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# PD-L1 Expression, T-lymphocyte Subpopulations and Langerhans Cells in Cutaneous Squamous Cell Carcinoma and Precursor Lesions

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**Abstract.** *Background/Aim: The role of immune cells and PD-L1 in cutaneous squamous carcinogenesis is unclear. This study examines T-cell populations, Langerhans cells (LCs) and PD-L1 in invasive squamous cell carcinoma (inSCC), adjacent precursors and normal skin (NS) to investigate their participation in tumorigenesis. Materials and Methods: Cases of cutaneous inSCC with adjacent precursors (n=125) were selected. In situ SCC (isSCC) and actinic keratosis (AK) were observed in 53 and 123 cases, respectively, whereas NS was present in 123 lesions. Immunohistochemistry was performed for CD3, CD8, Foxp3, CD1a and PD-L1. Results: T-cells, LCs and PD-L1 gradually increase during the evolution from AK to isSCC and inSCC, with statistical significance between all lesions, except for CD3+ and CD8+ cells between isSCC and inSCC. Epithelial PD-L1 expression correlates with tumor diameter and thickness. Conclusion: The progressive increase of T-cells, LCs and PD-L1 in cutaneous squamous carcinogenesis provides rationale for immunotherapy and identification of predictive biomarkers.*

Cutaneous invasive squamous cell carcinoma (inSCC), the second most common invasive skin cancer, usually evolves from precursor lesions, namely actinic keratosis (AK) and *in situ* squamous cell carcinoma (isSCC) (1). Although early stage inSCC is rarely fatal, advanced disease or the treatment can produce significant patient disfigurement (1, 2). AK, a common skin lesion, has an estimated risk of 1-10% to progress to inSCC. Since no progression parameters are defined, each AK should be treated, to prevent development of inSCC and related morbidity (2, 3).

During carcinogenesis, tumor-specific neoantigens are recognized by the immune system and an immune response is orchestrated against the tumor (4). Tumor infiltrating lymphocytes (TILs), mostly CD3+ T-cells, CD8+ cytotoxic T-cells (CTLs) and Foxp3+ regulatory T-cells (Tregs), accumulate in the tumor microenvironment (TME) in various tumors, including inSCC (5). Langerhans cells (LCs), the antigen-presenting cells (APCs) of the epidermis, participate in adaptive immunity, which is crucial for tumor elimination (6), but have not been studied in inSCC. T-cell stimulation requires binding of the T-cell receptor (TCR) to the antigen, presented by APCs *via* molecules of the major histocompatibility complex (MHC). Additional second signals are provided by APC-bound ligands to co-stimulatory receptors on T-cells. These signals, known as immune checkpoints, propagate or inhibit T-cell activation. Programmed Death-1 (PD-1), a member of the CD28/cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) family of co-stimulatory receptors, after binding to its ligands, PD-L1 or PD-L2, inhibits T-cell activation, impedes the immune response in the TME and allows tumor survival (4). PD-L1 is expressed on lymphocytes, macrophages, APCs (7) and tumor cells of various tumors (4, 8, 9). Accumulated knowledge on the role of immune checkpoints in cancer evolution led to the development of immune checkpoint inhibitors (ICIs). Antibodies targeting CTLA-4, PD-1 and PD-L1 have been approved for metastatic

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*Key Words:* Cutaneous invasive squamous cell carcinoma, in situ squamous cell carcinoma, actinic keratosis, tumor infiltrating lymphocytes/TILs, Langerhans cells, programmed death-ligand 1/PD-L1.

melanoma and several other malignancies. Recently, the US Food and Drug Administration (FDA) approved cemiplimab, the first PD-1 blocking antibody for advanced cutaneous SCC (10). PD-L1 expression is an effective predictive biomarker for immunotherapy, used as a criterion for treatment approval (11, 12) or an indicator of expected response to PD-1/PD-L1 ICIs in certain malignancies (7).

A limited number of studies have investigated TILs (5, 13-15), LCs (16) and PD-L1 (17-24) in cutaneous inSCC. These studies mainly examined small numbers of inSCCs and did not provide data on the precursor lesions. The present study exploits the natural cutaneous squamous carcinogenesis model, examining a large number of inSCCs with adjacent precursors, to evaluate T-cells, CTLs, Tregs, LCs and PD-L1 during the evolution of inSCC from precursors. The hypothesis to be investigated is that the immune system increasingly responds to the evolution of precursor lesions to inSCC, but the tumor silences the immune cells *via* PD-L1 expression.

## Materials and Methods

*Patient selection*. The Pathology Department electronic files of our University Hospital were retrospectively searched for inSCC cases during the years 2000-2005. Immunosuppression, prior radiation therapy (RT), chronic cutaneous inflammatory processes and reccurent tumors were exclusion criteria. Hematoxylin-eosin slides of recovered inSCCs were reviewed, to retrieve cases of inSCC with adjacent coexisting precursor lesions. Thus, 125 cases of inSCC were collected with adjacent isSCC, AK and NS in 53, 123 and 123 lesions, respectively. Patients clinical and tumor data are presented in Table I, including TNM stage (8$^{th}$ edition of the American Joint Committee for Cancer classification), the risk factors for local recurrence or metastases, as defined by the National Comprehensive Cancer Network (NCCN) 2018 guidelines for squamous cell skin cancer (25) and total number of high risk factors. High risk factors include poor differentiation, specific tumor subtypes (acantholytic, adenosquamous, desmoplastic or metaplastic), thickness ≥2 mm or Clark level IV or V, perineural, lymphatic or vascular involvement, location/size, such as Area L/≥20 mm, Area M/≥10mm or Area H.

*Immunohistochemistry*. Selected paraffin tissue blocks were cut at 3 μm. Immunohistochemistry was performed as previously described (5) with the following primary Abs: anti-CD3 (rabbit polyclonal Ab, A0452, Dako, CA, USA, dilution 1:300); anti-CD8 (mouse monoclonal Ab (mAb), clone C8/144B, Dako, dilution 1:50); anti-Foxp3 (mouse mAb, clone 236A/E7, Abcam, Cambridge, UK, dilution 1:100); anti-CD1a (mouse mAb, clone 010, Dako, dilution 1:60); anti-PD-L1 (rabbit mAb, clone E1L3N, Cell Signaling Technology, Danvers, MA, dilution 1:100). For all antibodies, tonsil served as a positive control. For negative controls, the primary antibody was substituted with TBS.

The immunostained slides were simultaneously evaluated on a double-headed light microscope by a pathologist (EK) and an investigator (AS). CD3+, CD8+ and Foxp3+ cells: The peritumoral "stromal" TILs (sTILs) were semi-quantitatively evaluated using the Klintrup-Makinen grading scheme as previously described (5, 26).

Table I. *Patient characteristics and clinicopathological characteristics of the lesions*.

| | inSCC [number (n)=125] |
|---|---|
| Age (Mean, range) | 77.2, 43-101 |
| Gender | |
| Male | 82 |
| Female | 43 |
| Diameter (cm) (Mean, range) | 1.37, 0.3-5.5 |
| Differentiation (n, %) | |
| Good | 87, 69.6% |
| Moderate | 29, 23.2% |
| Poor | 9, 7.2% |
| Location (n, %) | |
| H | 62, 49.6% |
| M | 40, 32% |
| L | 12, 9.6% |
| Unknown | 11, 8.8% |
| Histologic type (n, %) | |
| Infiltrative/common | 76, 60.8% |
| Acantholytic/adenoid | 12, 9.6% |
| Clear cell | 1, 0.8% |
| Spindle cell/metaplastic | 1, 0.8% |
| Verrucous | 8, 6.4% |
| Keratoacanthoma-like | 22, 17.6% |
| Lymphoepithelioma-like | 2, 1.6% |
| Mucin-producing/ adenosquamous | 1, 0.8% |
| Unknown | 2, 1.6% |
| Depth of invasion (mm) | |
| (Mean, range) | 4.84, 0.5-19 |
| Clark level (n, %) | |
| 2 | 9, 7.2% |
| 3 | 14, 11.2% |
| 4 | 40, 32% |
| 5 | 60, 48% |
| Unknown | 2, 1.6% |
| Lymphatic/Vascular invasion (n, %) | |
| Yes | 10, 8% |
| No | 114, 91.2% |
| Unknown | 1, 0.8% |
| Perineural invasion (n, %) | |
| Yes | 20, 16% |
| No | 103, 82.4% |
| Unknown | 2, 1.6% |
| Number of high risk factors | |
| 0 | 4 |
| 1 | 16 |
| 2 | 53 |
| 3 | 29 |
| 4 | 6 |
| 5 | 0 |
| Unknown | 17 |

inSCC: Invasive squamous cell carcinoma; n=number; H, M, L: high, medium, low risk classification by location, according to NCCN v.2018, independent of size.

The intraepithelial TILs (eTILs) of inSCC were assessed as: 0-2 for absent, scattered or prominent eTILs, respectively, and 3, for infiltration with tumor cell destruction. The term "intraepithelial" rather than "intratumoral" was used, to identify TILs within or

Table II. *CD3+, CD8+ and Foxp3+ T-lymphocytes, CD1a+ Langerhans cells and PD-L1 expression on epithelial and immune cells in inSCC and adja-cent lesions.*

T-lymphocyte subpopulations in inSCC and adjacent lesions[a]

| | CD3 (m/md/SD) | CD8 (m/md/SD) | Foxp3 (m/md/SD) |
|---|---|---|---|
| sTILs in inSCC | 1.8/2/0.85 | 1.64/1/0.8 | 1.4/1/0.68 |
| sTILs in isSCC | 1.66/2/0.63 | 1.53/2/0.67 | 1.22/1/0.59 |
| sTILs in AK | 1.28/1/0.52 | 1.13/1/0.57 | 0.83/1/0.61 |
| sTILs in NS | 0.82/1/0.46 | 0.58/1/0.56 | 0.34/0/0.47 |
| eTILs in inSCC | 1.87/2/0.78 | 1.71/2/0.86 | 1.31/1/0.83 |

CD1a+ cells in the epithelial and stromal compartments of the lesions

| | eCD1a cell counts/40×/Hot Spot (m/md/SD) | sCD1a cell counts/40×/Hot Spot (m/md/SD) |
|---|---|---|
| InSCC | 66.77/48/60.91 | 69.59/50/64.1 |
| IsSCC | 34.98/32/21.49 | 30.18/20/27.36 |
| AK | 33.22/30/16.24 | 22.02/11/27.69 |
| NS | 27.78/26/12.77 | 11.39/6/16.78 |

PD-L1 expression on epithelial and stromal immune cells in inSCC and adjacent lesions[b]

| | ePD-L1 expression (m/md/SD) | sPD-L1 expression (m/md/SD) |
|---|---|---|
| inSCC | 1.2/0/1.8 | 3/3/1.84 |
| isSCC | 0.44/0/1.24 | 1.69/2/1.3 |
| AK | 0.1/0/0.52 | 1.12/0/1.43 |
| NS | 0/0/0 | 0.36/0/0.94 |

AK: Actinic keratosis, eCD1a: intraepithelial CD1a+ cells, ePD-L1: epithelial PD-L1+ cells eTILs: intraepithelial tumor infil-trating lymphocytes, inSCC: invasive squamous cell carcinoma, isSCC: *in situ* squamous cell carcinoma, m/md/SD: mean/median/standard deviation, NS: normal skin, sCD1a: stromal CD1a+ cells, sPD-L1: stromal PD-L1+ immune cells, sTILs: stromal tumor infiltrating lymphocytes. [a]Klintrup-Makinen grading scheme values range 0-3. [b]Allred score values range 0-8.

touching malignant cell nests. CD1a+ cells: The hot spot area of CD1a expression was identified in low power magnification, and the absolute number of CD1a+ LCs was counted in the epithelial (eCD1a) and stromal (sCD1a) compartments at 40x magnification. PD-L1: Expression was evaluated in the tumor cells and stromal immune cells, by assessing: A. the percentage of positive tumor cells (ePD-L1) or stromal immune cells (sPD-L1) over the entire tumor or stromal immune cell populations, respectively. For analysis of positivity, the cut-off of ≥1% was employed; B. the Allred score, ranging 0-8, and calculated as the sum of points obtained for percentage (0-5 for 0%,<1%, 1-10%, 11-33%, 34-66% and 67-100%, respectively) and intensity of positive cells (0-3, for absent, weak, moderate or strong intensity, respectively). For comparisons between groups the more detailed Allred score data were used.

*Statistical analysis*. The clinical and biomarker data were summarized using descriptive statistics and exploratory data analysis. Continuously scaled parameters were summarized with descriptive statistical measures [mean (m), standard deviation (SD) and median (md) (range)], and categorical data were described using contingency tables. Paired comparisons of continuous and categorical variables were performed using Wilcoxon signed-rank test and Marginal Homogeneity test, respectively. Intergroup comparisons were performed using the Mann-Whitney nonparametric test for continuous and ordinal variables and chi-square test for categorical variables. Correlations between the biomarkers were performed using the Spearman's Rank Correlation Test. All *p*-value determinations were 2 sided, at a significance level of 0.05. Analyses were performed with the use of IBM SPSS Statistics for Windows, Version 21.0 (IBM Corp., Armonk, NY, USA).

## Results

*CD3+, CD8+ and Foxp3+ TILs in inSCC, adjacent lesions and NS*. The results including descriptive statistics and statistical significance of comparisons for CD3+, CD8+ and Foxp3+ T-cell subpopulations in inSCC, isSCC, AK and NS are presented in Table II and Figure 1A. Representative images of the corresponding immunostains are shown in Figure 2. sCD3+, sCD8+ and sFoxp3+ TILs increased significantly and stepwise, from NS to AK and isSCC/inSCC. No significant differences were noted between inSCC and isSCC, except for sFoxp3+ TILs which were more numerous in inSCC compared to isSCC (Figure 1A). eCD3+ TILs in inSCC outnumbered eCD8+ and eFoxp3+ TILs ($p$=0.002 and <0.001, respectively). In all lesions, CD8+ TILs outnumbered

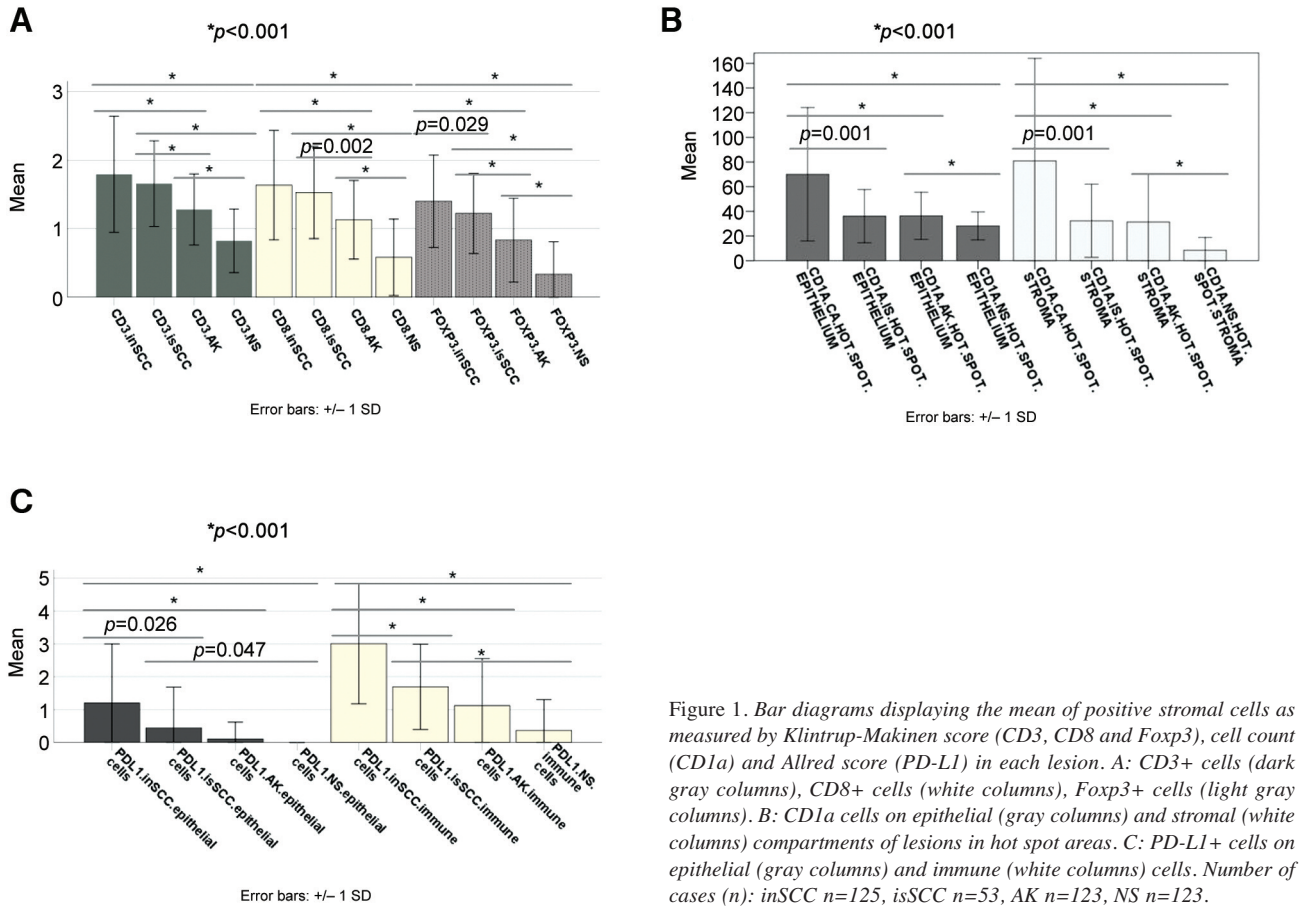


Figure 1. *Bar diagrams displaying the mean of positive stromal cells as measured by Klintrup-Makinen score (CD3, CD8 and Foxp3), cell count (CD1a) and Allred score (PD-L1) in each lesion. A: CD3+ cells (dark gray columns), CD8+ cells (white columns), Foxp3+ cells (light gray columns). B: CD1a cells on epithelial (gray columns) and stromal (white columns) compartments of lesions in hot spot areas. C: PD-L1+ cells on epithelial (gray columns) and immune (white columns) cells. Number of cases (n): inSCC n=125, isSCC n=53, AK n=123, NS n=123.*

Foxp3+ TILs (inSCC: $p$=0.004; isSCC: $p$=0.002; AK: $p$<0.001 and NS: $p$<0.001). Concerning the correlation of TIL subpopulations with the patients' and lesion characteristics, eCD3+ and eCD8+ TILs were inversely correlated with the number of high risk factors ($p$=0.034 and 0.031, respectively). eCD3+ TILs were more numerous in poorly differentiated tumors ($p$=0.019) and eFoxp3+ cells were more numerous in thinner tumors ($p$=0.037).

*CD1a+ Langerhans cells*. eCD1a and sCD1a expression in inSCC, isSCC, AK and NS are presented in Table II and Figure 1B and representative immunostaining is shown in Figure 3. The number of eCD1a+ and sCD1a+ LCs, was higher in the hot spots of inSCC compared to isSCC, AK and NS ($p$-Values in Figure 1B). The number of sCD1a+ LCs was higher in thinner tumors ($p$=0.04) and was positively correlated with sCD8+ and sFoxp3+ TILs ($p$=0.02 and 0.004, respectively).

*PD-L1 expression by epithelial neoplastic and immune cells*. PD-L1 expression results in tumor cells and TILs are shown in Table II and Figure 1C. Representative images are shown in Figure 4. Allred score ePD-L1 and sPD-L1 values were higher in inSCC compared to isSCC, AK and NS (Table II). Applying a cut-off ≥1%, 20/120 (16.7%) of inSCC, 2/43 (4.65%) of isSCC and 1/117 (0.86%) of AK were positive for ePD-L1 expression. Correlation analysis revealed that ePD-L1 and sPD-L1 values positively correlated with inSCC thickness ($p$=0.029 and 0.024, respectively). ePD-L1 positively correlated with tumor diameter ($p$=0.033), sPD-L1 ($p$<0.001), sCD3 ($p$=0.025), eCD3+ ($p$<0.001), sCD8+ ($p$=0.044) and eCD8 TILs ($p$<0.001). sPD-L1 inversely correlated with perineural invasion ($p$=0.007) and positively correlated with sCD3, eCD3, sCD8, eCD8 and sFoxp3+ TILs ($p$<0.001 for all comparisons), eFoxp3+ TILs ($p$=0.009), sCD1a+ ($p$=0.012) and eCD1a+ LCs ($p$=0.002).

## Discussion

Accumulation of TILs around precancerous AK and isSCC suggests early engagement of the immune system in inSCC carcinogenesis, as previously shown (5) but despite the presence of immune cells, the tumors continue to grow, indicating a functional impairment of the immune system.

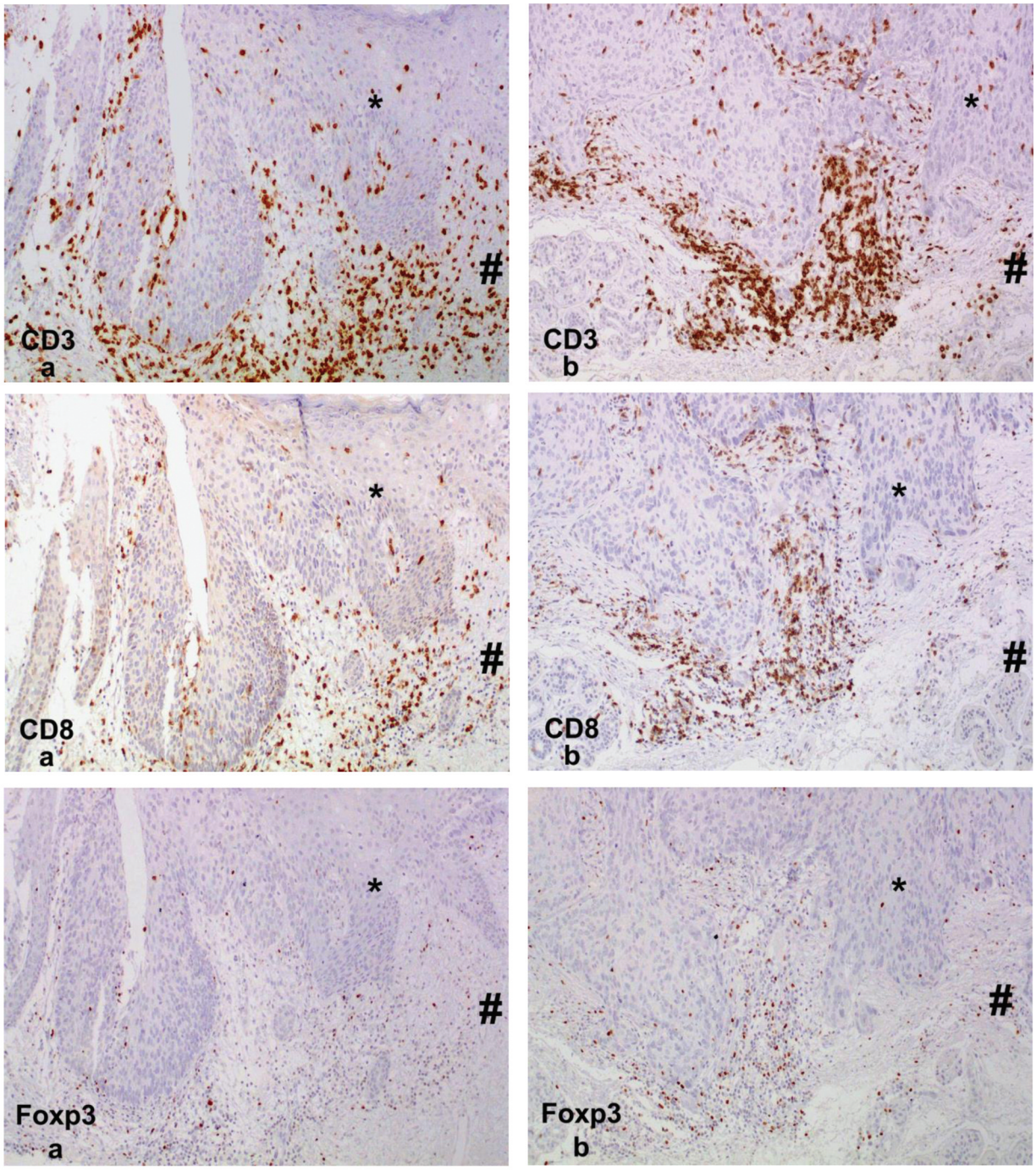


Figure 2. *A case of inSCC (panels b) with adjacent AK (panels a) immunostained for CD3, CD8 and Foxp3. Note the increased TILs in inSCC compared to AK (*corresponds to epithelial and #to stromal compartment).*

The aim of this study was to map and describe the various immune elements of the TME in inSCC and adjacent to each tumor precursor lesion. Exploiting the natural model of cutaneous squamous carcinogenesis will contribute to understanding the role of immune TME and provide rational for appropriate immunotherapeutic modalities.

The Klintrup-Makinen score is easily applicable and adequately informative (5) and was applied herein for TIL

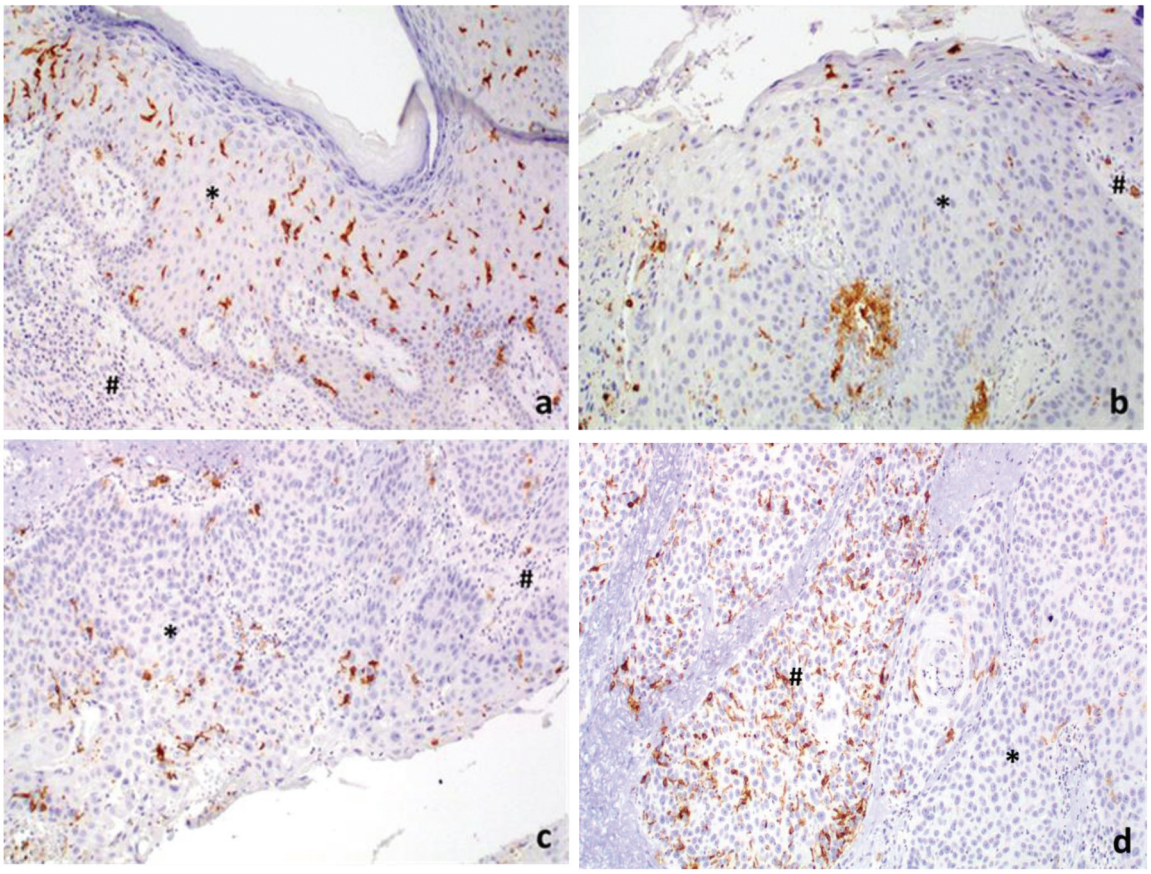


Figure 3. *A case of inSCC with adjacent precursors immunostained for CD1a, depicting the epithelial compartment hot spots (×10). (a) normal skin, (b) actinic keratosis, (c) in situ squamous cell carcinoma, (d) invasive squamous cell carcinoma. The * and # signs indicate the epithelial and stromal compartments, respectively.*

subpopulation analysis. CD3+, CD8+ and Foxp3+ TILs gradually and significantly increase as the carcinogenesis progresses. Although CD3+ and CD8+ TILs do not differ significantly from isSCC to inSCC, Foxp3+ cells are significantly higher in inSCC compared to isSCC, suggesting involvement of Foxp3+ TILs in inSCC *via* blunting the immune response. Notably, this finding is shown for the first time, and was not observed in previous reports, including our pilot study (5, 13). Likely explanations include the larger number of cases and the different cohort that includes evolutionally related precursor lesions along with the adjacent inSCC. In the evolution from AK to isSCC, T-cell populations exhibited a gradual increase of statistical significance, further supporting engagement of the immune system during the development of isSCC. Higher numbers of Foxp3+ cells were also reported by Jang in isSCC compared to AK (13).

The absolute number of CD1a+ LCs was found to gradually increase during transition from AK to isSCC and inSCC. Takahara *et al*. in a smaller study of 12 NS, 15 AK, 15 isSCC, 15 inSCC, observed opposite results (16). Unlike the present study, where the precursor lesions were contiguous to each inSCC, in the study of Takahara *et al*., each precursor lesion derived from different patients, thus a safe comparison is not feasible. LCs, as APCs of the epidermis, have an important role in orchestrating the antitumor immune response. The tumor cells suppress the maturation of dendritic cells (DCs), resulting in defective antigen presentation (27). Consequently, LC elimination from the TME allows tumor escape from immune surveillance (28, 29). The correlations of stromal LCs with sCD8+ and sFoxp3+TILs suggest communication of these cells in the TME of inSCC. Higher number of stromal LCs was found associated with thinner tumors. This finding leads to the hypothesis that more efficient immune stimulation by higher numbers of LCs leads to better control and inhibition of cancer growth. LCs were found to be decreased in basal cell carcinoma (28), but in melanoma the results are contradictory.

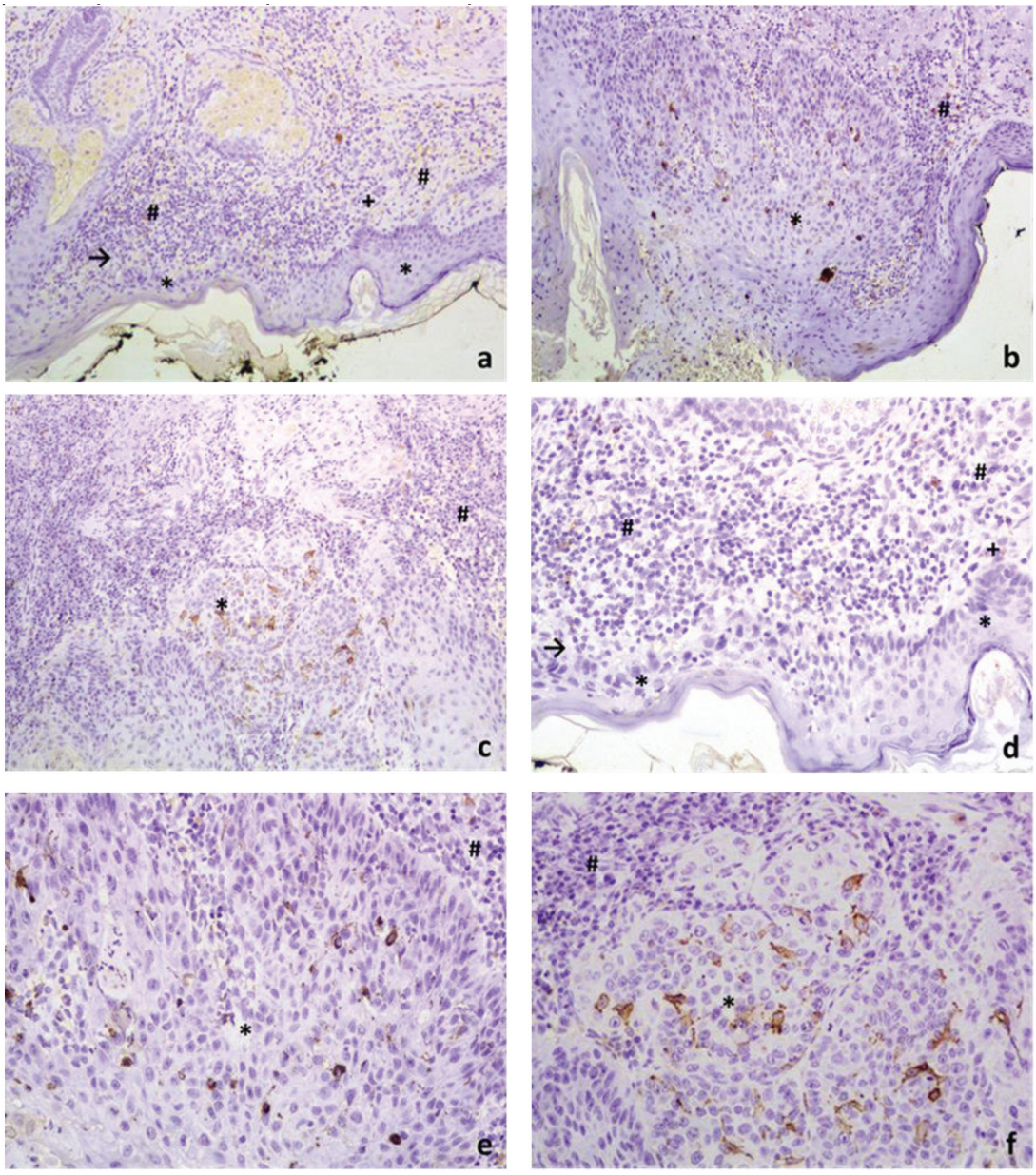


Figure 4. *Images of a case of inSCC with adjacent precursors immunostained for PD-L1, depicting the epithelial expression of PD-L1. (a) Actinic keratosis (arrow) and normal skin (+ sign) (×10); (b) in situ squamous cell carcinoma (×10); (c) invasive squamous cell carcinoma (×10); (d) Actinic keratosis (arrow) and normal skin (+ sign) (×20); (e) in situ squamous cell carcinoma (×20); (f) invasive squamous cell carcinoma (×20). The * and # signs indicate the epithelial and stromal components, respectively. In panels (b), (c), (e), and (f), the epithelial components correspond to tumor tissue.*

Dyduch *et al*. observed no difference in epidermal and dermal CD1a+ cell density in melanoma and dysplastic nevi (30), but Vermi *et al*. found significant increase of LCs in melanomas compared to melanocytic nevi and NS (31). The mechanisms involved in tumor depletion of LCs during carcinogenesis are not yet resolved. In line with our findings, Fujita et al reported that LCs from human cutaneous SCC induced a stronger TH1 immune response with CD4+, CD8+ and interferon-γ production, compared to LCs from surrounding peritumoral skin (32). Nonetheless, the precise differentiation and role of LCs may vary due to their plasticity, according to microenvironmental influences (33).

Adaptive PD-L1 expression on tumor cells is induced by inflammatory cytokines in the TME, such as IFN-γ, IL-10,

IL-4, TNF-a, VEGF and GM-CSF (34, 35) and is considered to better predict response to immunotherapeutic agents than constitutive expression, particularly when accompanied by immune cell infiltration of the tumor. The "next generation" of immune biomarkers combines TIL subpopulations and PD-L1 expression (7). Up to now, a limited number of studies investigating PD-L1 expression in cutaneous inSCC are available (17-19, 21-24, 36-41) and only one has reported PD-L1 expression in 26 inSCC and precursors, namely 20 isSCCs and 26 AKs (17).

In our study, the expression of PD-L1 in tumor cells was higher in inSCC compared to isSCC and AK, in accordance to the study by Gambichler *et al*. (17), whereas there was no significant difference between PD-L1 expression in isSCC and AK. PD-L1 expression was not observed in NS, and although a difference between AK and NS was observed, it was only marginal, given close to zero values noted in AK. AK and isSCC are preinvasive lesions and it is possible that epithelial cells have not yet developed immune escape mechanisms. The expression of PD-L1 in inSCC epithelium, applying a cut-off ≥1%, was positive in 16.7% of the cases. García-Pedrero *et al*. (18) and Schaper *et al*. (22) using the same PD-L1 clone and positivity cut-off in 100 and 68 inSCCs respectively, identified higher percentages of positive cases, namely 41% and 26%. Notably, García-Pedrero *et al*. studied metastatic cutaneous SCCs, whereas none of our cases was metastatic; the difference in tumor stage likely reflects different tumor biology and explains the difference in PD-L1 positivity (18). PD-L1 expression on tumor cells of inSCC positively correlated with intraepithelial and stromal CD3+ and CD8+ TILs, as previously reported (39). Similarly, Schaper *et al*. reported a correlation between PD-L1 expression and intratumoral CD8+ cells (22). In inSCC, PD-L1 expression is gradually increasing stepwise from precursors, along with TILs. This is similar to the findings of TIL infiltration along with PD-L1 expression in other carcinomas, such as breast (reviewed by Dieci *et al*.) (43). PD-L1 expression is thought to suppress TILs and facilitate tumor escape and growth. This hypothesis is supported by two findings in our study; firstly, increased numbers of TILs are observed in isSCC but they are not accompanied by PD-L1 expression, unlike inSCC; secondly, PD-L1 expression in inSCC correlates with greater tumor diameter and thickness. Whether PD-L1 expression is related to tumor mutation burden (TMB) is an additional point of interest that merits to be investigated.

Immune cell sPD-L1 expression correlates not only with CD3+, CD8+ and Foxp3+ TILs but also with CD1a+ cells. All these cell types are able to express PD-L1 on their cell membrane following activation by pro-inflammatory cytokines, such as IFN-γ and TNF-a (42). In view of the intense lymphocytic infiltration in inSCC, the induction of PD-L1 expression on these cells is not surprising. Previous studies in SCC have also correlated PD-L1 expression with the degree of inflammation (18) and TILs (21, 22).

The positive correlation of ePD-L1 expression with risk factors for metastasis, such as tumor diameter and thickness, has also been observed by Slater and Googe (24) but not by other investigators (22, 40). ePD-L1 expression has been correlated with tumor stage (12) and the risk of nodal metastasis (9) and is found to be increased in metastatic or advanced disease (38, 40, 41). This association can be explained as a reflection of the tumor evasion facilitated by the PD-L1 "mantle".

The weaknesses of our study include its retrospective nature, and due to the "innocuous" nature of early cutaneous SCC, the inability to maintain long term patient follow-up.

## Conclusion

In conclusion, and to the best of our knowledge, this study is the largest on TILs, LCs and PD-L1 in cutaneous inSCC and precursor lesions. Three factors relating to the immune components of the TME were identified that may assist the escape of tumor cells from immune surveillance and contribute to the evolution of precursor lesions to inSCC: increased infiltration by Foxp3+ immunosuppressive T-cells, antigen presenting LCs and PD-L1 expression by the tumor cells. These findings, along with the intense presence of CD8+ CTLs in both inSCC and precursors, provide support for immunotherapeutic targeting with immune checkpoint inhibitors in selected cases. PD-L1, CD8+ and Foxp3+ TILs, singly or in combination merit to be tested as predictive biomarkers in future large prospective clinical studies.

## Conflicts of Interest

Eleni P. Kourea has received honoraria as invited speaker by Bristol Myers Squib (BMS) and Merck Sharp & Dohme (MSD), Greece.

## Authors' Contributions

Conceptualization: Eleni P. Kourea; Methodology: Aristea Stravodimou, Eleni P. Kourea; Data collection: Aristea Stravodimou, Stavros Balasis; Formal analysis and investigation: Aristea Stravodimou, Vasiliki Tzelepi; Writing - original draft preparation: Aristea Stravodimou; Writing - review and editing: Vasiliki Tzelepi, Sophia Georgiou, Helen Papadaki, Athanasia Mouzaki Maria Melachrinou, Eleni P. Kourea; Resources: Maria Melachrinou, Eleni P. Kourea; Supervision: Eleni P. Kourea.

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