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CONCISE COMMUNICATION

Alterations in the inflammatory cells infiltrating basal cell carcinomas during immunocryosurgery

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Abstract

Immunocryosurgery, the combination modality of a cryosurgery session at day 14 of a 5-week daily imiquimod treatment cycle, has shown remarkable efficacy in the treatment of basal cell carcinoma (BCC). The modality was designed to exploit synergy of antitumor effects, including the induction of immune responses, elicited by imiquimod and cryosurgery. Herein, we report on the infiltration of the BCC by selected inflammatory cell species during an immunocryosurgery treatment cycle. The density of tissue infiltrating $CD68^+$, $CD3^+$ and $Foxp3^+$ cells was studied by immunohistochemistry in 56 BCC biopsies from 28 treated sites (26 patients) at baseline and at days 12, 16 or 28 during treatment. Immunocryosurgery induces statistically significant alterations in all three cell species ($p < 0.003$): The density of $CD68^+$ increased already by day 12 and remained at a higher level during the treatment thereafter. The density of $CD3^+$ cells increased significantly between days 12 and 16 of treatment. The density of T_{reg} ($Foxp3^+$) cells increased in the early phase of treatment (highest at day 12) to decrease significantly already 2 days after the cryosurgery session (day 16) and thereafter up to day 28 of the treatment cycle ($p = 0.033$). Within the tumor tissue, these alterations result in an abrupt increase in the $CD3^+/Foxp3^+$ ratio, a finding suggesting that the cryosurgical perturbation may probably play a decisive modulating role in the cellular composition of the inflammatory infiltrate during immunocryosurgery, eventually heralding the induction of an effective tumor-destructing immune response.

Keywords Basal cell carcinoma · Immunocryosurgery · Imiquimod · Cryosurgery · Tumor-infiltrating inflammatory cells

Introduction

Cryoablation of tumor tissue in animal models in vivo is followed by a massive release of functional tumor antigens and their detection in considerable quantities in the afferent lymph node stations [1]. In the clinical setting, however, specific immune responses after tumor cryoablation are usually of unpredictable intensity and in general too weak to attain clinical relevance [2]. It has been anticipated that the immune response could be augmented if cryoablation is combined with immunotherapies that target antitumor immunity, and a number of studies combining immunomodulation with cryosurgery have shown antineoplastic synergy against target as well as remote tumor foci [3, 4]. Immunocryosurgery was also designed to explore synergy in the antitumor immune responses elicited by the Toll-like receptor agonist imiquimod and cryosurgery [5]. This distinct combination modality is applied in five-week treatment cycles of once-daily topical imiquimod 5% cream on the skin lesion and a session of a rather mild cryosurgery at the end of the second treatment week [6]. The modality has shown remarkable efficacy particularly in the treatment of basal cell carcinoma (BCC) [7, 8]. Induction of vivid local inflammation, sometimes accompanied by mild systemic 'flu-like' symptoms, is a hallmark side effect of this modality and a finding that seems to link to a more favorable treatment outcome. The fact that inefficient boosting of this reaction, for

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example in immunocompromised patients, correlates with reduced efficacy of the modality to control BCC growth (own unpublished data) further underlines the pivotal antineoplastic role of the observed peritumoral inflammatory exaggeration during treatment.

Herein, we present preliminary immunohistochemical findings of the density alterations in the numbers of selected inflammatory cell subpopulations that infiltrate the BCC tissue during a therapeutic immunocryosurgery treatment cycle.

Materials and methods

Patients and treatment

The study was approved by the Research and Ethics Committee of the University Hospital of Ioannina according to the 1964 Helsinki Declaration principles [approval no. 4/8-4-2009 (θ18)]. Immunocompetent patients with biopsy-proven nodular BCC, informed about treatment with immunocryosurgery as an alternative to surgical excision of their tumors, were enrolled in this study. The inclusion criteria were: adults (> 18 years), tumor size > 1 cm maximal diameter, histological type: nodular (ulcerative). Patients with known imiquimod or cryosurgery intolerance were excluded.

Immunocryosurgery treatment consisted of all-night application of imiquimod on the site of the tumor and a surrounding skin zone of approximately 0.5 cm beyond the macroscopic tumor margins. Two weeks after treatment onset, a session of cryosurgery (liquid N_2, open spray, two freeze–thaw cycles of 20 s freezing time each) was applied to the skin area enclosing the tumor and a 0.5 cm skin rim around it. Once daily imiquimod was continued for additional 3 weeks (a total of 5 weeks) and post-treatment care with fusidic acid cream and strict sun protection was advised (for treatment details see: [9]).

Study design

A baseline biopsy with the diagnosis of BCC was prerequisite for inclusion. Pretreatment tissue probes were available for all treated tumors. At the day of treatment initiation, patients were randomly assigned to one of the three study groups, according to the day of the second biopsy of the treated lesion during treatment: Group 1 was scheduled for a 2nd biopsy at day 12 (late 2nd week of treatment, 2 days prior to cryosurgery), Group 2 (3rd week of treatment, 2 days after cryosurgery) and finally Group 3 at the end of the 4th week of treatment (day 28 of treatment). For randomization, the method of a fixed three-class list of random numbers was applied. After treatment, a scheduled follow-up scheme (1, 3 and 6 months after treatment and every 6 months thereafter) was recommended for all the patients. At the first visit, the tumor size was measured, and standardized photo-documentation was performed at baseline and during the protocol and follow-up visits.

Tissue processing: immunohistochemistry

Biopsies were performed with a 3 mm punch under local anesthesia (Xylocaine 2%). Tissue probes were fixed in buffered 10% formalin, dehydrated in graded concentrations of ethanol, xylole and finally embedded in paraffin. Serial tissue slices 3 μm thick were prepared and floated onto glass slides. A hemotoxylin- and eosin-stained section was obtained from each tissue block.

Immunohistochemical staining according to the standard technical procedure was carried out for the following antigens: CD3 for T lymphocytes (CELLMARK, 1:150), CD68 for macrophages (DAKO, 1:500) and forkhead box P3 [Foxp3] for regulatory T cells, T_{reg} (ABCAM, 1:100).

Histopathological evaluation

After consensus revision of all histopathological sections by two physicians experienced in skin histopathology, a pathologist (EL) conducted standardized photography of representative tissue areas at magnification × 200, employing an image analysis system composed of the Olympus BX43 upright microscope and digital camera Olympus Cam-SC30 supported by the analySISH software. For inclusion of a section for the analysis, inflammatory cellular elements and stroma cells should be easily identifiable (even if scarce) in the vicinity of tumor nests (a representative part of BCC tissue or tissue formations indicative of past nests should be included in each photo) and the whole picture ought to have been taken from a coherent tissue section (excluding empty artefacts). Only biopsies for which at least 3 (3–5) non-overlapping such screens were available were included in further analysis.

Image analysis (color segmentation)

Histopathological images were processed, and stained cells were automatically identified using a color clustering image analysis method. More specifically, a two-level clustering approach was implemented. At the first level, a binary tree quantization algorithm has been used to extract cell structures in the RGB color space [10]. At the second level, the *k*-means clustering algorithm was applied to extract the target stained cells further [11]. Cell images were converted to YIQ colorspace and the I, Q color components have been used to cluster image pixels. An illustrative example is given in Online resource 1 (Online resource 1, ESM_1; Fig. S1). Results are displayed as pixel numbers/histopathological

image, which is proportional to the numerical concentration of the corresponding cell species in the tissue.

Statistical analysis

Average pixel counts for the different cell species per biopsy were calculated as well as ratios of counts of two cell species and were compared with the Kruskal–Wallis test with Dunn's test for post hoc pairwise comparisons employing SPSS soft ware at significance level $p < 0.05$.

Results

Twenty-six patients (10 males, 16 females; median age 78.5 years, range 58–91 years) with 28 biopsy-diagnosed BCC (median maximal diameter 1.2 cm; range 0.8–2.5 cm) were enrolled (Online resource 2, ESM_2, Table). Most tumors (26/28) were located on the head and neck, and almost half of them (13/28) were of the 'ulcerative' type. All lesions responded completely to the treatment, and the tumor sites had healed and were clinically (including dermoscopy) tumor free at 3-month follow-up examination (clinical treatment examples in Online resource 1, ESM_1; Fig. S2).

A total of 56 tissue samples were available for evaluation: 28 baseline samples (all tumors), ten samples from day 12, ten from day 16 and eight from day 28 of the immunocryosurgery treatment cycle. The material of two BCC collected at day 28 was non-evaluable and was excluded from further analysis.

In the BCC sites studied during immunocryosurgery, statistically significant alterations in the tissue concentration of core immunologically relevant cell species ($CD68^+$, $CD3^+$, $Foxp3^+$; all $p < 0.003$) were recorded as a function of time (Figs. 1, 2). Already at day 12 of treatment, as compared to baseline, the tissue concentration of $CD68^+$ cells (Fig. 1a, 2a) increased significantly and remained at similarly higher levels at the two subsequent time points (days 16 and 28 of the treatment cycle; for all selection points: $p < 0.022$ compared to baseline). The degree of BCC tissue infiltration by $CD3^+$ cells (Fig. 1b, 2b) remained initially rather stable during treatment to significantly increase abruptly 2 days after the cryosurgery session (day 16; $p < 0.021$ for the comparison of day 16 level to baseline and day 12 of treatment). On the contrary, the infiltration by $Foxp3^+$ cells increased significantly early during treatment (Fig. 1c, 2c; $p = 0.002$ for the comparison of day 12 to baseline) to return thereafter progressively to baseline levels ($p = 0.006$ for the comparison of days 12 and 28 of the treatment cycle).

Notably, the ratio of $CD3^+$ to $Foxp3^+$ cells (r) that infiltrate the BCC varied significantly during treatment ($p = 0.033$; Fig. 3). Initially, this ratio tended to decrease from the baseline value (median) from $r = 16.2$ to $r = 15.4$ at day 12 of treatment. However, it abruptly increased by almost 50% from $r = 15.4$ to $r = 22.0$ between days 12 and 16 of treatment and remained thereafter at significantly higher levels up to the end of the observation period ($r = 22.8$ at day 28).

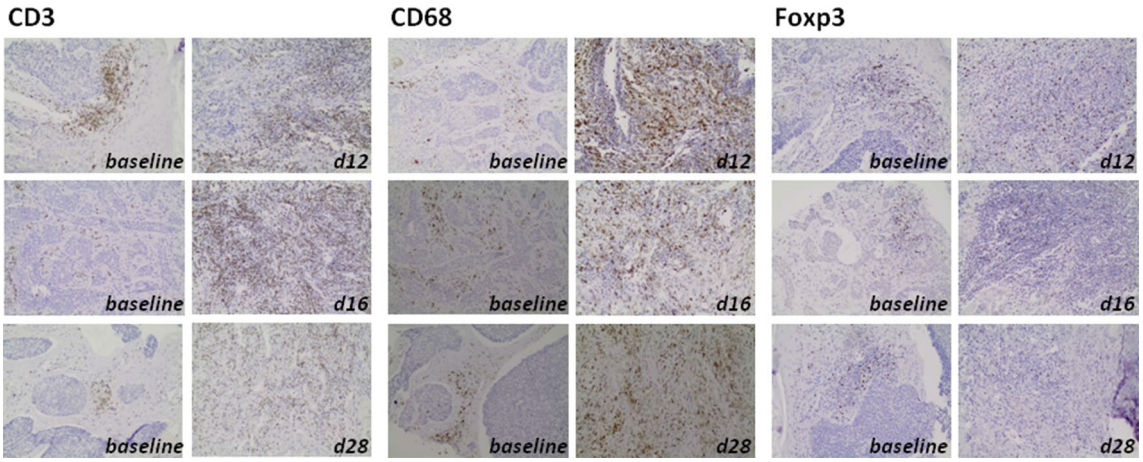

Fig. 1 Representative immunohistological sections of basal cell carcinomas during immunocryosurgery (original magnification x200) demonstrating tissue infiltration by $CD3^+$ cells (left pairs of columns), $CD68^+$ cells (middle pairs of columns) and $Foxp3^+$ cells (right pairs of columns). Within each panel, horizontal dyads of pictures represent biopsies at different time points during the treatment of the same tumor: the pictures at left correspond to baseline and at right to tissue biopsied at the indicated days after treatment onset (d12, d16 and d28 corresponding to 12th, 16th and 28th days of a 35-day immunocryosurgery treatment cycle)

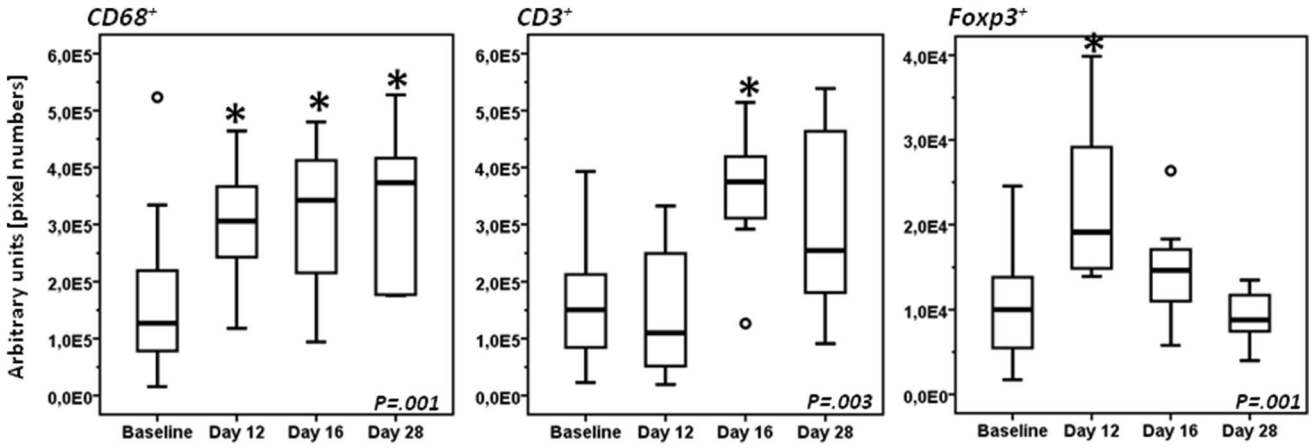

Fig. 2 Boxplot presentation of the infiltration levels (arbitrary pixel number units) of $CD68^+$ (left), $CD3^+$ (middle) and $Foxp3^+$ (right) cells into the BCC tissue site as a function of the duration of immunocryosurgery treatment cycle

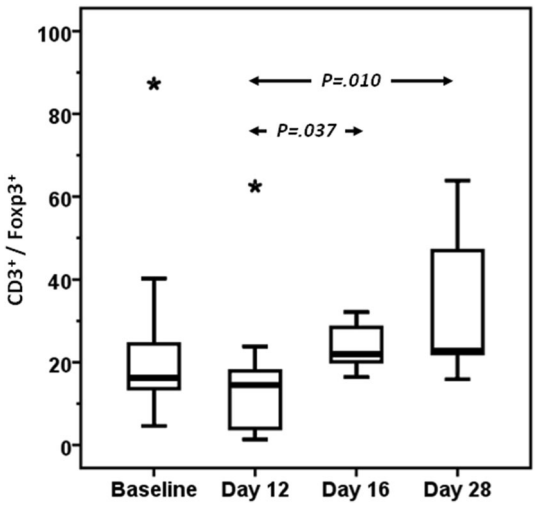

Fig. 3 Boxplot presentation of the ratio of $CD3^+$ to $Foxp3^+$cell densities as a function of time during an immunocryosurgery treatment cycle

Discussion

The tissue concentration of T_{reg} cells in the treated BCC increased significantly during the early phase of immunocryosurgery, i.e., during the monomodal imiquimod application and prior to the cryosurgery session. T_{reg} cells are typically found in large numbers within the unperturbed BCC tissue [12, 13], particularly within peritumoral inflammatory cell foci. It is suggested that they mediate a Th2-skewed immune suppressive tissue milieu permissive for cancer growth [13–15]. The application of imiquimod, a potent TLR7 agonist, to BCC is anticipated to cause a shift toward an immunologically Th1-dominated tumor tissue environment [16] which, characterized by a reduction in the density of the tumor-infiltrating T_{reg} population [17], heralds the inhibition of tumor growth [18, 19]. Imiquimod is currently approved for the topical treatment of non-invasive superficial BCC. However, treatment outcome with this substance is quite unpredictable when monomodal imiquimod is applied to thicker and more invasive BCC [20]. It has been hypothesized that the loss of treatment effectiveness in larger tumors may result from a counteracting homeostatic immune suppressive effect of TLR agonists that runs in parallel to their well-known immune stimulating action [21]. This immunosuppressive effect seems to be mediated by an induction of T_{reg} cells activities both in human [22] and in murine tumors [23, 24]. Our present finding of increased concentration of $Foxp3^+$ cells infiltrating the tumor tissue after 12 daily imiquimod applications is in accordance with these later observations, suggesting the stimulation of a rather tumor tolerance tissue milieu early during the immunocryosurgery treatment of nodular BCC.

Concerning cryosurgery, our findings underscore its decisive immunomodulatory role within the context of immunocryosurgery for BCC. The cryosurgery session after a period of imiquimod pretreatment ('cryosurgery during imiquimod application' [25, 26]) seems to reverse the local immunological balance in favor of a tumor-destructing proinflammatory immune response. We described significant alterations in the composition of inflammatory cell species that infiltrate the tumor site between the 12th and 16th day of the treatment cycle, i.e., 2 days after compared to 2 days before the cryosurgery session. There is an influx of T cells in parallel to the decline of T_{reg} tissue concentration corresponding to a shift to a proinflammatory tissue milieu. Li et al. [27] also found that the antitumor immune response evoked by cryosurgery was mediated by a T_{reg} decrease and

Waitz et al. [28] reported a significant increase in the ratio of intra-tumoral T_{eff}: T_{reg} cells after a combination of cryosurgery with anti-CTLA-4 therapy. It is worth noting that cryosurgery of 'moderate' intensity, typical for immunocryosurgery, seems to be more effective than 'intense' session in inducing antitumor immune responses [29].

In conclusion, we present tissue level evidence highlighting the key role of the cryosurgery session (day 14th of the treatment cycle) for the induction of an effective antitumor immune response in the course of an immunocryosurgery treatment cycle (Fig. S3; Online resource 1, ESM_1). Future studies should expand these findings with the inclusion of further cell species and correlative investigations of cytokine levels locally in the tissue and in the blood.

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Compliance with ethical standards

Conflict of interest Author IDB has received honoraria for speaking at symposia from Companies (Roche Hellas, Meda Hellas) and financial support for attending symposia relevant to paper's topic (Meda Hellas, Leo Hellas). All other authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee (University Hospital of Ioannina Research and Ethics Committee; approval no. 4/8-4-2009 [θ18]) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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