

**Progression of cutaneous squamous cell carcinoma in immunosuppressed patients is associated with reduced CD123+ and FOXP3+ cells in the perineoplastic inflammatory infiltrate**

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## **Abstract**

**Background:** Squamous cell carcinoma of the skin (SCC) increases dramatically in organ transplant recipients (OTRs). We assumed that qualitative and quantitative differences in perineoplastic inflammation in OTRs contribute to the increased carcinogenesis.

**Material and Methods:** We studied perineoplastic SCC inflammatory infiltrate assessing depth, density and phenotype (CD3, 4, 8, FOXP3, CD123, and STAT1) by immunohistochemistry in paired biopsies of intraepithelial and invasive SCC in immunocompetent patients and OTRs.

**Results:** Considerable inflammation was observed in all intraepithelial SCC (inflammatory infiltrate depth 2.80 mm  $\pm$ 2.21 immunocompetent pts, 2.15 mm  $\pm$ 2.95 OTRs). Inflammation was more pronounced in invasive SCC of immunocompetent patients (4.60  $\pm$ 4.67 mm) and OTRs (3.30  $\pm$ 5.90 mm) respectively ( $p < 0.005$ ). The density of perineoplastic inflammatory infiltrates increased from intraepithelial to invasive SCC ( $p = 0.005$ ). OTRs show a lower density of perineoplastic inflammatory infiltrate ( $p = 0.041$ ). OTRs also show reduced CD3+ T-lymphocyte and CD8+ cytotoxic T-lymphocyte proportions in intraepithelial SCC ( $p = 0.025$  and  $0.027$ , respectively). FOXP3+ regulatory T-lymphocyte proportions in OTRs' invasive SCC are markedly diminished ( $p = 0.048$ ). CD123+ plasmacytoid dendritic cells increase in the progression from intraepithelial to invasive SCC in immunocompetent patients ( $p = 0.040$ ). CD123+ cells are reduced in all SCC of OTRs ( $p = 0.036$ ).

**Conclusions:** Perineoplastic inflammation in intraepithelial SCC is pronounced both in immunocompetent patients and OTRs. Inflammation increases further in invasive SCC. OTRs show reduced proportions of FOXP3+ regulatory T cells and CD123+ plasmacytoid dendritic cells. This distinct inflammatory infiltrate may result in the

increased cutaneous carcinogenesis and more aggressive behaviour of SCC in  
OTRs.

## Introduction

Squamous cell carcinoma of the skin (SCC) develops from atypical keratinocytes within sun-damaged Caucasian epidermis, clinically visible as actinic keratosis or Bowen's disease<sup>1,2</sup>. Only 10 to 25% of these intraepithelial lesions, however, progress to invasive SCC<sup>3,4</sup>. An inflammatory response eliminates most intraepithelial lesions before invasive SCC develops<sup>5,6</sup>. Recognition and elimination of tumour antigen-bearing cells may be one mechanism of tumour control<sup>7</sup>. Topical therapies for intraepithelial lesions are numerous and to a large part effective, all directly or indirectly eliciting considerable inflammatory response<sup>8</sup>. The magnitude of the inflammatory response overall seems clinically important for clearance of intraepithelial lesions<sup>9</sup>. Likewise, lack of tumour surveillance by the immune system is of importance<sup>5</sup>: Drug-induced immunosuppression in organ transplant recipients (OTRs) dramatically increases cutaneous carcinogenesis 60- to 100-fold<sup>10,11</sup>. In opposition to the immune system's beneficial function in tumour surveillance, chronic, smouldering inflammation on the other hand is suggested to be a significant promoter of tumour development<sup>6,12</sup>.

Carcinogenic stimuli such as ultraviolet radiation typically affect not only individual spots of skin, but rather whole anatomical regions such as the face which give rise to SCC repeatedly, a phenomenon called field cancerization<sup>13-15</sup>. Subsequent lesions thus probably result from the same pathogenic driving forces. Studying intraepithelial lesions and invasive lesions from the same field in the same patient might provide insight into inflammation and its relationship to human neoplasm progression in particular to the beneficial or detrimental role of chronic inflammation for neoplasm development. We chose patients who had first developed an intraepithelial lesion and subsequently an invasive lesion of SCC in the same anatomical region.

Immunocompetent patients' lesions were compared to OTRs' lesions to detect potentially important differences in this immunosuppressed population with greatly increased development of squamous cell carcinoma of the skin.

We studied perineoplastic inflammatory infiltrate assessing depth, density and phenotype (CD3, 4, 8, FOXP3, CD123, and STAT1) by immunohistochemistry.

Human Papilloma Virus (HPV) is known to potentially transform keratinocytes and prevent apoptosis thus contributing to cancer development. Particularly in immunosuppressed OTRs, viral origin of squamous cell carcinoma of the skin is discussed<sup>16</sup>. To assess the importance of HPV in our population, HPV protein expression was considered.

## **Material and Methods**

### **Neoplasm selection**

Our goal was to obtain tissue material from an area of field cancerization which had first given rise to an intraepithelial and at least one year later to an invasive SCC. Following approval by the local ethical committee, we randomly identified 43 immunocompetent patients and 42 immunosuppressed OTRs. All patients had first had an intraepithelial lesion such as actinic keratosis or Bowen's disease removed and archived as formalin-fixed paraffin-embedded tissue block, followed by an invasive SCC on the same anatomical region at least one year later and again present as a formalin-fixed paraffin-embedded tissue block in our archive. The immune status of all patients was assessed by reviewing patients' history: OTRs with systemic immunosuppression to prevent transplant rejection were identified. Actinic keratosis was considered as an in-situ squamous cell carcinoma of the skin as discussed elsewhere<sup>1,17</sup> and grouped with Bowen's disease as intraepithelial squamous cell carcinoma of the skin as opposed to invasive squamous cell carcinoma of the skin. Distinction between in-situ and invasive forms of squamous cell carcinoma was made according to the recent WHO skin tumour classification<sup>18</sup>. Of the 42 OTRs, 32 patients had received a kidney, 4 had received a lung, and 6 a heart, respectively. Patient characteristics, lesions, and numbers are provided in table 1.

### **Immunohistochemistry**

3- to 5- $\mu$ m adjacent sections were used for haematoxylin and eosin (HE) staining and immunohistochemistry. The deparaffinised sections were heated in a 100-W household microwave oven at maximum power for three times 5 minutes each in 10 mmol/L citric acid for antigen retrieval. Primary antibody was then applied for 60

minutes at room temperature. Immunohistochemical stainings were performed with monoclonal IgG mouse antibodies specifically binding human CD3 (A 0452; Dako, Glostrup, Denmark), CD4 (NCL-1F6; Novocastra, Newcastle upon Tyne, UK), CD8 (M 7103; Dako), and Cytoactiv HPV L1 monoclonal mouse antibody ACA1910 (cytoimmun diagnostics GmbH, Pirmasens, Germany). According to the company's specifications, ACA1910 monoclonal antibody stains all HPV types and is not limited to genital HPV types. FOXP3 was stained using rabbit polyclonal ab10563 (Abcam Limited, Cambridge, United Kingdom). CD123 was stained using mouse monoclonal antibody clone 6H6 (eBioscience, San Diego, CA, USA) in a dilution of 1:100. STAT-1 was stained using mouse monoclonal antibody clone 9H2 (Cell Signaling Technology, Danvers, MA, USA) in a dilution of 1:50. Secondary staining was performed using the alkaline phosphatase anti-alkaline phosphatase method<sup>19</sup>. Antibodies were chosen because of previously published studies and for their availability within our laboratory. Normal epidermal cells, lymphocytes, fibroblasts as well as other cells of the subcutaneous tissue served as internal negative controls, sections of known positive tissue such as tonsils for lymphocyte antigens as external positive controls.

Immunoreactivity was assessed over the perineoplastic inflammatory infiltrate and rated as 0-5%, 6-25%, 25-50%, 51-75%, 76-100% of all inflammatory cells. The maximum depth of inflammatory infiltrate was measured perpendicularly to the surface in mm. Density of inflammatory infiltrate was rated as dense, when more inflammatory than stromal cells were present within the inflammatory perineoplastic infiltrate, or as scarce, when there were fewer inflammatory cells than stromal cells within the perineoplastic inflammatory infiltrate. All samples were reviewed by a dermatohistopathologist unaware of the patients' diagnosis or immune status.

Statistical analysis was performed using Microsoft Excel 2000 and SPSS for Windows 11.5.



## **Results**

### **Patient characteristics**

43 immunocompetent patients and 42 immunosuppressed OTRs with first an intraepithelial and at least one year later an invasive SCC within the same anatomical region and with formalin-fixed paraffin-embedded tissue from both lesions available for haematoxylin & eosin and immunohistochemical staining qualified. These paired samples should reflect evolution of squamous cell carcinoma within the same region of field cancerization<sup>13-15</sup>. Gender of the immunocompetent patients was adjusted to 74% male, as this is the proportion of male organ transplant recipients at Zurich University Hospital in order to avoid confounding data by gender inequality. Age was nearly matched, as well as distribution of actinic keratoses, Bowen's disease and invasive SCC. Patient characteristics are summarized in table 1.

### **Perineoplastic inflammatory infiltrate increases with progression from intraepithelial to invasive SCC**

Perineoplastic infiltrate was measured as described on H&E stains (Fig. 1). Maximum depth of perineoplastic inflammatory infiltrate was 2.80 mm (SD  $\pm$  2.21 mm) for intraepithelial and 4.60 mm (SD  $\pm$  4.67 mm) for invasive SCC in immunocompetent patients and 2.15 mm (SD  $\pm$  2.95 mm) for intraepithelial and 3.30 mm (SD  $\pm$  5.90mm) mm for invasive SCC in immunosuppressed OTRs (Fig. 2). Both immunocompetent and immunosuppressed patients taken together showed a marked increase in depth of inflammatory infiltrate in the progression from intraepithelial to invasive SCC ( $p < 0.005$ ). Maximum depth of perineoplastic inflammatory infiltrate, however, was not significantly different between immunocompetent patients and OTRs ( $p = 0.22$ ).

**Density of perineoplastic inflammatory infiltrate increases with progression from intraepithelial to invasive SCC**

A high density perineoplastic inflammatory infiltrate was observed in 35% of intraepithelial and in 65% of invasive lesions in immunocompetent patients (Fig. 3). In OTRs, a high density was seen in 29% of intraepithelial and in 41% of invasive SCC. Immunocompetent patients and OTRs together showed a marked increase in density of inflammatory infiltrate in the progression to invasive SCC ( $p=0.005$ ).

**OTRs show a lower density of perineoplastic inflammatory infiltrate**

Intraepithelial and invasive lesions taken together showed a high density perineoplastic inflammatory infiltrate in 50% of all immunocompetent patients' samples and in 35% of all OTRs' samples ( $p=0.041$ ; Fig. 4).

**Quality of perineoplastic inflammatory infiltrate does not change in the progression from intraepithelial to invasive SCC**

CD3+, 4+, 8+ and FOXP3+ cell proportions do not change in the progression to invasive SCC in immunocompetent patients and OTRs, respectively. Distributions of immunoreactive cells within the perineoplastic inflammatory infiltrate are displayed in figure 5 (a), (b), (c), and (d).

**OTRs show markedly diminished proportions of CD3+ and CD8+ cells in intraepithelial SCC**

Intraepithelial SCC in OTRs is accompanied by a lesser proportion of CD3+ T-lymphocytes and CD8+ cytotoxic T-lymphocytes in the perineoplastic inflammatory infiltrate ( $p=0.025$  and  $0.027$ , respectively).

**OTRs show a markedly diminished proportion of FOXP3+ cells in invasive SCC**

Invasive SCC in organ transplant recipients is accompanied by a lesser proportion of FOXP3+ regulatory T-lymphocytes (Tregs) in the perineoplastic inflammatory infiltrate ( $p=0.048$ ).

**CD123+ plasmacytoid dendritic cells increase from intraepithelial to invasive SCC in immunocompetent patients and are reduced in OTRs**

The progression to invasive SCC in immunocompetent patients was accompanied by a significant increase of CD123+ plasmacytoid dendritic cells (pDCs) in the perineoplastic inflammatory microenvironment ( $p=0.040$ ). SCC of OTRs showed a significant reduction of CD123+ plasmacytoid dendritic cells in their perineoplastic inflammatory infiltrate ( $p=0.036$ ).

**STAT1 immunoreactivity increases in invasive SCC of OTRS**

STAT1 is mainly induced by interferon gamma as well as by other ligands and is generally thought to be anti-proliferative and pro-inflammatory in cancer<sup>20, 21</sup>.

Compared to invasive SCC of immunocompetent patients, OTRs showed a significant increase of STAT1+ cells in their perineoplastic inflammatory infiltrate ( $p=0.032$ ).

Exemplary stainings for CD3, FOXP3, CD123, and STAT1 are shown in figure 1b. Distributions of immunoreactive cells within the perineoplastic inflammatory infiltrate are displayed in figure 5.

### **HPV protein expression occurs in a minority of SCC**

In order to control for the potential role for HPV, a random subset of neoplasms was chosen for HPV protein expression analysis by immunohistochemistry (14 immunocompetent patients' neoplasms, 13 OTRs' neoplasms). Prevalence of HPV protein expression was low in all groups assessed by immunohistochemistry (data not shown).

## Discussion

43 immunocompetent and 42 immunosuppressed OTRs with first an intraepithelial and at least one year later an invasive SCC within the same anatomical region could be identified for analysis. These paired samples are expected to reflect evolution of SCC within the same region of field cancerization, i.e. under similar oncogenic stimuli and within the same individual. These samples form a highly select group which has not been studied before.

Inflammatory activity was seen in all lesions, intraepithelial or invasive. A fair amount of chronic inflammatory infiltrate is therefore present in intraepithelial lesions already at the elimination and equilibrium stages of tumour immunoediting<sup>16</sup> which is particularly surprising in the profoundly immunosuppressed organ transplant recipient group. Porokeratoses as human intraepithelial lesions are the only ones to have been studied in a similar manner so far. In these lesions, the inflammatory infiltrate was moderately dense in the dermis and showed T helper cells as majority of lymphocytes<sup>22</sup>. Similar infiltrates have been reported in ovine actinic keratoses<sup>23</sup>. Interestingly in our series, immunocompetent patients and OTRs had virtually indistinguishable depths of inflammation in both intraepithelial and invasive squamous cell carcinoma of the skin, but of lower overall density in OTRs. Invasion of SCC through the dermoepidermal junction results in increased depth and density of inflammatory infiltrate. One potential explanation is exposure of the neoplasm to better recognition by the immune system, once the neoplasm has breached the basement membrane and enters the evasion stage in tumour immunoediting<sup>5</sup>. Here, influence of immunosuppression is seen through markedly reduced density of inflammatory infiltrate which others have also observed in OTRs before<sup>24</sup>.

In the progression from intraepithelial to invasive lesions in our highly select groups of patients, the quantity of the inflammatory infiltrate in immunosuppressed patients starts lagging behind the immunocompetent patients' infiltrate. This distinction in quantity of inflammation has not been reported yet and is an exciting finding. For invasive SCC, inflammatory infiltrate seems thus neoplasm-protective rather than neoplasm-promoting at this stage of tumour evasion: Low density of inflammatory infiltrate in many neoplasms has been shown to negatively impact prognosis<sup>6</sup>. OTRs with lesser infiltrate display more aggressive growth and metastasis<sup>11, 25</sup>.

Beyond depth and density of inflammatory perineoplastic infiltrate, composition of the inflammatory infiltrate matters. Our sample population did not show significant differences for CD3+, 4+, 8+ subpopulations or FOXP3+ regulatory T cells (Tregs) in the course from intraepithelial to invasive squamous cell carcinoma of the skin.

Again, little data in man is available to date, while in ovine squamous cell carcinoma development, CD3, CD8 and MHC II have been shown to be upregulated during tumour progression<sup>23</sup>. Perineoplastic inflammatory infiltrate in squamous cell carcinoma of the skin has been reported to contain 30-40% CD3+ cells within mononuclear cells with the majority being CD4+<sup>26</sup>. In OTRs, our samples show a higher proportion of inflammatory cells expressing CD3, and we observe a predominance of CD4+ over CD8+ cells as well (figure 6 (a), (b), and (c)). OTRs as a model for immunosuppression show significantly lower proportions of CD3+ and CD8+ cells in all their lesions compared to immunocompetent patients, while CD4+ cell proportions do not change noticeably. A reduction in T-lymphocytes overall and cytotoxic T lymphocytes in particular may thus lie at the heart of the observed lesser density of inflammatory infiltrate under immunosuppression.

Lack of T cells and cytotoxic T cells in particular will compromise anti-tumour response<sup>5</sup>. Tumour-associated antigens show different expression patterns in this

immunocompromised patient group indicating impaired tumour immunoediting<sup>7</sup>.

Interestingly, intraepithelial lesions in OTRs show significantly reduced CD3+ and CD8+ proportions (figure 5).

Lately, regulatory T cells have gained attention for their function in suppressing immune responses. Many cancers have been shown to display higher proportions of CD4+CD25+ regulatory cells which are thought to down-regulate the anti-tumour response<sup>27, 28</sup>. In invasive SCC, however, OTRs display a significantly lower proportion of FOXP3+ Tregs within the perineoplastic inflammatory infiltrate.

Generally, a reduction in Tregs should favour the anti-tumour response<sup>27</sup>. The calcineurin inhibitor Cyclosporine A is known to negatively impact regulatory T cell generation and function<sup>29, 30</sup> and is part of the immunosuppressive regimen in our immunosuppressed patients. This may well be the reason for the lower proportion of FOXP3+ Tregs in invasive lesions of OTRs. However, the increase of TGF-beta and VEGF following Cyclosporine A administration<sup>31</sup> and other factors may well outweigh the reduced amount of FOXP3+ Tregs in favour of neoplasm progression, thus assigning only a minor role to Tregs.

CD123+ plasmacytoid dendritic cells constitute an important local cell population mediating immune responses by antigen presentation and interferon signalling. pDCs increased in immunocompetent patients with the progression from intraepithelial to invasive lesions in our study. This may reflect increased antigen presentation with invasive SCC. OTRs show significantly lesser proportions of CD123+ pDCs in their SCC inflammatory infiltrate and do not significantly increase CD123+ pDCs with neoplasm progression. Impaired antigen presentation by OTRs may contribute to the more aggressive course of SCC in these patients. Recently, reduced antigen-presenting capacity of perineoplastic DCs from SCC in OTRs has been reported and

may add dysfunction to the relative paucity of APC in the inflammatory microenvironment of SCC in OTRs <sup>32</sup>.

STAT1 is mainly induced by interferon gamma as well as by other ligands and is generally thought to be anti-proliferative and pro-inflammatory in cancer <sup>20, 21</sup>. STAT1 immunoreactivity is seen in a large proportion of the inflammatory infiltrate in intraepithelial and invasive SCC in immunocompetent patients and OTRs in our study. While immunocompetent patients show no difference in STAT1 expression during neoplasm progression, STAT1 immunoreactivity in the perineoplastic inflammatory infiltrate of invasive SCC in OTRs is significantly upregulated compared to immunocompetent patients, reflecting activation of the immune response in OTRs. The IFN/STAT1 pathway thus does not seem impaired in our samples judging by STAT1 immunoreactivity in the perineoplastic inflammatory infiltrate.

Human Papilloma Virus (HPV) is known to potentially transform keratinocytes and prevent apoptosis thus contributing to cancer development. Particularly in immunosuppressed OTRs, viral origin of SCC is discussed <sup>16</sup>. Stainings for HPV protein expression were performed to assess importance of HPV in our sample population. Prevalence of HPV protein expression was found to be low (data not shown). We interpret the contribution of HPV to the inflammatory infiltrate in our population to be of minor importance. Beyond inflammation, other factors such as increasing allelic imbalance may decide about the progression from intraepithelial to invasive SCC and will be addressed in separate studies <sup>33</sup>.

In summary, we see a sustained perineoplastic inflammatory infiltrate in intraepithelial SCC which potentially contributes to neoplasm progression rather than fighting neoplasm effectively. Neoplasm invasiveness is distinguished by increase in depth and density of inflammatory infiltrate. OTRs as a model for immunosuppression show on the one hand marked inflammation in intraepithelial



lesions and on the other hand a clear compromise of the quantity and quality of inflammatory infiltrate in invasive lesions compared to immunocompetent patients (CD3, CD8, FOXP3, CD123). We conclude that these differences in inflammatory infiltrate potentially mediate the increased incidence and aggressive growth of squamous cell carcinoma of the skin in the high-risk population of OTRs.

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## References

1. Ackerman AB, Mones JM. Solar (actinic) keratosis is squamous cell carcinoma. *Br J Dermatol* 2006;**155**:9-22.
2. Cockerell CJ. Histopathology of incipient intraepidermal squamous cell carcinoma ("actinic keratosis"). *J Am Acad Dermatol* 2000;**42**:11-17.
3. Bloch B. Cancers and precancerous affections from the dermatological viewpoint. *Cancer Review* 1932;**7**:65 - 98.
4. Salasche SJ. Epidemiology of actinic keratoses and squamous cell carcinoma. *J Am Acad Dermatol* 2000;**42**:4-7.
5. Dunn GP, Old LJ, Schreiber RD. The three Es of cancer immunoediting. *Annu Rev Immunol* 2004;**22**:329-360.
6. Bui JD, Schreiber RD. Cancer immunosurveillance, immunoediting and inflammation: independent or interdependent processes? *Curr Opin Immunol* 2007;**19**:203-208.
7. Mühleisen B, Schärer L, Dummer R, Burg G, Hofbauer GF. Cancer/testis antigen MAGE-A4 expression pattern differs in epithelial skin tumors of organ-transplant recipients and immunocompetent patients. *J Cutan Pathol* 2007;**34**:1-6.
8. Hadley G, Derry S, Moore RA. Imiquimod for actinic keratosis: systematic review and meta-analysis. *J Invest Dermatol* 2006;**126**:1251-1255.
9. Jorizzo JL. Current and novel treatment options for actinic keratosis. *J Cutan Med Surg* 2004;**8 Suppl 3**:13-21.
10. Berg D, Otley CC. Skin cancer in organ transplant recipients: Epidemiology, pathogenesis, and management. *J Am Acad Dermatol* 2002;**47**:1-17; quiz 18-20.

11. Euvrard S, Kanitakis J, Claudy A. Skin cancers after organ transplantation. *N Engl J Med* 2003;**348**;1681-1691.
12. Gebhardt C, Riehl A, Durchdewald M *et al.* RAGE signaling sustains inflammation and promotes tumor development. *J Exp Med* 2008;**205**;275-285.
13. Slaughter DP, Southwick HW, Smejkal W. Field cancerization in oral stratified squamous epithelium; clinical implications of multicentric origin. *Cancer* 1953;**6**;963-968.
14. Braakhuis BJ, Tabor MP, Kummer JA, Leemans CR, Brakenhoff RH. A genetic explanation of Slaughter's concept of field cancerization: evidence and clinical implications. *Cancer Res* 2003;**63**;1727-1730.
15. Jonason AS, Kunala S, Price GJ *et al.* Frequent clones of p53-mutated keratinocytes in normal human skin. *Proc Natl Acad Sci U S A* 1996;**93**;14025-14029.
16. Dang C, Koehler A, Forschner T *et al.* E6/E7 expression of human papillomavirus types in cutaneous squamous cell dysplasia and carcinoma in immunosuppressed organ transplant recipients. *Br J Dermatol* 2006;**155**;129-136.
17. Guenther ST, Hurwitz RM, Buckel LJ, Gray HR. Cutaneous squamous cell carcinomas consistently show histologic evidence of in situ changes: a clinicopathologic correlation. *J Am Acad Dermatol* 1999;**41**;443-448.
18. LeBoit PE, Burg G, Weedon D, Sarasain A. World Health Organization Classification of Tumours. Pathology and Genetics of Skin Tumours. Lyon: IARC Press, 2006;30-32.
19. Cordell J, Falini B, Erber WN, Ghosh AK, Abdulaziz Z. Immunoenzymatic labeling of monoclonal antibodies using immune complexes of alkaline

phosphatase and monoclonal anti-alkaline phosphatase (APAAP complexes).  
*J Histochem Cytochem* 1984.

20. Klampfer L. The role of signal transducers and activators of transcription in colon cancer. *Front Biosci* 2008;**13**;2888-2899.
21. Yoshimura A. Signal transduction of inflammatory cytokines and tumor development. *Cancer Sci* 2006;**97**;439-447.
22. Jurecka W, Neumann RA, Knobler RM. Porokeratoses: immunohistochemical, light and electron microscopic evaluation. *J Am Acad Dermatol* 1991;**24**;96-101.
23. Mozos E, Mendez A, Martin MP, Herraes P, Perez J. Ovine cutaneous squamous cell carcinoma: immunohistochemical expression of CD3, CD4, CD8 and MHC class II antigens in the associated inflammatory infiltrate. *Vet Immunol Immunopathol* 1998;**61**;221-228.
24. Hoyo E, Kanitakis J, Euvrard S, Thivolet J. Proliferation characteristics of cutaneous squamous cell carcinomas developing in organ graft recipients. Comparison with squamous cell carcinomas of nonimmunocompromised hosts by counting argyrophilic proteins associated with nucleolar organizer regions. *Arch Dermatol* 1993;**129**;324-327.
25. Smith KJ, Hamza S, Skelton H. Histologic features in primary cutaneous squamous cell carcinomas in immunocompromised patients focusing on organ transplant patients. *Dermatol Surg* 2004;**30**;634-641.
26. Smith KJ, Hamza S, Skelton H. Topical imidazoquinoline therapy of cutaneous squamous cell carcinoma polarizes lymphoid and monocyte/macrophage populations to a Th1 and M1 cytokine pattern. *Clin Exp Dermatol* 2004;**29**;505-512.

27. Zou W. Regulatory T cells, tumour immunity and immunotherapy. *Nat Rev Immunol* 2006;**6**;295-307.
28. Betts GJ, Clarke SL, Richards HE, Godkin AJ, Gallimore AM. Regulating the immune response to tumours. *Adv Drug Deliv Rev* 2006;**58**;948-961.
29. Kawai M, Kitade H, Mathieu C, Waer M, Pirenne J. Inhibitory and stimulatory effects of cyclosporine A on the development of regulatory T cells in vivo. *Transplantation* 2005;**79**;1073-1077.
30. Coenen JJ, Koenen HJ, van Rijssen E *et al.* Rapamycin, not cyclosporine, permits thymic generation and peripheral preservation of CD4(+)CD25(+)FoxP3(+) T cells. *Bone Marrow Transplant* 2007.
31. Noris M, Casiraghi F, Todeschini M *et al.* Regulatory T cells and T cell depletion: role of immunosuppressive drugs. *J Am Soc Nephrol* 2007;**18**;1007-1018.
32. Carucci JA. The Immune Microenvironment Associated with SCC. *International Transplant Skin Cancer Collaborative Annual Meeting*. San Antonio, TX, 2008.
33. Nelson MA, Einspahr JG, Alberts DS *et al.* Analysis of the p53 gene in human precancerous actinic keratosis lesions and squamous cell cancers. *Cancer Lett* 1994;**85**;23-29.

## Figure legends

### Figure 1

(a) Exemplary characterization of perineoplastic inflammatory infiltrate in intraepithelial (upper line) and invasive (bottom line) squamous cell carcinoma of the skin. Infiltrate was assessed for its maximum depth in millimetres (left hand side) and density. (b) Exemplary characterization for low and high density of CD8, FOXP3, CD123, STAT1 (from left to right) immunoreactivity at low power on top and an insert at high power below.

### Figure 2

Depth of perineoplastic inflammatory infiltrate increases from intraepithelial to invasive squamous cell carcinoma of the skin. Maximum depth of perineoplastic inflammatory infiltrate is higher in invasive squamous cell carcinoma than in intraepithelial lesions ( $p < 0.005$ ) and similar between immunocompetent patients (closed boxplot) and organ transplant recipients (OTR, open box plot) ( $p = 0.22$ ). Upper and lower limits of box represent upper and lower quartiles. Median is represented by line within the box.

### Figure 3

Density of perineoplastic inflammatory infiltrate increases from intraepithelial to invasive squamous cell carcinoma of the skin ( $p = 0.005$ ). Percentages of samples with dense perineoplastic inflammatory infiltrate in intraepithelial lesions and invasive squamous cell carcinoma (SCC) in immunocompetent patients as closed bars and organ transplant recipients (OTR) as open bars are shown.

#### **Figure 4**

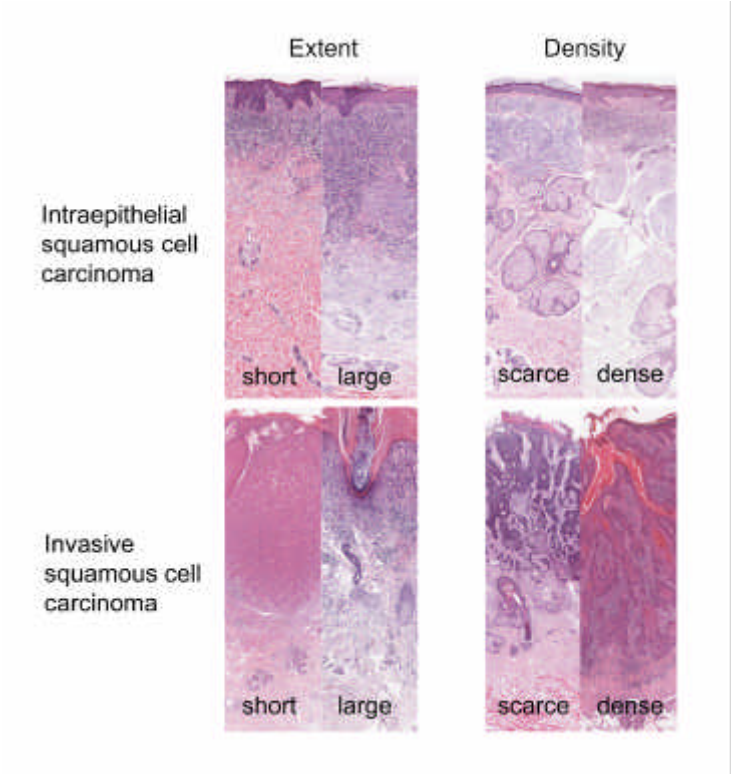
Immunosuppression results in reduced density of inflammatory infiltrate surrounding squamous cell carcinoma of the skin ( $p = 0.041$ ). Percentages of samples with dense perineoplastic inflammatory infiltrate in immunocompetent patients (closed bar,  $n = 86$ ) and in organ transplant recipients (OTR; open bar,  $n = 84$ ) are shown.

#### **Figure 5**

Proportions of CD3+ (a), CD4+ (b), CD8+ (c) and FOXP3+ (d) cells do not change significantly in perineoplastic infiltrate in the progression from intraepithelial to invasive squamous cell carcinoma of the skin. Immunosuppression reduces CD3+ (a) and CD8+ (c) cells within perineoplastic inflammatory infiltrate, particularly in intraepithelial squamous cell carcinoma of the skin. FOXP3+ regulatory cells (d) are decreased perineoplasticly in invasive squamous cell carcinoma of the skin under immunosuppression. CD123+ cells (e) increase significantly with invasiveness of SCC in immunocompetent patients and are significantly different between immunocompetent patients and organ transplant recipients. STAT1+ cells (f) are significantly increased in invasive SCC of organ transplant recipients compared to invasive SCC of immunocompetent patients. Top half immunocompetent patients' neoplasias (Im.compt., closed bars), bottom half organ transplant recipients' neoplasias (OTR, open bars); i.ep. intraepithelial, inv. Invasive SCC.

**Figure 1**

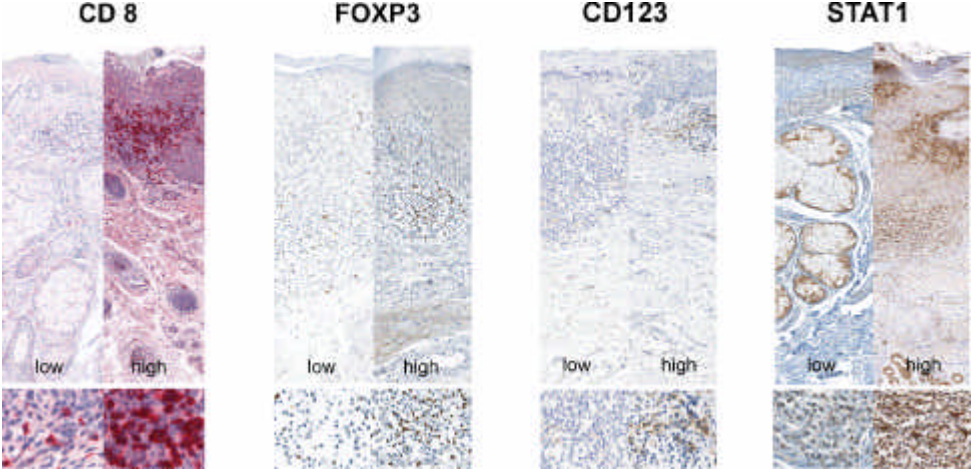
(a) Exemplary characterization of perineoplastic inflammatory infiltrate in intraepithelial (upper line) and invasive (bottom line) squamous cell carcinoma of the skin. Infiltrate was assessed for its maximum depth in millimetres (left hand side) and density (right hand side).





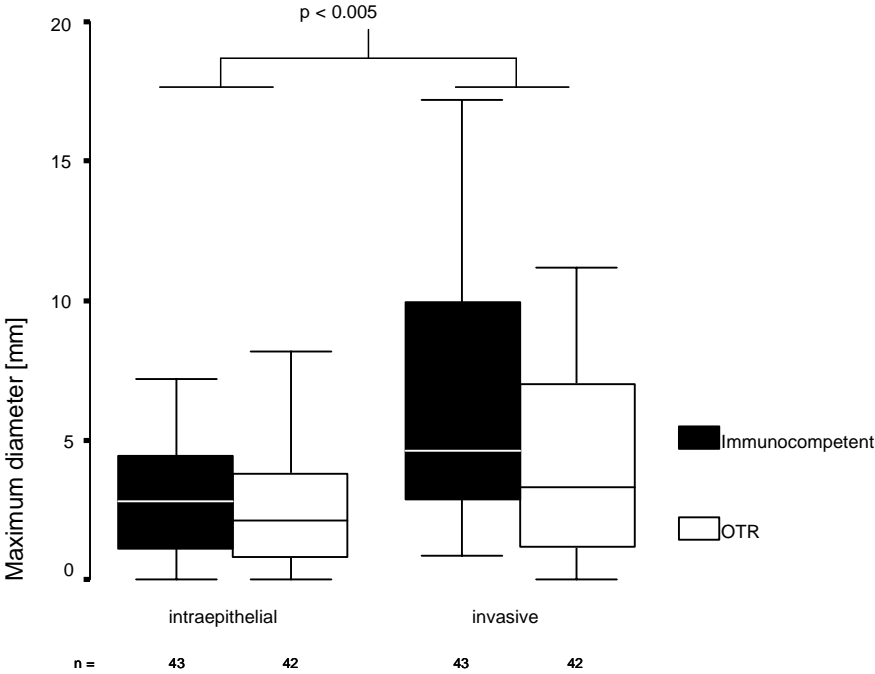
**Figure 1 (b)**

Exemplary characterization for low and high density of CD8, FOXP3, CD123, STAT1 (from left to right) immunoreactivity at low power on top and an insert at high power below.



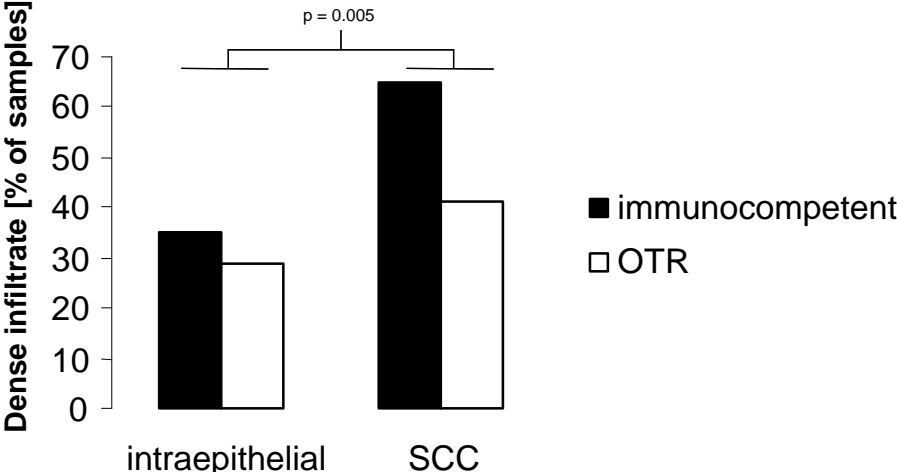
**Figure 2**

Depth of perineoplastic inflammatory infiltrate increases from intraepithelial to invasive squamous cell carcinoma of the skin.



**Figure 3**

Density of perineoplastic inflammatory infiltrate increases from intraepithelial to invasive squamous cell carcinoma of the skin.



**Figure 4**

Immunosuppression results in reduced density of inflammatory infiltrate surrounding squamous cell carcinoma of the skin.

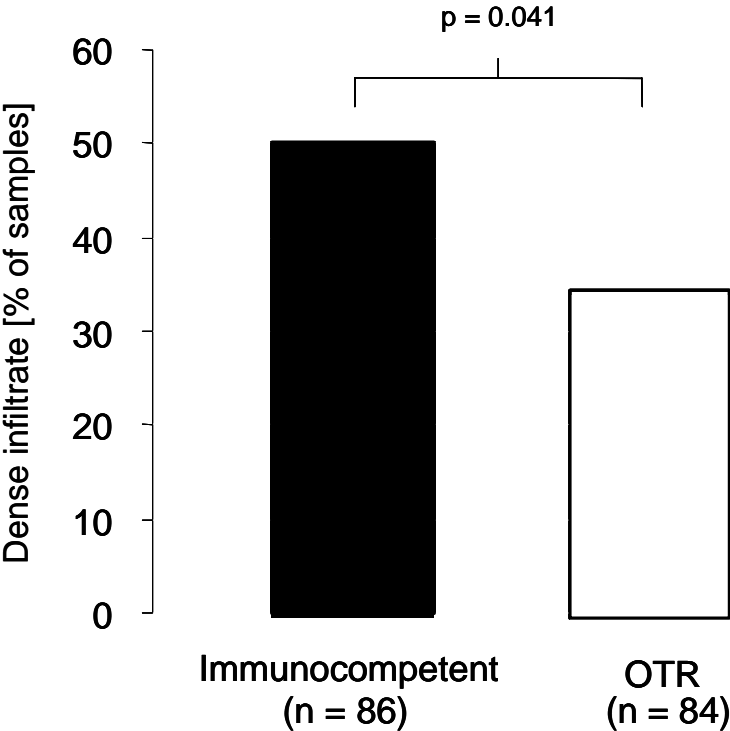
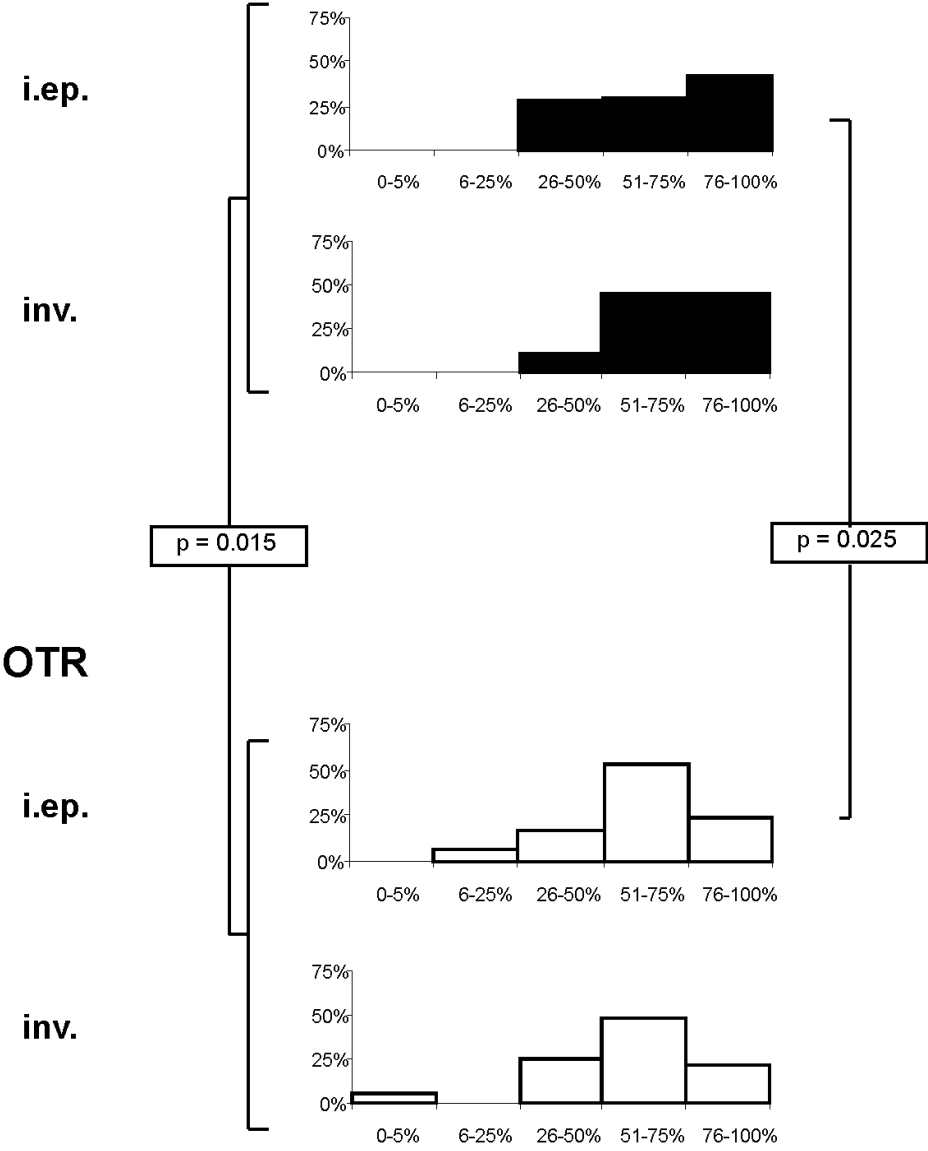


Figure 5

(a)  
Im.compt.

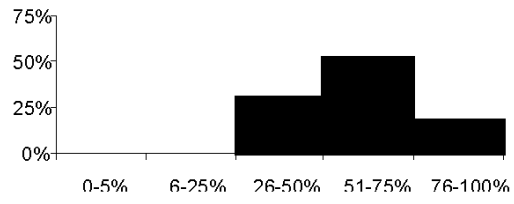
### CD3



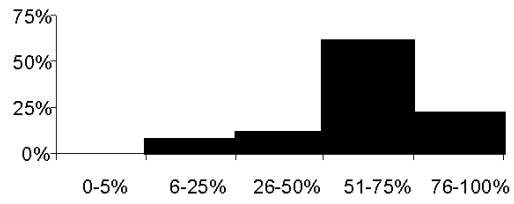
<sup>(b)</sup>  
**Im.compt.**

## CD4

**i.ep.**

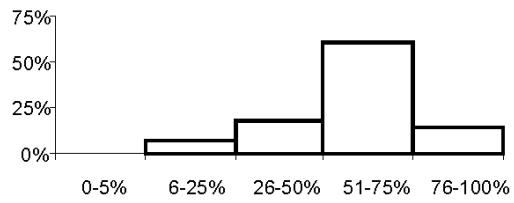


**inv.**

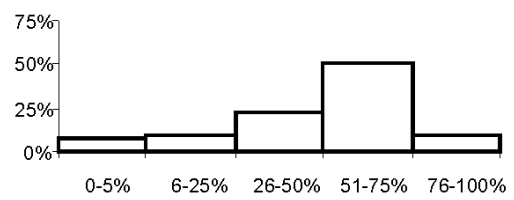


## OTR

**i.ep.**

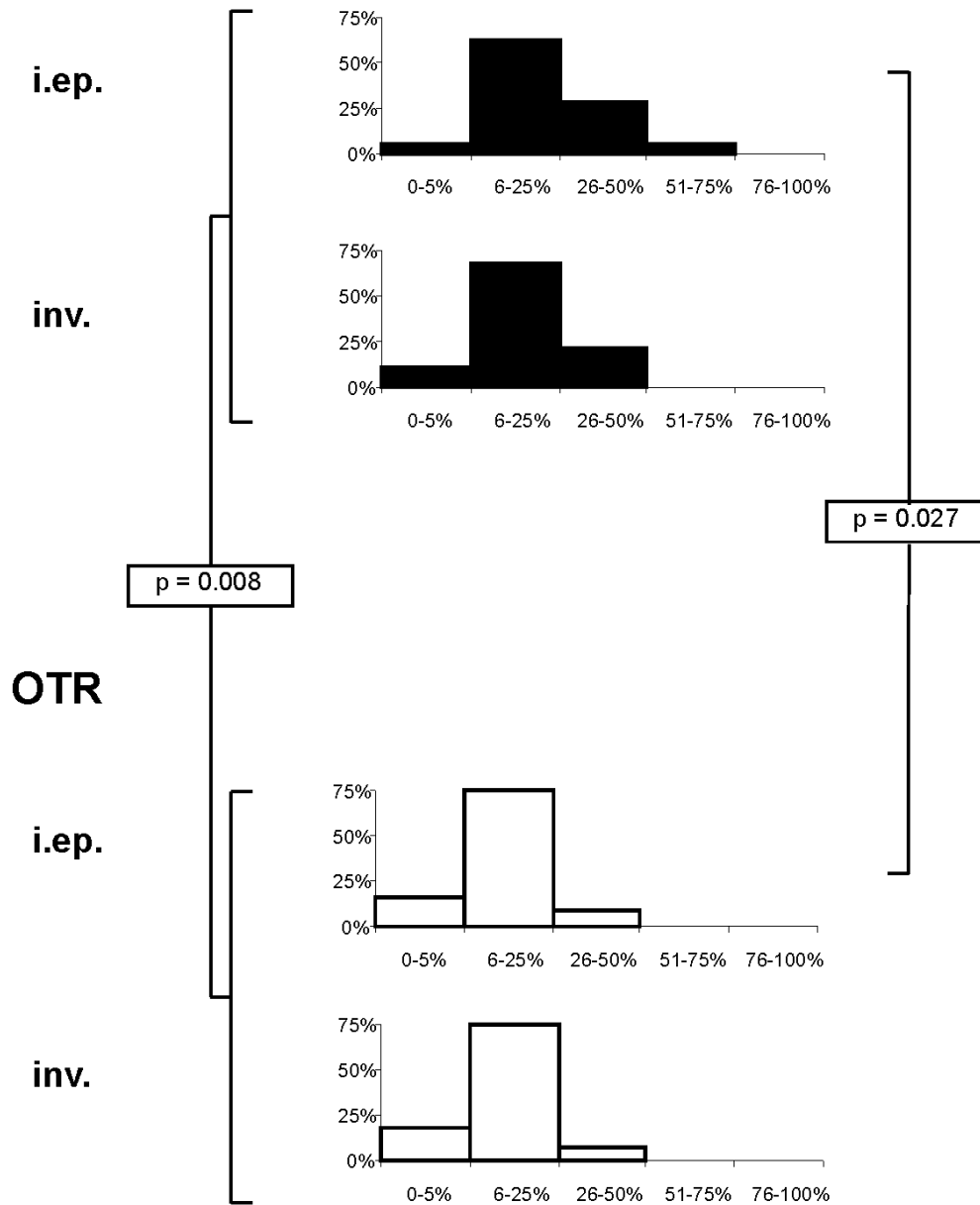


**inv.**



<sup>(c)</sup>  
**Im.compt.**

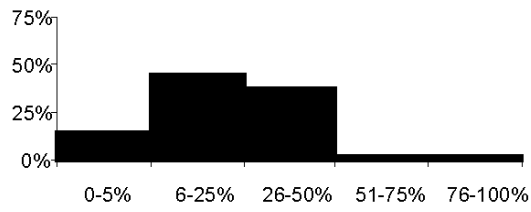
# CD8



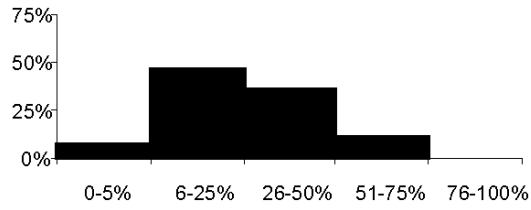
<sup>(d)</sup>  
**Im.compt.**

# FOXP3

**i.ep.**



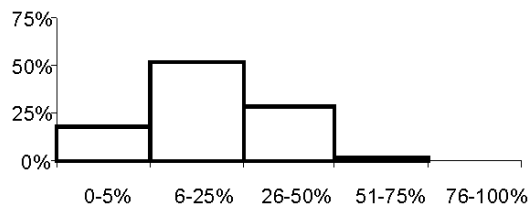
**inv.**



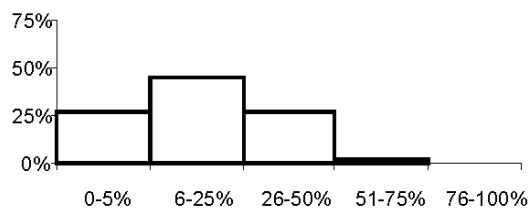
p = 0.048

**OTR**

**i.ep.**



**inv.**

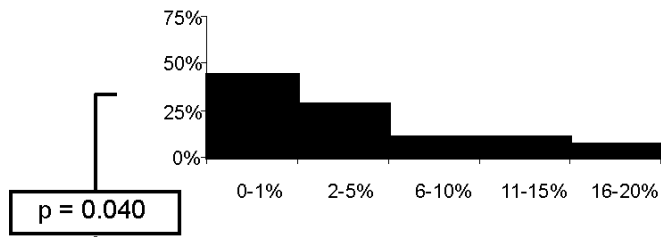




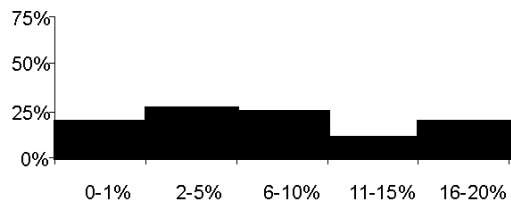
<sup>(e)</sup>  
Im.compt.

# CD123

i.ep.



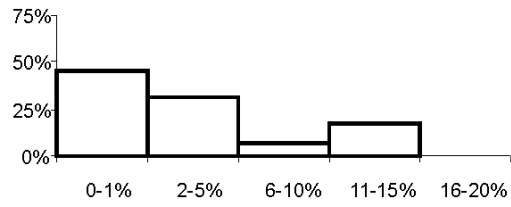
inv.



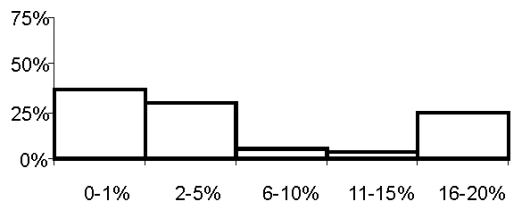
p = 0.036

OTR

i.ep.



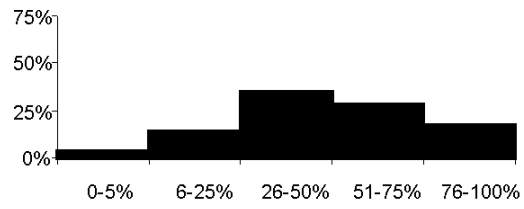
inv.



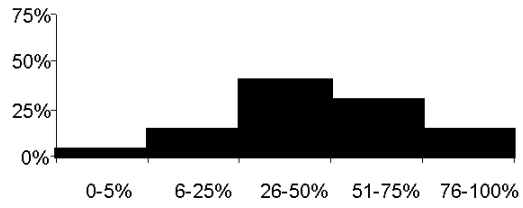
<sup>(f)</sup> Im.compt.

# STAT1

**i.ep.**



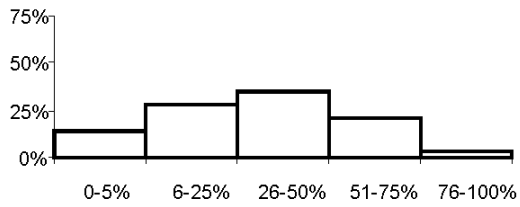
**inv.**



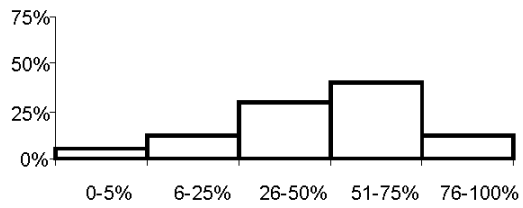
p = 0.032

**OTR**

**i.ep.**



**inv.**



## **Table legends**

### **Table 1**

Patient characteristics.

**Table 1**

## Patient characteristics

	Immunocompetent	OTR	Total
Patients	43	42*	85
Gender:			
male [n (%)]	32 (74)	36 (86)	68 (80)
Age [mean ( $\pm$ SD)]	66.0 ( $\pm$ 0.74)	61.3 ( $\pm$ 1.07)	63.7 ( $\pm$ 0.67)
Age [Min / Max]	47.6 / 81.5	36.1 / 79.5	36.1 / 81.5
Diagnoses [n (%)]			
- Actinic keratosis	34 (40)	29 (34)	63 (37)
- Bowen's disease	9 (11)	13 (16)	22 (13)
- Squamous cell carcinoma	43 (50)	42 (50)	85 (50)

\* kidney: 32, heart: 6, lung: 4