC. This may offer an explana-
tion for the selective increase of SCC incidence in OTR. Topical application of imiquimod, a Toll-like receptor-7 agonist, results in total regression of the lesion in a large proportion of NMSC patients. Interestingly, we found a significant upregulation of MHC-I expression in BCC on imiquimod treatment, which adds another possible mode of action to the presumed ones, including stimulation of innate immunity and induction of apoptosis in tumor cells (14, 15).

Materials and Methods

Patient samples
All patients enrolled in the study were treated at the Der-
matology Department of the University Hospital of Zur-
ich. The study was approved by the cantonal ethics committee (EK no. 1017). All patients signed informed consent. Specimens of patients receiving imiquimod treatment had been published previously (15, 16). Eight pa-
tients were analyzed, which applied 5% imiquimod cream (Aldara, 3M Pharmaceuticals) once daily 5 times per week for a maximum of 6 weeks. Patients were evaluated weekly until the tumor began to show signs of ero-
sion. At this point, the tumor was surgically excised and stored in formalin.

For PCR extraction, primary cutaneous SCC (including the noninvasive forms actinic keratosis and morbus bo-
en to represent the whole facet of the disease), BCC, and healthy skin samples were obtained during Mohs micro-
graphic surgery and frozen in liquid nitrogen. We ob-
tained 113 biopsies from immunocompetent patients (Supplementary Table S1) and 20 SCC biopsies from OTR receiving immunosuppressive treatment (Supple-
mentary Table S2); histologic classification was done ac-
cording to LeBoit et al. (17).

Sera were collected from 23 immunocompetent BCC and 2 OTR BCC and 8 immunocompetent SCC and 1 OTR SCC.

Cell culture

BCC biopsies were minced into ≤2-mm2 pieces and di-
gested in collagenase type IV (Sigma-Aldrich) for 1 to 2 hours at 37°C. Fragments were digested with 0.25% tryp-
sin (Invitrogen) for 15 to 30 minutes. Debris was filtered through 100- and 40-μm nylon filters (BD Biosciences) and single cells were cultured in Keratinocyte SFM Medi-
um (Invitrogen) with 0.5 units/mL penicillin (Invitrogen) and 0.5 μg/mL streptomycin (Invitrogen). On reaching confluence, cells were trypsinized and cultured as described above.

In vitro imiquimod and IFN-γ treatment

BCC short-term cultures (passage 1) were incubated in Keratinocyte SFM Medium (Invitrogen) containing either 100 units/mL IFN-γ (PeproTech, Inc.) or 30 μmol/L imi-
quimod (Aldara Crème 5%, MEDA Pharma) for 36 hours at 37°C.

Flow cytometry

Samples were stained with FITC-anti-β2-microglobulin, allophycocyanin-anti-HLA-A,B,C (BD Biosciences), and LIVE/DEAD violet fixable dead cell stain kit (Invitrogen). Samples were measured on a Cyan ADP (Beckman Coul-
ter) and analyzed with Flow Jo Software (Tree Star).
RNA isolation and reverse transcription-PCR

Total RNA/DNA extraction from frozen samples was done using the AllPrep DNA/RNA Mini Kit (Qiagen) and RNA was subjected to DNase I digestion (Invitrogen). Total RNA concentration and purity were evaluated using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). RNA (150 ng) was transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosciences).

Reverse transcription-PCR (RT-PCR) was carried out using Taq DNA Polymerase (NEB) with primers specific for the individual CT-antigens or for β-actin as internal control (Supplementary Table S3). The cDNA samples were amplified using an Eppendorf Mastercycler Gradient (Eppendorf AG) using the following conditions: an initial denaturation step for 10 minutes at 95°C, 35 amplification cycles (denaturation for 1 minute at 95°C, annealing for 1 minute at variable temperatures (Supplementary Table S3), elongation for 1 minute at 72°C), and a final elongation step for 10 minutes at 72°C. All primers were tested on at least five independent control healthy skin samples. All kits were used according to the manufacturers' recommendations.

Immunohistochemistry

Paraffin-embedded tissue sections were stained with mouse anti-human monoclonal antibodies against CD8 (1:100; DAKO A/S), CD56 (1:50; Novoceastra Laboratories Ltd.), CT-45/KiA10 (1:50; a generous gift from H.J. Heidebrecht, University of Kiel, Kiel, Germany), MAGE-A1 (1:200; a generous gift from the Ludwig Institute for Cancer Research, New York, NY), MHC-I (1:1,000; KAG Research Diagnostics, Inc.), NY-ESO-1 (1:50; Zymed Laboratories, Inc.), rabbit anti-human polyclonal antibody against PRAME (5 μg/mL; Abcam Ltd.), and the mouse hybridoma supernatant 57B (13) that predominantly recognizes MAGE-A4 (undiluted; a generous gift from Prof. Giulio C. Spagnoli, Department of Surgery).
University Hospital Basel, Basel, Switzerland). Primary antibodies were detected using the ultraVIEW DAB detection kit (Ventana Medical Systems). Sections were counterstained with hematoxylin, dehydrated, and mounted. All sections were stained with the Ventana Benchmark automated staining system (Ventana Medical Systems) using Ventana reagents for the entire procedure.

Image analysis and quantification

Images of the stained paraffin sections were acquired on a Zeiss Axiophot HAL100 (Carl Zeiss MicroImaging GmbH) with the KYF70 digital camera (JVC) and the software analySIS™ (Olympus). Adobe Photoshop version 10.0 (Adobe Systems, Inc.) was used for image analysis.

The intensity of immunoreactivity was scored in arbitrary units as negative (0) or weakly (1), moderately (2), or strongly (3) positive as illustrated in Supplementary Fig. S1. This panel of figures was compiled before the systematic evaluation of the tumor cohort was commenced and served as reference. Quantification of CD8+ cells present in intratumoral and peritumoral regions as well as invasive front was done by counting cells in high-power fields (HPF) of ×40 magnification. In case of a heterogeneous histology, up to 3 HPF were counted and the average is displayed in the figures. A pathologist advised on and supervised the evaluation of immunohistochemistry.

Western blot analysis

We tested sera from 34 patients for the presence of CT-antigen–specific IgG by Western blotting as described (18),
using the recombinant proteins CT-45, NY-ESO-1, and PRAME. All proteins were overexpressed in E. coli; NY-ESO-1 was produced as previously described (18), CT-45 was a generous gift from H.J. Heidebrecht (University of Kiel, Kiel, Germany) and PRAME was a generous gift from C. Melief (University of Leiden Medical Center, Leiden, the Netherlands).

ELISA
ELISA screening was carried out as recently described (19). Thirty-four NMSC sera were screened for the presence of IgG-specific responses for 36 different tumor antigens (for a detailed list of antigens, see Supplementary Fig. S2).

Statistical analysis
Statistical analysis was done with SPSS, version 17.0 (SPSS, Inc.). P values <0.05 were considered significant. The t test, Wilcoxon test, Fisher exact test, and Pearson correlations were used for analysis, depending on the data set.

Results and Discussion
CT-antigens are broadly expressed in NMSC
We investigated the expression of 23 CT-antigens in biopsies from 113 immunocompetent NMSC patients and 13 corresponding healthy tissues with RT-PCR and immunohistochemistry.

Overall, CT-antigens were broadly expressed in NMSC on the mRNA level. PRAME was the most frequently expressed antigen with 55% overall expression in NMSC, followed by MAGE-A4 (25.6%), MAGE-A9 (23.9%), and NY-ESO-1 (14.2%). We found no expression of non-X-chromosome-linked CT-antigens such as Boris or BRDT (Fig. 1) and no expression of any CT-antigen in the healthy skin samples.

We did not observe any difference in CT-antigen expression between noninvasive and invasive SCC; hence, the various histologic types are presented together as one group (SCC) in all the figures.

Analysis of CT-antigen expression by immunohistochemistry on paraffin sections confirmed our RT-PCR results in all cases investigated (Fig. 1C). Most CT-antigens were homogeneously expressed within the entire tumor; however, the expression of MAGE-A4 and PRAME varied in intensity between patients, and a stronger staining was often observed in cells detaching from the tumor structure, so-called acantholytic cells (Fig. 1C).

CT-antigens are frequently coexpressed in various malignancies (20–22). Our results in NMSC confirmed these observations, as more than 40% of NMSC biopsies coexpressed ≥2 antigens and we observed examples of coexpression of as much as 11 antigens (Fig. 2C).

All CT-antigens were more frequently expressed in BCC than in SCC, except for MAGE-A3 (Fig. 1B). In addition, BCC expressed more antigens per biopsy on average than SCC (Fig. 2). The ratio of SCC to BCC in patients is usually
1:4, which means that BCC represents 80% of NMSC and SCC represents 20%. However, in patients under immunosuppressive treatment (OTR), the ratio of SCC to BCC is reversed to be 10:1, meaning that BCC represents only approximately 10% of NMSC in this population of patients, whereas more than 90% are SCC. This makes SCC the most common cutaneous malignancy in OTR (3, 23), suggesting a role for adaptive immunity in the control of SCC. Immunologic pressure in SCC may explain why the percentage of CT-antigen+ biopsies as well as the number of CT-antigens per biopsy is lower in SCC compared with BCC (24). However, we did not observe a significant difference in CT-antigen mRNA expression in SCC from immunocompetent patients compared with SCC from OTR with respect to the number of CT-antigens per biopsy or to the percentage of biopsies expressing one or more CT-antigens (Fig. 3). This observation had previously been made for MAGE-A4 (13). This finding may be unexpected at first glance, but may be explained as follows: The majority of OTR develop SCC within the first 5 years after start of immunosuppressive treatment (3), which argues against de novo formation of the disease and in favor of uncontrolled outgrowth of subclinically preexisting malignancies. Before immunosuppressive treatment removed immunologic control and allowed pathogenic outgrowth of the malignancy, subclinically existing SCC were presumably subjected to immunologic pressure, resulting in selective outgrowth of tumors that express fewer CT-antigens.
No spontaneous humoral immune responses to CT antigens in NMSC patients

CT-antigens often cause spontaneous humoral responses in patients with cutaneous malignancies like melanoma (18, 25) and cutaneous T-cell lymphoma (26). We used Western blotting and ELISA to investigate whether also NMSC patients display humoral immune responses to tumor antigens.

We screened 34 sera from NMSC patients (9 SCC, 25 BCC) for antibodies against an array of CT-antigens by Western blot (data not shown) and ELISA (Supplementary Fig. S2). The absence of tumor antigen-specific IgG in sera from patients with NMSC was in contrast with the data from most other malignancies and was therefore unexpected. We explain the virtual absence of tumor-specific humoral immune responses by the relatively small tumor load of NMSC in comparison with other tumor entities. It has been well documented that titers of tumor-specific IgG increase with progressive disease (i.e., with increasing tumor load and/or the occurrence of metastases; refs. 27, 28), both of which are rare in NMSC.

Low expression of MHC-I and paucity of infiltrating CD8+ cells in BCC suggest limited immunosurveillance

The selective increase of the SCC risk in immunosuppressed individuals suggests a more pronounced role of
immunosurveillance in SCC than in BCC. One possible explanation may be the relative absence of suitable tumor-associated antigens in BCC; however, our results show the opposite (Figs. 1 and 2). The relative absence of MHC-I molecules on BCC, which precludes recognition by CT-antigen-specific CD8+ effector T cells, may be another possibility. We thus compared the expression of MHC-I in SCC and BCC and correlated the amount of infiltrating CD8+ cells to MHC-I expression by immunohistochemistry. We found that both the expression of MHC-I and the infiltration of the invasive front and peritumoral and intratumoral regions by CD8+ cells were drastically reduced in most BCC biopsies when compared with SCC biopsies (Figs. 4 and 5), which confirms previous observations (7, 8). Both the expression of MHC-I as well as the number of infiltrating CD8+ cells were most prominent in the invasive front of SCC biopsies, and statistical analysis revealed a significant correlation between these two parameters (Fig. 5C). Thus, we propose that the relative absence of MHC-I molecules from tumor cells and the ensuing absence of infiltrating CD8+ cells in BCC make this malignancy comparably resistant to the adaptive immune response. Natural killer cells were virtually absent from both BCC and SCC (assessed by CD56 staining; data not shown).

Nevertheless, although MHC-I staining does not differ between SCC OTR and SCC patients, the numbers of CD8+ cells in the intratumoral, peritumoral, and invasive regions of the tumor are decreased in SCC OTR compared with immunocompetent patients (Fig. 5B). This coincides with a recent study showing an overall decrease of cytotoxic T cells in OTR (29) and further outlines the role of CD8+-mediated immune control of SCC and probably not of BCC. These results fit the fact that the activated sonic hedgehog pathway presumably is the driving force in BCC formation (for review, see ref. 30) and that immunologic tumor defense, therefore, has a minor effect on BCC formation.

**Local treatment with imiquimod results in upregulation of MHC-I and increased peritumoral CD8+ T-cell infiltration in BCC**

Repeated topical application of imiquimod, a Toll-like receptor-7 agonist, results in regression of superficial skin cancers, presumably through the induction of innate immunity and apoptosis of tumor cells (14, 15). To investigate the possibility that imiquimod treatment affects the adaptive tumor-specific immune response, we compared the expression of MHC-I molecules and the presence of CD8+ T cells in BCC biopsies before and after imiquimod treatment.

The expression of MHC-I by tumor cells was significant-
ly stronger in biopsies after imiquimod treatment, which was accompanied by an increase of peritumoral CD8+ T cells (Fig. 6). Because BCC do not express Toll-like receptor 7 (31, 32), the observed upregulation of MHC-I molecules on tumor cells presumably is an indirect effect of imiquimod, which may be mediated through type I and type II IFNs produced by infiltrating plasmacytoid dendritic cells (16) or by tumor stroma, or through stimulation of tumoricidal activity of inflammatory myeloid dendritic cells and plasmacytoid dendritic cells (33). Our assumption that upregulation of MHC-I by imiquimod does not result from a direct interaction with BCC is supported by our observation that in vitro treatment of BCC cultures with 30 μmol/L imiquimod does not upregulate surface expression of HLA class I molecules. In contrast, treatment with IFN-γ (100 units/mL) induced increased surface expression of β2-microglobulin as well as HLA-A,B,C molecules at least by 2-fold (assessed by measuring mean fluorescence intensity by flow cytometry; data not shown).

Alternatively, treatment with imiquimod may induce changes in the microenvironment other than upregulation of MHC-I molecules that support local tumor-specific CD8+ T cells, as has been reported for SCC (5, 34). Because the CD8+ T cells of imiquimod-treated BCC patients remain in the peritumoral region and do not seem to penetrate the BCC nests (Fig. 6), their tumor-antigen specificity and antitumor effector functions remain to be confirmed for BCC.

Taken together, we found that BCC expresses no or low levels of MHC-I molecules and that—probably as a result thereof—significantly lower numbers of CD8+ immune effector cells infiltrate the tumor. We thus propose that BCC is less subjected to immunologic pressure than SCC, and our finding that BCC express more immunogenic CT antigens as well as the fact that the incidence of SCC but not of BCC increases in individuals under immunosuppressive treatment supports this hypothesis. Furthermore, we propose that the therapeutic effect of local imiquimod treatment may involve, besides stimulation of the innate defense and induction of tumor cell apoptosis, the upregulation of MHC-I on tumor cells and the concomitant influx of CD8+ cells.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Acknowledgments**

We thank Claudia Matter, Alexandro Landshammer, and Christa Dudli-Furrer for excellent technical assistance; Günther Hofbauer for discussion, help with the manuscript, and providing material; and Mirjana Maiwald-Urosevic for samples of imiquimod-treated patients.

**Grant Support**

Ludwig Institute for Cancer Research/Cancer Research Institute (Cancer Antigen Discovery Collaborative), Atlantic Philanthropies, the Cancer Research Institute, the Hanne Liebemann Foundation, the Hartman Müller Foundation, the Terry Fox Foundation, and the Zürcher Krebsliga. M.J. Barysch and R. Dummer were supported by funds obtained from the Swiss National Foundation (grant no. 310040-103671) and the Gottfried and Julia Bangerter Rhyner Stiftung. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received 11/30/2009; revised 03/16/2010; accepted 03/16/2010; published OnlineFirst 06/02/2010.
References

# Clinical Cancer Research

## Cancer-Testis Antigens and Immunosurveillance in Human Cutaneous Squamous Cell and Basal Cell Carcinomas

Anne Walter, Marjam J. Barysch, Silvia Behnke, et al.


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