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ORIGINAL ARTICLE

Oral Squamous Carcinoma Cells Express B7-H1 and B7-DC Receptors in Vivo

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Abstract B7-H1 and B7-DC ligands are members of the B7 family with important regulatory functions in cell-mediated immune response. Both receptors are ligands of the programmed death receptor PD-1. B7-H1 expression has been detected in the majority of human carcinomas in vivo. B7-H1 mediated signals are able to negatively regulate activated T cell functions and survival, and enable tumor cells to overcome host response. The aim of this study was to investigate the expression of B7-H1 and B7-DC proteins in oral squamous cell carcinomas (OSCC) in vivo. Tissues from 15 samples were cryo-sected and following histological routine staining (HE), incubated with antibodies against human B7-H1 and B7-DC. Immuno-staining of pan-cytokeratin was performed to ascertain the epithelial origin of the tissue and CK 19 to demonstrate the proliferating stage. Confocal laser scanning microscopy confirmed the presence of both B7-H1 and B7-DC in all 15 OSCC. The B7-H1 and B7-DC staining was located in areas of the tissue that were identified as cancerous lesions in the previously stained HE sections before. Staining with Pan-CK and CK19 provided evidence for the epithelial origin and the proliferating stage of the tissue. The in vivo expression of the B7-H1 and B7-DC receptors in oral squamous cell carcinomas suggest that general mechanisms for immune evasion of tumors are also found in OSCC.

Keywords Oral squamous cell carcinomas · B7-H1 · B7-Dc · In vivo · Immune evasion

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Introduction

B7-H1 (also called PD-L1) is a member of the B7 family with important regulatory functions in cell-mediated immune response [1, 2]. B7-H1 ligands are constitutively expressed on APCs such as macrophages and dendritic cells (DCs), and are induced on activated T cells, B cells, endothelial cells and epithelial cells [2–5]. The counter-receptor of B7-H1 is the programmed death-1 (PD-1) receptor, a CD28/CTLA-4 like molecule expressed on activated T cells, B cells, monocytes and macrophages which belongs to the immunoglobulin (IG) superfamily [2, 6]. B7-H1 mediated signals play a critical role in co-signaling the regulation of T cell activation and tolerance [7]. B7-H1 signals are also able to negatively regulate activated T cell functions and survival [2, 8, 9]. Modulation of immune responses in tumor sites is a critical mechanism attributed to tumor immune evasion. Soluble factors and membrane-bound molecules have been found to be up-regulated in tumor sites, which potentially inhibit immune responses [10, 11]. Data suggest that the PD-1-PD-L1 (B7-H1) pathway regulates the organ-specific tolerance in normal tissue and may contribute to immune evasion by cancer cells [2, 12, 13]. Interactions between PD-L1 and PD-1 in the tumor microenvironment protect the tumor through several distinct pathways including ligation of PD-1 by PD-L1 on antigen specific T cells leading to functional anergy and/or apoptosis of these effector T cells, possible promotion of tolerance by

ligation of PD-1 by PD-L1 and direct protection of tumor from apoptosis by reverse signaling through PD-L1 [8, 14–16]. The role of the PD-1: PD-L1 pathway in squamous cell carcinomas of the head and neck is reviewed in Zandberg and Strome, 2014, who concluded that antibodies and fusion proteins, capable of blocking PD-L1: PD-1 interactions have tremendous promise in phase treatment trials for advanced solid tumors [17]. Currently a number of clinical trials for PD-1 or PD-L1 blockade is ongoing (reviewed in Ritprajak and Azuma, 2015) [18]. PD-L1 blockade by a mAb efficiently augmented the effects of adaptive T cell immunotherapy in a murine model of PD-L1-transfected SCC and inhibited the growth of de novo induced PD-L1positive SCC [19, 20]. These results suggested the potential utility of PD-L1 blockade therapy in clinical situations. In esophageal SCC, the PD-L1 and PD-L2 expressions were closely correlated and it was demonstrated that PD-L1 and PD-L2 positive patients experienced significantly poorer prognosis than those who expressed neither form of PD-L, but no significant correlation between PD-L1 expression and the number of tumor- infiltrating lymphocytes was evident [21]. The up-regulation of B7-H1 in host cells may contribute to the chronicity of inflammatory disorders that frequently precede the development of human cancers [22]. In cells originating from cancers of lung, ovary, colon, skin, brain, kidney, esophagus, stomach and breast, expression of B7-H1 was up-regulated [8, 20, 21, 23–26]. These cancers are accompanied by chronic inflammation. Oral cancers belong to the ten most common neoplasms [27]. Besides tobacco and alcohol, further risk factors like infections and poor oral hygiene seem to be important [27–30]. B7-H1 ligands mediate co-stimulatory signals that can lead to anergy and apoptosis of activated T cells. This in turn may enable tumors to evade the immune response [8, 9, 31]. It has been demonstrated that the blockade of B7-H1 or B7-DC induces an anti-tumor effect in a mouse pancreatic cancer model [32]. Das et al. (2006) reported a high level of B7-H1 expression on gastric epithelial cells during chronic *Helicobacter pylori (H. pylori)* infection [33]. Periodontal infections are one of the most common bacterial infections. In previous studies by Tezal et al. (2005, 2007) a significant association between periodontitis and oral neoplasms was reported [34, 35]. The aim of this study was to investigate, if expression of B7-H1 and B7-DC is present in oral squamous cell carcinomas that possibly provides a tumor protective mechanism for immune evasion or suppression.

Materials and Methods

Tissue

Tissues from 15 patients with oral squamous cell carcinomas (OSCC) were investigated. The samples were harvested while the patients underwent diagnostic or therapeutic surgery. After surgical excision the tissue was embedded in TissueTec (Sakura Finetek, Staufen, Germany) embedding medium and stored frozen at −20 °C. For the immunofluorescence analysis the samples were sectioned using a cryo-microtome (thickness 4–8 μm) and adhered to Superfrost® slides (Menzel GmbH, Braunschweig, Germany). After overnight drying, sections were fixed in 4 °C cold 90 % methanol (Merck, Darmstadt, Germany) for 30 min. Before immuno-staining the kryosections were washed three times with phosphate-buffered saline (PBS) (Invitrogen, Darmstadt, Germany) for 5 min.

Haematoxylin Eosin (HE) Staining

The sections were washed in aqua dest. for 5 min. The cell nuclei were stained for 10 min in Mayer's acid-haematoxylin solution (Merck, Darmstadt, Germany). The staining was stabilized by incubation in running water (because of its slight alkaline pH) for 10 min. The sections were washed 1 min in aqua dest. and counter-stained with1% eosin (Merck, Darmstadt, Germany) in alcoholic solution for 15 min. After washing 1 min in aqua dest., the sections were dehydrated in alcoholic solutions (2-propanole) (Merck, Darmstadt, Germany) with ascending concentrations (70 %, 80 %, 96 %, 100 %), then they were incubated 2 x in 100 % xylene (Merck, Darmstadt, Germany) and embedded with DePex (VWR, Darmstadt, Germany) under a cover slip. The HE sections were examined at 400 x magnification.

Immunostaining

Cryosections

For the immuno-staining the sections were pre-treated with 10 % goat serum (Gibco, Invitrogen, Darmstadt, Germany) for 1 h at room temperature (RT) to block non-specific antibody-binding. Anti-human-B7-H1 and B7-DC antibodies (murine) (eBioscience, Frankfurt/M, Germany) were used in a dilution of 1:50 and mouse-anti-human cytokeratin (CK) 19 and pan CK (abcam®, Cambridge, UK) were used in a dilution of 1:40. The antibodies were incubated 1 h at RT in a humidified chamber. The CK 19 and pan CK staining was performed to prove the squamous epithelial origin. After washing three times for 10 min in PBS, they were incubated with B7-H1/DC-antibodies and afterwards with biotinylated-anti-mouse IgG (eBioscience, Frankfurt/M, Germany) 1:200 in PBS for 60 min at RT. The tissue incubated with CK antibodies was immuno-stained with fluorescein-iso-thio-cyanat (FITC) labeled goat anti-mouse-Ig (eBioscience, Frankfurt/M, Germany) in a dilution of 1:40 for 1 h at RT in a humidified chamber.

After washing three times for 10 min in PBS, streptavidin-FITC (eBioscience, Frankfurt/M, Germany) was applied at 1:200 for 1 h at RT in a humidified chamber to those samples previously incubated with biotinylated secondary antibody. As negative controls 1. staining was performed using secondary antibodies only, 2. tumor free areas were identified and analyzed in comparison to the tumorous areas. Cell nuclei were counterstained using To-Pro-3 (Invitrogen, Darmstadt, Germany) fluorescent dye 1: 1000 in PBS. The tissue samples then were washed three times for 10 min in PBS and coated with Vectashield (Vector Labs, Burlingame CA, USA) mounting media under a coverslip.

Analysis

The HE stained sections were analyzed in an Olympus AH2 microscope.

The cancerous areas were identified as well as the non-cancerous and marked. These marked areas were analysed after the immuno-staining and the expression of the markers was examined. Immunofluorescence was visualized with the Leica LSM DM LFSA laser scanning confocal imaging system (543 HeNe Laser) (Leica Microsystems, Wetzlar, Germany). Analysis was focused on areas previously identified as cancerous lesions. The intensity of the green staining was analyzed on three different areas using ImageJ and designated as arbitrary units (AU). The localization of the staining was identified as cytoplasmatic and the staining pattern was classified in fine to coarsely granular if the appearance was granular or dense in case the staining was so intense that the cells appeared completely filled with fluorescent material. In two tumor samples within the sections we found tissue areas that could be classified as tumor-free, so these areas were analyzed for their expression of B7-H1 and B7-DC as well to obtain an internal negative control.

Statistical Analysis

The fluorescence intensity of each marker was quantified in arbitrary units (AU), mean values and standard deviation of the tumor samples were calculated as well as of the negative controls and of the non- tumor areas. The samples were grouped dependent on the value of AU in three categories: Group 1 = 20–39 AU, group 2 40–50 AU, group 3 = 51–70 AU, the mean values and standard deviations of every group was calculated and shown in relation to the negative controls. The AU values of the three groups were analyzed against the negative controls and the non-tumor areas using independent two-sample Student's t-test. The character of the evaluation was explorative. Probability of error was set at 5 % and shown as *p*-values.

Results

Characteristics

The clinical and histo-pathological characteristics of B7-H1 and B7-DC expressing tumor tissues are shown in Table 1. Tissues were obtained from patients with OSCC and were collected from 3 women and 12 men, ranging from 40 to 79 years, median age was 61.53 years (±10.06). Most of the tumors were modestly differentiated and graded using the UICC (Union internationale contre le cancer) classification [36] as follows: One OSCC was G1, nine were G2, four were G2–3, one was G3. Staging using the TNM Classification of Malignant Tumor describing tumor size (T), lymph nodes (N) and distant metastasis (M) showed that one OSCC was T1, eleven were T2 and one was T3. Two tumors were not classified by TNM. The nodular status was N0 in eight, N1 in one and N2 in 4 tumors.

Immunostaining of B7-H1 and B7-DC

All of the 15 oral squamous cell carcinomas investigated showed a positive expression of the B7-H1 receptor in the cancerous areas (Figs. 2 and 4). The intensity of the green staining ranged from 25.5 to 59.8 arbitrary

Table 1 Clinical and histopathologic characteristics of B7-H1 and B7-DC expressing tumour tissues for 15 patients with OSCC

TG No.	male/female	Age	Ca	Grade	TNM
TG1	m	55	SCC		
TG2	m	66	SCC	G3	
TG3	f	64	SCC	G2	T2 N0 Mx
TG4	m	63	SCC	G2	T2 N0 Mx
TG5	m	40	SCC	G2	T2N0Mx
TG6	m	69	SCC	G2	T2N2Mx
TG7	m	64	SCC	G2–3	T2N2Mx
TG8	f	79	SCC	G2	T2N2Mx
TG9	m	48	SCC	G2–3	T2 N0 Mx
TG10	m	72	SCC	G2–3	T2 N0 Mx
TG12	m	72	SCC	G1	T2 N0 Mx
TG12	m	52	SCC	G2	T3N1Mx
TG13	m	62	SCC	G2	T1N2Mx
TG14	f	60	SCC	G2	T2N0Mx
TG15	m	57	SCC	G2–3	T2N0Mx

units (AU). All 15 carcinomas were positive for B7-DC expression in identical areas (Figs. 2 and 4). The green staining showed intensities between 17.6 and 56.1. The staining patterns of B7-H1 and B7-DC exhibited a fine granular intra-cytoplasmatic appearance with some more coarsely stained granular spots. In Figs. 1-5 the results of two representative tumor tissues are depicted. The areas further analysed are marked in the HE staining and shown in increasing magnifications (Fig. 1). Immuno-staining for B7-H1 (Fig. 2 and 4 a-c), B7-DC (Fig. 2 and 4 d-f), Pan-CK (Fig. 3 and 5 a-c) and CK19 (Fig. 3 and 5 d- f) are shown in increasing magnifications.

Negative Control

In the non-tumour areas (Fig. 6-8, HE staining is shown in Fig. 6 in increasing magnifications) of two tissues samples, no detectable expression of B7-H1 (Fig. 7 and 8 a-c, increasing magnifications) and B7-DC (Fig. 7 and 8 d-f, increasing magnifications) was demonstrated. In the immuno-staining of B7-DC, only a faint non-specific fluorescence around the cells is visible. The staining intensities of the negative controls were 10 to 22 AU and of the non-tumor areas 13 to 18.4 AU.

Immunostaining of Pan-CK and CK19

Of the 15 OSCC, 13 could be investigated for the expression of Pan-CK and CK 19. In all 13 OSCC, the epithelial origin could be ascertained by expression of Pan-CK and CK19. The intensity of the green staining was 21.9 to 68.2 AU (Pan CK) and 18.3 to 66.6 AU (CK 19). The pattern of Pan-CK and CK 19 appeared as dense intra-cytoplasmatic staining, sometimes with a fine or coarse granular appearance, in the epithelial cell cones.

The results from the analysis of the staining intensity are summarized in Table 2.

The mean values of the tumor group 1 (20–39 AU) were 34.8 ± 5.6 AU (B7-H1, n = 15), 30.4 ± 6.7 AU (B7-DC, n = 21), 28.3 ± 7.4 AU (Pan-CK, n = 15) and 28.0 ± 9.6 AU (CK 19, n = 12). The mean values of the tumor group 2 (40–50 AU) were 45.8 ± 4.6 AU (B7-H1, n = 18), 43.8 ± 3.3 AU (B7-DC, n = 18), 46.6 ± 3.3 AU (Pan-CK, n = 6) and 45.2 ± 4.6 AU (CK 19, n = 9) and the mean values of the tumor group 3 (51–70 AU) were 56.8 ± 7.7 AU (B7-H1, n = 12), 56.2 ± 6.7 AU (B7-DC, n = 6), 62.8 ± 12.3 AU (Pan-CK, n = 18) and 61.3 ± 12.0 AU (CK 19, n = 15). The values of staining intensities in all three groups from all 4 markers (B7-H1, B7-DC, Pan-CK, CK 19) were statistically significant higher than the values of the negative controls and the non-tumor areas ($p < 0.01$). These results are shown in Fig. 9.

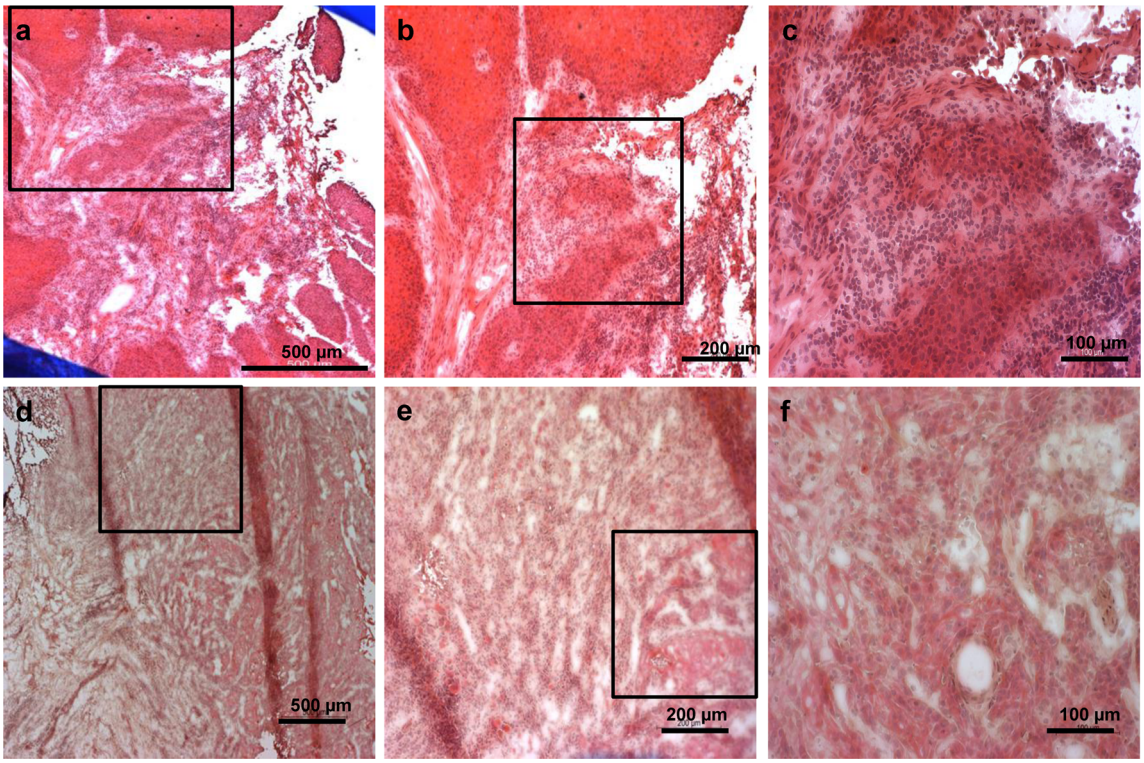

Fig. 1 Tissues from two human OSCC, a -c = HE staining of first OSCC in thre increasing magnifications with marked area for the immunostaining, **d** - **f** = HE staining of second OSCC in three increasing magnifications with marked area for the immunostaining

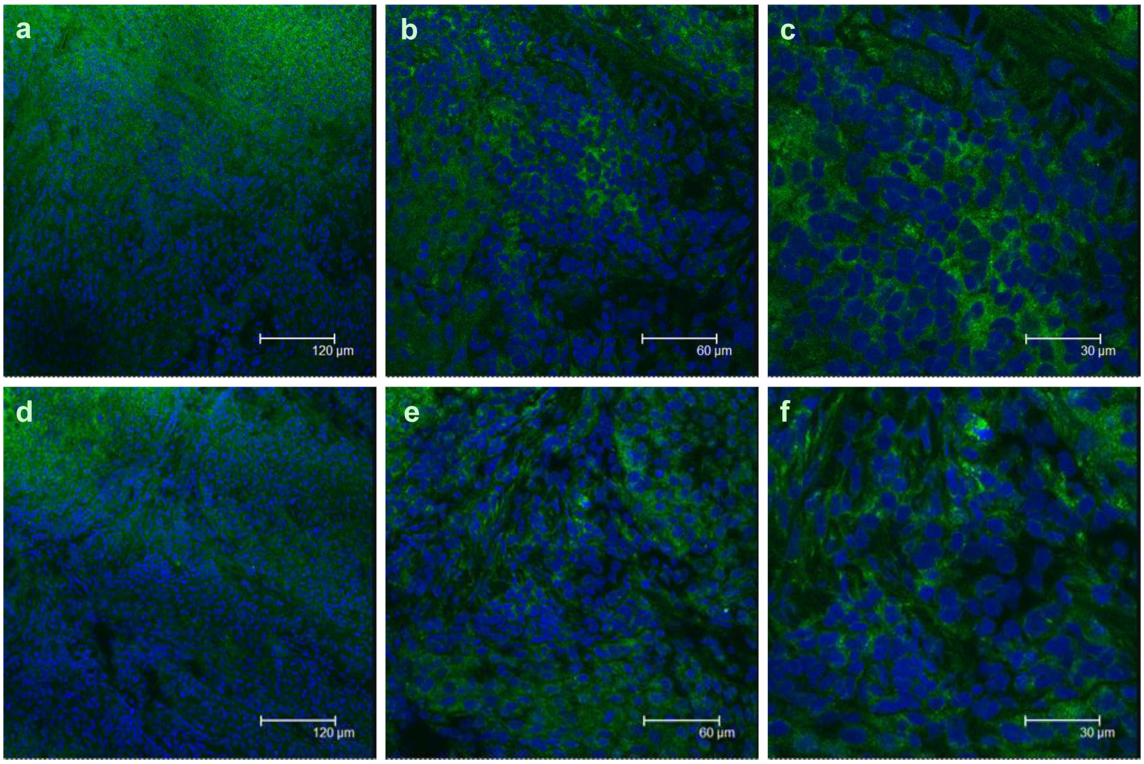

Fig. 2 Tissue from the first human OSCC, **a** -**c** = immunostainig of B7-H1 in three increasing magnifications, **d** - **e** = immunostainig of B7-DC in three increasing magnifications

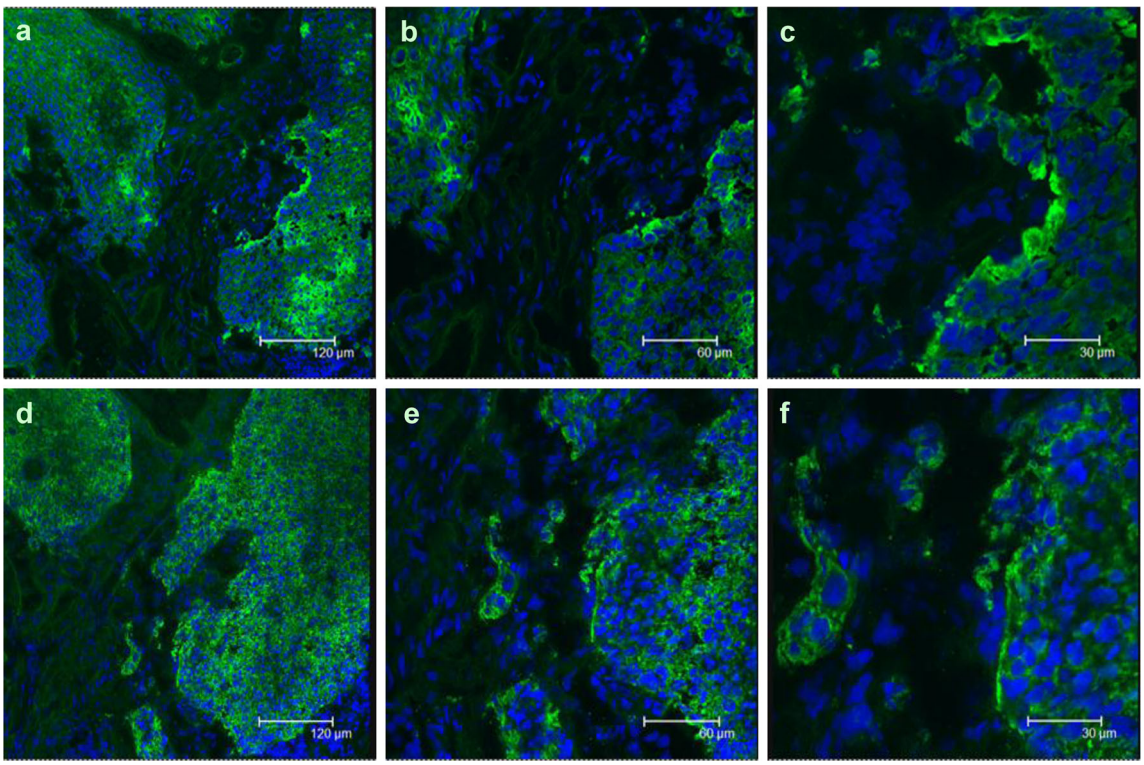

Fig. 3 Tissue from the first human OSCC, **a** -**c** = immunostainig of Pan-CK in three increasing magnifications, **d** - **e** = immunostainig of CK19 in three increasing magnifications

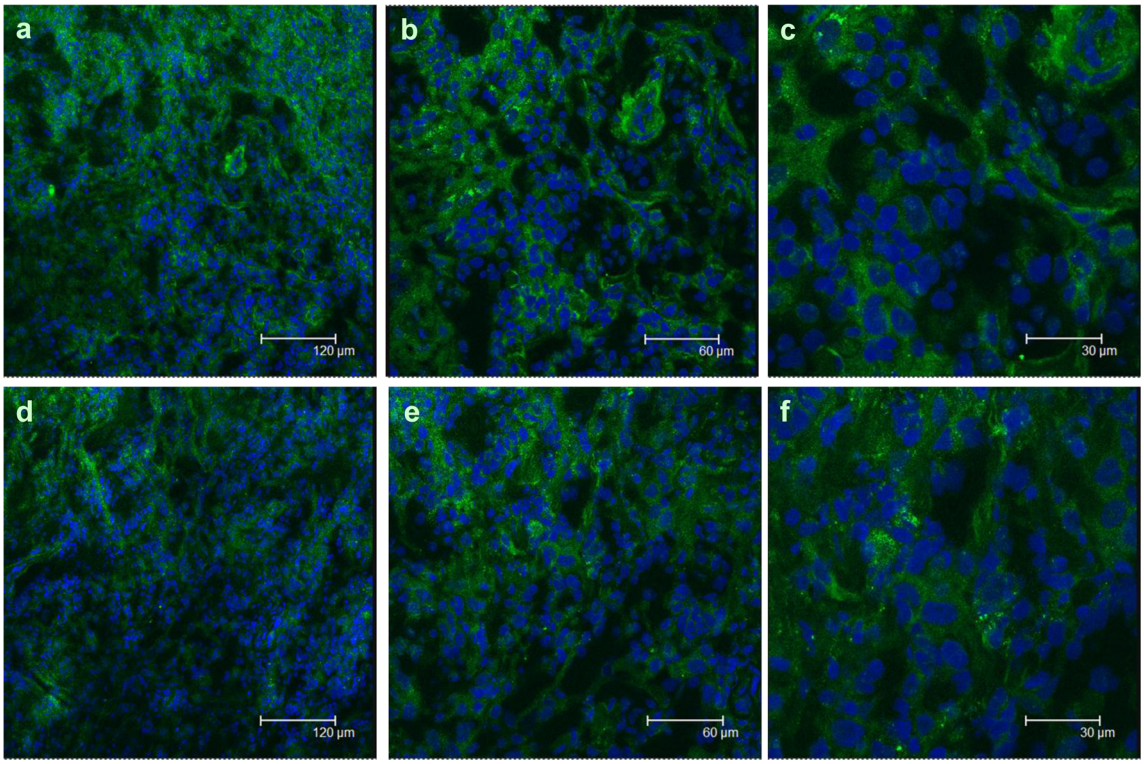

Fig. 4 Tissue from the second human OSCC, **a** -**c** = immunostainig of B7-H1 in three increasing magnifications, **d** - **e** = immunostainig of B7-DC in three increasing magnifications

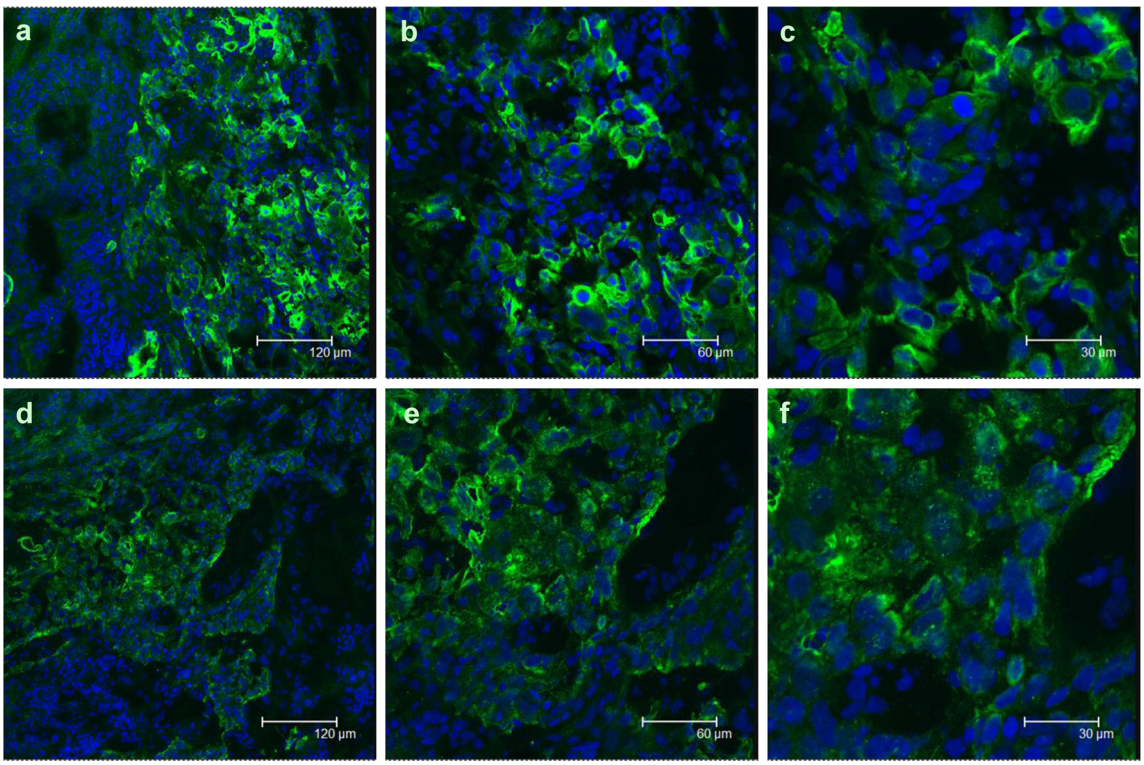

Fig. 5 Tissue from the second human OSCC, **a** -**c** = immunostainig of Pan-CK in three increasing magnifications, **d** - **e** = immunostainig of CK19 in three increasing magnifications

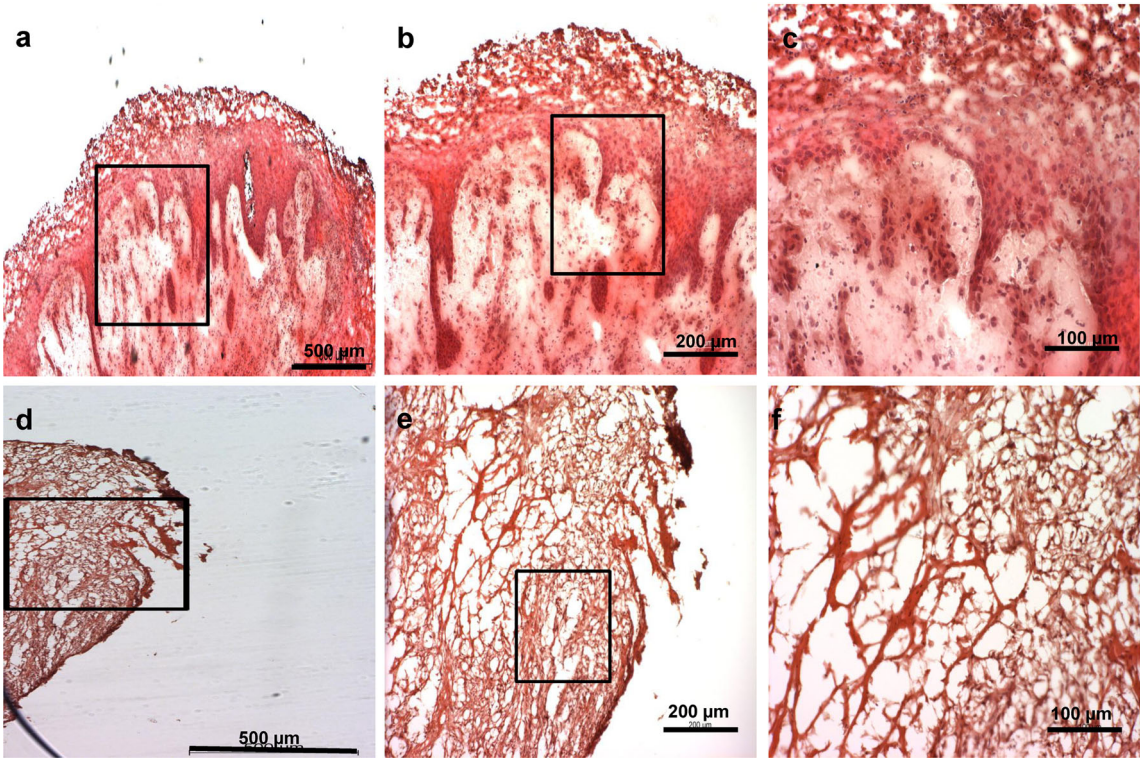

Fig. 6 Tissues from two human OSCC in non-cancerous areas, **a** -**c** = HE staining of first OSCC in thre increasing magnifications with marked area for the immunostaining, **d** - **f** = HE staining of second OSCC in three increasing magnifications with marked area for the immunostaining

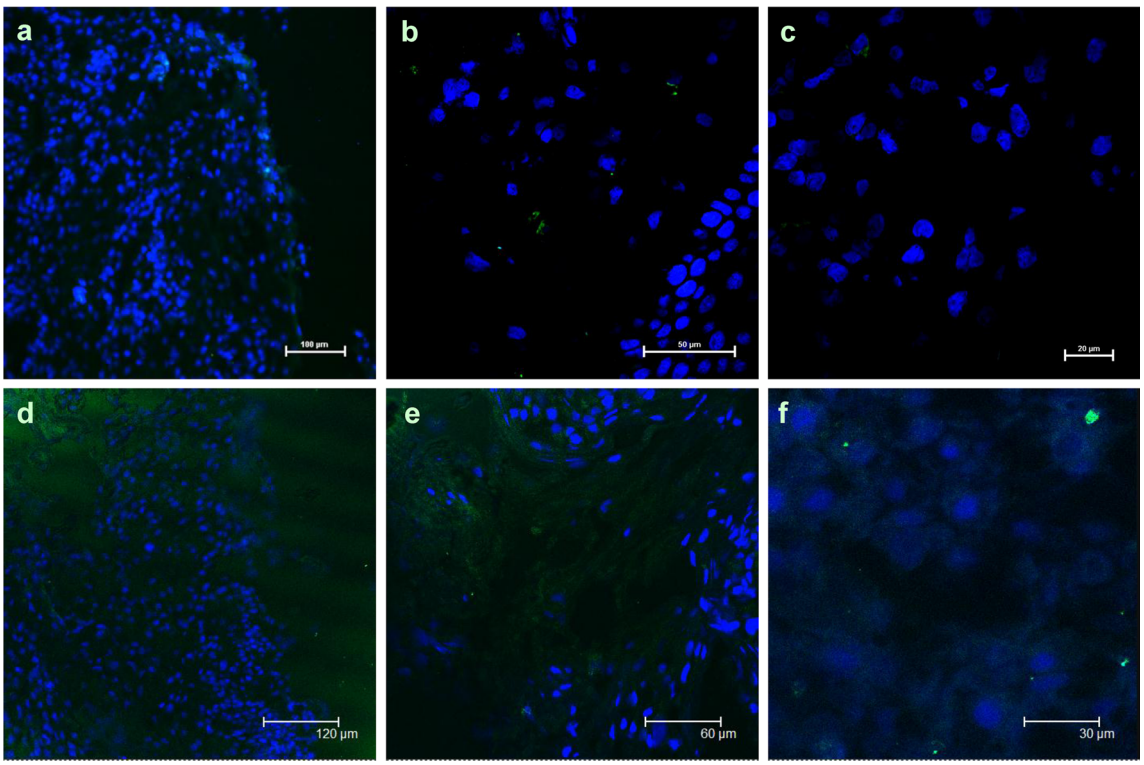

Fig. 7 Tissue from the first human OSCC of non-cancerous areas, **a** -**c** = immunostainig of B7-H1 in three increasing magnifications, **d** - **e** = immunostainig of B7-DC in three increasing magnifications

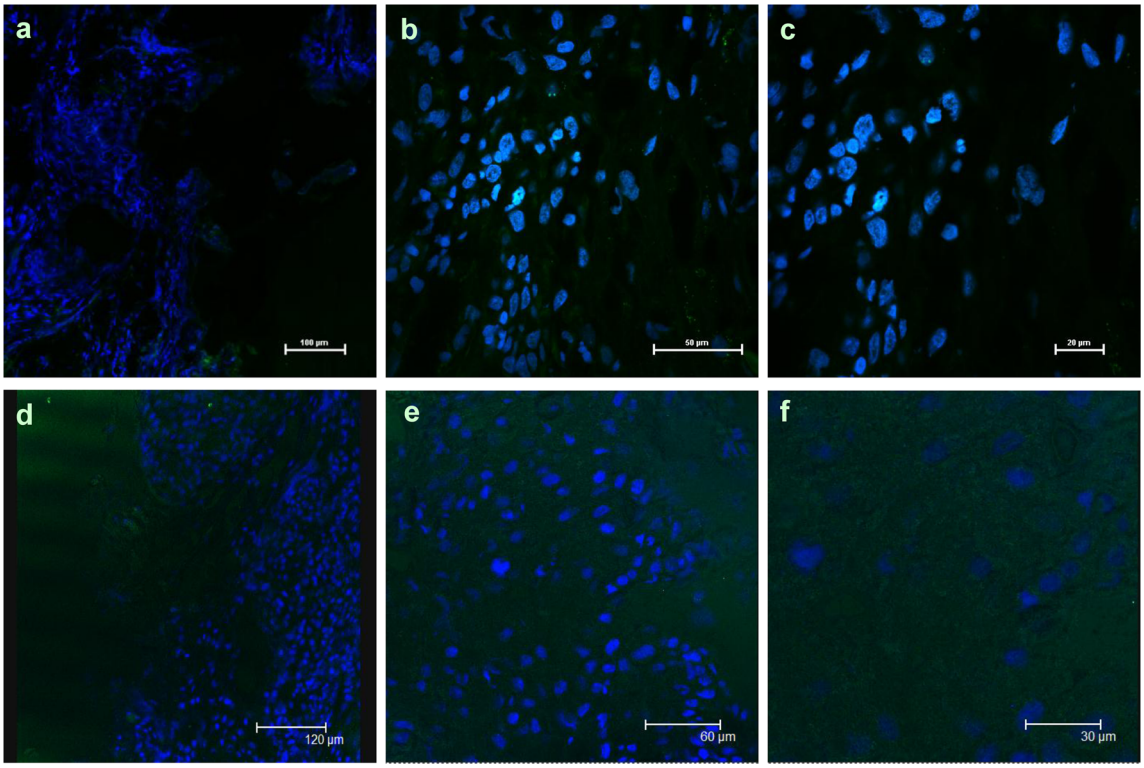

Fig. 8 Tissue from the second human OSCC of non-cancerous areas, **a** -**c** = immunostainig of B7-H1 in three increasing magnifications, **d** - **e** = immunostaining of B7-DC in three increasing magnifications

Table 2 Values of the green staining intensities of the immunofluorescences of B7-H1, B7-DC, Pan-CK and CK 19 and the negative controls and non-tumor areas in arbitrary units of all OSCC (n = 15) analyzed with ImageJ

	Mean staining intensity in arbitrary units (AU)			
Tumor No.	B7-H1	B7-DC	Pan-CK	CK19
T1	59.8 ± 5.1	56.1 ± 8.7	n.d.	n.d.
T2	59.8 ± 11.7	30.4 ± 0.8	n.d.	n.d.
T3	41.9 ± 8.7	31.2 ± 7.0	59.2 ± 3.7	45.4 ± 6.0
T4	48.2 ± 2.3	44.6 ± 3.7	67.8 ± 5.6	80.4 ± 28.7
T5	54.1 ± 3.4	33.1 ± 2.9	n.d.	n.d.
T6	33.7 ± 2.5	35.0 ± 3.5	34.3 ± 4.8	45.9 ± 2.5
T7	37.8 ± 0.8	33.3 ± 6.4	37.9 ± 0.9	32.6 ± 9.3
T8	54.4 ± 9.9	56. 3 ± 2.8	68.2 ± 17.7	66.5 ± 11.6
T9	48.2 ± 1.2	32.4 ± 5.3	57.1 ± 8.1	61.3 ± 11.2
T10	46.8 ± 1.7	45.6 ± 2.2	57.2 ± 11.0	62.2 ± 23.4
T11	38.8 ± 3.4	42.1 ± 4.0	46.5 ± 3.4	38.7 ± 3.0
T12	47.1 ± 4.8	45.1 ± 2.6	62.6 ± 12.7	44.7 ± 6.4
T13	25.5 ± 3.6	17.6 ± 1.5	25.1 ± 5.5	18.3 ± 4.4
T14	38.1 ± 2.1	46.0 ± 1.4	37.0 ± 6.9	22.3 ± 1.6
T15	43.5 ± 4.3	39.7 ± 1.7	46.7 ± 1.4	52.7 ± 4.5
Non tumor areas	14.8 ± 2.7			
Negative controls	19.5 ± 4.6			

Postoperative Prognosis

36 month after surgery, 4 of the 15 patients (26.67 %) had deceased. The survival rate of the patients, whose tissues were positive for B7-H1 and B7-DC expression, was 73.33 % (11 of 15).

Discussion

The mechanisms of tumor escape from immune recognition and destruction are multifactorial, including down-regulation of MCH class I molecules, [37–39] loss of tumor antigens, [40, 41] defective death receptor signaling, [42–45] lack of co-stimulation, [46] production of immune suppressive cytokines [47] and suppressive cells [48–50]. Recent studies have suggested that tumors may evade host immune response through expression of B7-H1. These receptors have been thought to be involved in negative regulation of cellular and humoral immune responses by engaging PD-1 receptor on activated T and B cells [2, 51]. In tumor immunity, tumor-associated B7-H1 has been proposed to induce apoptosis of tumor-reactive T cells [8]. The role and importance of B7-H1 expression in clinical human cancers is poorly understood. In this study, we investigated oral squamous cell carcinomas (OSCC). The results show that B7-H1and B7-DC are expressed in

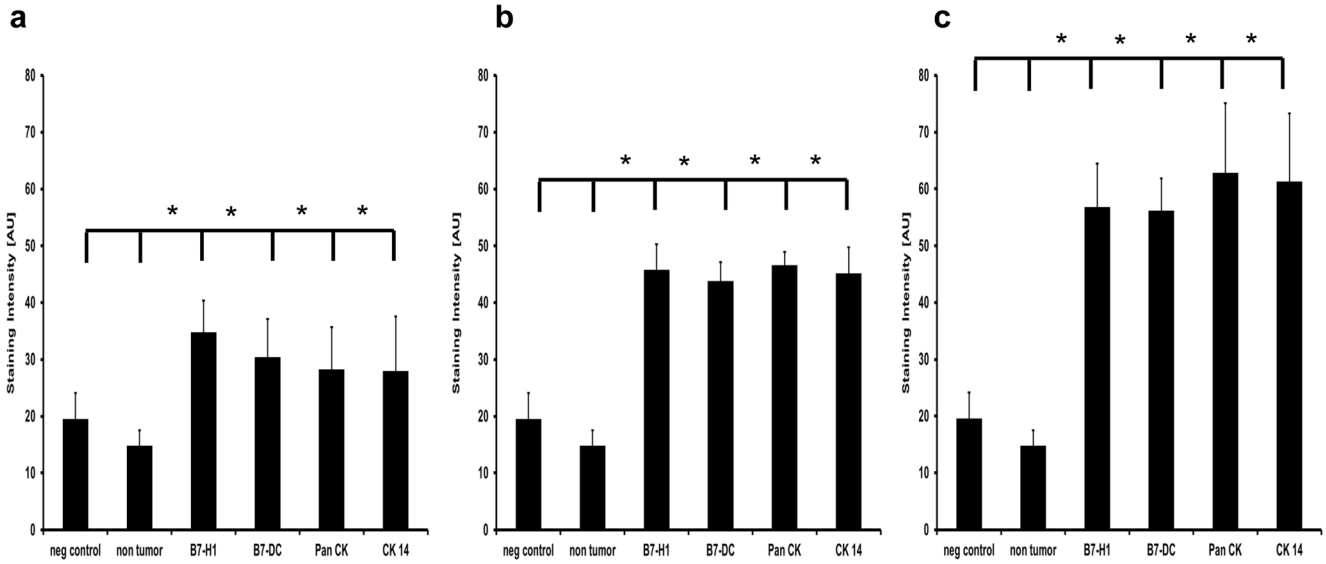

Fig. 9 The mean values of staining intensities the tumor group 1 (20–39 AU) (a) for B7-H1 $n = 15$, B7-DC $n = 21$, Pan-CK n = 15 and CK 19 $n = 12$; tumor group 2 (40–50 AU) (b) B7-H1 $n = 18$, B7-DC n = 18, Pan-CK $n = 6$, CK 19 $n = 9$; tumor group 3 (51–70 AU) (c) B7-H1 n = 12, B7-DC n = 6, Pan-CK n = 18 and CK 14 n = 15; * = $p < 0.01$ against negative control and non-tumor areas

OSCC in vivo. Under physiological and non-inflammatory conditions, B7-H1 expression is mainly observed on professional antigen-presenting cells such as monocytes and dendritic cells (DCs) [1, 8]. However, B7-H1 expression is not restricted to antigen-presenting cells, but also found on other cell types in non-lymphoid tissues, e.g., endothelial cells and muscle [1, 2, 52–55]. Positive correlation of high level expression of B7-H1 with poor prognosis has been demonstrated in patients with renal and urothelial carcinomas, esophageal cancer, and pancreatic carcinomas [21, 56–59]. In two studies of renal carcinomas, survival of patients 3 years after surgery was 71.5 % and 69.1 % when B7-H1 expression was positive, in comparison to 84.9 % and 91.8 % survival of B7-H1 negative patients [57, 58]. The survival rate 36 month after surgical treatment of patients with high B7-H1 expressing urothelial carcinomas was less than 80 % compared to B7-H1 low expressing cases (survival 100 %) [56]. These findings confirm the survival rate of 73.33 % found in our study and provide the conclusion, that expression of B7-H1 may be a prognostic marker in oral squamous cell carcinomas. In a study analyzing the expression levels of PD-L1 and PD-L2 in 52 surgically resected non-small cell lung carcinoma (NSCLC) patients including those with SCC and adenocarcinoma, it was found that there was no correlation of the expression levels of PD-L1 and PD-L2 with clinical and pathological variables or postoperative survival but in the significantly fewer tumor-infiltrating lymphocytes (TILs) were observed in PD-L1-positive tumor regions, and the proportions of PD-1+ TILs were significantly lower in these regions [60]. Therapies that block the PD-1: PD-L1 interaction, have demonstrated promising clinical results in several tumor types. Preliminary data on the antitumor activity of monoclonal PD-L1 antibody from the HNSCC dose expansion cohort, in which patients had to have at least 1 % PD-L1 expression in their tumor samples to participate, were recently demonstrated. Sixty eligible patients were treated with the antibody. The best overall response rate was 19.6 % (95 % CI, 10.2–32.4) [61]. The expression of B7-H1 also has been shown in human OSCC cell lines in vitro [20]. Up-regulation of B7-H1 after infection with the periodontal pathogen *Porphyromonas gingivalis* could be demonstrated in both, OSCC cell lines and primary and immortalized human gingival keratinocytes in vitro [62]. Our study provides evidence for the expression of B7-H1 and B7-DC in OSCC of different sites of the oral cavity in vivo. Studies provided evidence for the existence of a $CD4^+CD25^+$ population of regulatory/suppressor T cells that actively and dominantly prevent the activation and function of effector T cells [63–65]. B7-H1 expression promotes the development of Tregs, which are key mediators of peripheral tolerance actively suppressing effector T cells and inhibiting immune-mediated tissue damage [66–68]. Recent studies also provided evidence for the involvement of these cells in immune evasion mechanisms used by tumors [64, 69–71]. A positive correlation between the increased numbers of Treg cells and tumor progression in experimental as well as clinical settings provided the first indirect evidence that these cells may play an important role in tumor immune evasion [64, 69, 70], which also could play a role in oral squamous carcinoma cells. In patients with oral squamous cell carcinomas, increased numbers of Tregs were demonstrated in carcinoma

tissue, blood and tumor microenvironment [72, 73]. Tumor infiltrating Tregs were shown to relate to the tumor grade where tumors with poor differentiation were more infiltrated [74]. Induction of Treg differentiation by enhanced B7-H1 expression in oral squamous cell carcinomas could be an important local mechanism to undermine anti-tumor immunity.

Future studies are planned to investigate the markers described to clarify the underlying mechanisms.

Compliance with Ethical Standards

Disclosure/Conflict of Interest The authors declare that they have no conflict of interest to disclose.

Ethical Considerations The study was approved by the ethical committee of the University of Giessen.

(Number of the request: 23/12). Informed consent was obtained from all individual participants included in the study. All experiments followed the guidelines of good clinical/laboratory practice (GCP/GLP) and the WHO declaration from Helsinki 1964, latest update Seoul 2008 (59th WMA General Assembly, Seoul, October 2008).

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