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Decreased inflammatory profile in oral leukoplakia tissue exposed to cold physical plasma ex vivo

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Abstract

Background: Oral leukoplakia (OL) is an unfavorable oral disease often resistant to therapy. To this end, cold physical plasma technology was explored as a novel therapeutic agent in an experimental setup.

Methods: Biopsies with a diameter of 3 mm were obtained from non-diseased and OL tissues. Subsequently, cold atmospheric pressure plasma (CAP) exposure was performed ex vivo in the laboratory. After 20 h of incubation, biopsies were cryoconserved, and tissue sections were quantified for lymphocyte infiltrates, discriminating between naïve and memory cytotoxic and T-helper cells. In addition, the secretion pattern related to inflammation was investigated in the tissue culture supernatants by quantifying 10 chemokines and cytokines.

Results: In CAP-treated OL tissue, significantly decreased overall lymphocyte numbers were observed. In addition, reduced levels were observed when discriminating for the T-cell subpopulations but did not reach statistical significance. Moreover, CAP treatment significantly reduced levels of C-X-C motif chemokine 10 (CXCL10) and granulocyte-macrophage colony-stimulating factor in the OL biopsies' supernatants. In idiopathically inflamed tissues, ex vivo CAP exposure reduced T-cells and CXCL10 as well but also led to markedly increased interleukin-1β secretion.

Conclusion: Our findings suggest CAP to have immuno-modulatory properties, which could be of therapeutic significance in the therapy of OL. Future studies should investigate the efficacy of CAP therapy in vivo in a larger cohort.

KEYWORDS
inflammation, kINPen MED, mucosal diseases, T-cells

1 | INTRODUCTION

Cancers stemming from the oral cavity are often a consequence of precancerous lesions. The latter are usually referred to as potentially malignant disorders (PMD) residing in the mucosa of the oral cavity, as set by the World Health Organization.[1] Besides oral submucous fibrosis, oral erythroplakia, actinic cheilosis, dyskeratosis congenita, discoid lupus erythematosus, and oral lichen planus, oral leukoplakia (OL) appears to be a frequent PMD of the oral mucosa.[2,3] OL is characterized as a plaque of white color with indeterminate risk after

excluding the diagnosis of any other diseases based on histopathology, clinical considerations, and the patients' history. Leukoplakia can transform into cancer, and different probabilities between 0.13% and 34% (average: 3.5%) are reported in the study by Pinto and colleagues.[4,5] Typical risk factors of PMD are alcohol consumption, infection (fungal, bacterial, or viral), chronic irritation, oral restoration-related galvanism, and tobacco use of any kind, which is the pathognomic trigger for OL.[5] An important role is played by the immune system in the prognosis of potentially malignant lesions. Changes in the submucosal inflammation are under suspicion to develop dysplasia as well as its invasive properties of cells of epithelial origins as determined in lesions of premalignant and malignant origins.[6,7]

Surgical excision is a regular treatment modality besides ablative therapies using carbon dioxide (CO_2)-lasers in combinational photodynamic therapy regimens.[8] Unfortunately, treatment of widespread lesions is difficult and, in many cases, not feasible. Since the introduction of cold atmospheric pressure plasma (CAP), a physical modality generated at atmospheric pressure that exhibits only about body temperature, the usage of physical plasma as a new therapeutic mode of treatment has become feasible. Physical plasmas are systems made up of multiple mediators, producing mild heat, electrons, electric fields, ions, ultraviolet light, and reactive oxygen and nitrogen species (collectively referred to as ROS). Cold plasma-generated ROS are hypothesized to be the primary drivers of the biological effects observed.[9] Several cold plasma devices are approved and marketed to improve chronic wound healing.[10] However, only the kINPen MED (neoplas med) plasma system is a device of the so-called plasma-jet family that has advantages in treating uneven and fissured body surfaces such as in the oral cavity.[11] In addition, experimental and clinical evidence points to a therapeutic role of CAP in reducing precancerous skin lesions[12,13] and head and neck cancer in vivo and in the palliation of patients with infected tumor beds.[14,15]

Currently, gas plasma technology is investigated with international interest as a therapeutic model for various new medical applications, including the oral cavity.[16] On this basis, we examined the effects of cold physical plasma generated with the kINPen MED on OL tissue biopsies ex vivo, especially with regard to the inflammatory effects, hypothesizing to identify changes following CAP treatment.

2 | MATERIALS AND METHODS

2.1 | Patients

Patients were put under local anesthesia and, using a 3-mm hole punch biopsy, tissue was sampled from mucosal regions of healthy and diseased origins. Altogether, 25 patients were suspected of suffering from OL. In some cases, OL could not be confirmed in regular pathological diagnostics, and specimens of that subject were discarded. OL was confirmed histologically in 19 patients (67 ± 11.5 years; range 41–89 years, sex: 13 male, 6 female), and these samples were used for investigations ex vivo. Diagnostic confirmation is mandatory, during which the samples were taken, while primary medical consultation was done for regular pathological diagnostics. At least four biopsies were taken from each patient, referring to the groups (i) healthy tissues, (ii) OL, (iii) healthy tissues + CAP, and (iv) OL + CAP. Because of the limited amount of usable biopsies, not all stainings could be carried out for all subgroups and patients. The exposure of the tissue from the CAP groups was performed ex vivo. Written and informed consent to participate in the additional sample collection and its ex vivo investigations was received by all patients and volunteers. This regime was approved by the local ethics committee of the University Medical Center Greifswald (approval number: BB61/11b).

2.2 | Ex vivo plasma treatment

The 3-mm healthy mucosa or OL punch biopsies were exposed to an atmospheric pressure argon plasma jet (kINPen MED; neoplas med; Figure 1) approved as a Class IIa medical device in Europe.[10] The plasma treatment time was 3 min at a distance of 10 mm from the jet nozzle to the tissue surface and at a feed gas flux of three standard liters of argon (Air Liquide) gas per minute. The sample was wetted with water every 30 s to prevent effects from gas-drying due to the plasma jet and its argon gas. Treatment started immediately after surgical removal. After plasma treatment, samples were incubated in 500 µL of William's E medium (Lonza) in 24-well plates for 20 h at 37°C in a cell culture incubator. Subsequently, medium supernatants were collected, centrifuged at $1000 \times g$ for 10 min to remove unwanted cellular material and debris, and stored at −80°C until longitudinal analysis.

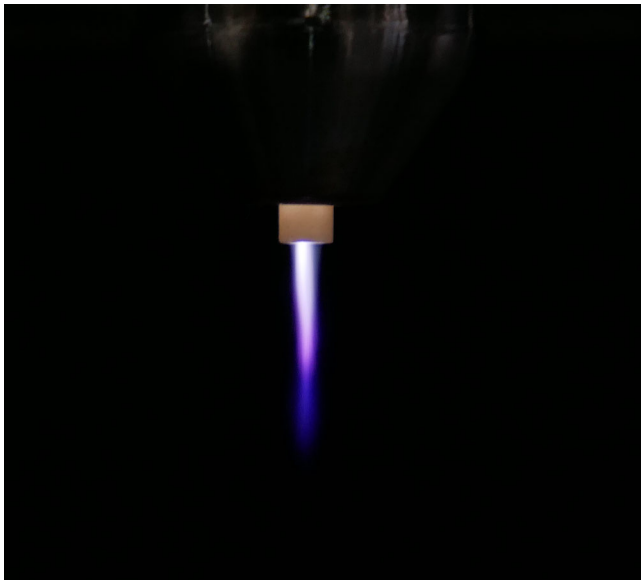

FIGURE 1 The atmospheric pressure argon plasma jet kINPen MED.

2.3 | Preparation and analysis of tissue staining

After 20 h culturing of the tissue biopsies, they were embedded in an optimal cutting temperature compound (Sakura Finetech) and frozen in liquid nitrogen. Tissues were cryo-sectioned at 5 μm thickness on superfrost microscopy slides (Thermo Fisher Scientific) using a microtome (Leica). Then, the samples were fixed in acetone. Subsequently, the tissue sections were incubated with a peroxidase block. Next, the material was separately stained with horseradish peroxidase-labeled antibodies targeted against cluster of differentiation 4 (CD4) (clone: SK3; Becton-Dickinson), CD8 (clone: SK1; BioLegend), CD45-RA (clone: HI100; eBioscience), and CD45-R0 (clone: UCHL1; eBioscience) for a total time of 40 min. Phosphate-buffered saline (PBS) was used to wash the sections. The tissue slides were then stained with peroxidase-labeled polymer and substrate chromogen (3,3′-diaminobenzidine; DAB), all submerged in an appropriate buffer (all Thermo Fisher Scientific) for a total time of 40 min. After washing with water, the slides were mounted for analysis using microscopy, which was carried out employing a high-content imaging device (Operetta CLS; PerkinElmer). The device harbors a slide holder that can house up to four slides for consecutive imaging. Each tissue slide was imaged fully (whole tissue section imaging) and facing downwards in separate channels. These included brightfield (λ_{ex} 785 nm, λ_{em} 655–760 nm), autofluorescence (for enhancing segmentation of tissue areas; λ_{ex} 505 nm, λ_{em} 570–650 nm), and an optimized DAB channel (creating pseudo-contrast; λ_{ex} 475 nm, λ_{em} 655–760 nm). An air objective (×20; NA 0.4) was employed for fluorescence microscopy (Zeiss) in a binning mode set to 1 and in widefield imaging mode. To retrieve quantitative results from this microscopy, all fields of view of each tissue slide were digitally converged to single images (stitching) before flat-field corrections, as well as brightfield corrections were used. The digital workflow was supported by Harmony 4.9 software (PerkinElmer). First, the tissue region was determined. For this, an image was calculated based on autofluorescence and brightfield channels. DAB^+ cell quantification was done by inverting the DAB-contrast channel and enhancing it using sliding parabola functions before DAB^+ cell segmentation was performed. Certain properties of the cells (e.g., the size and intensity) were employed to reject false-positive objects that were identified with the help of algorithms (e.g., objects >200 μm^2; roundness <0.4 on a 0–1 relative scale). With the help of this method, we could enumerate T-helper ($CD4^+$), cytotoxic T-cells ($CD8^+$), naïve T-cells ($CD45RA^+$), and memory T-cells ($CD45R0^+$) given as per mm^2 of entire microscopy slides with tissue from healthy and diseased mucosa. For TUNEL-labeling, a commercially available conjugate (Roche Diagnostics) was employed to identify apoptotic cells as recommended in the manual of the manufacturer. Specifically, the tissue slides were submerged in a humidified chamber at 37°C for 1 h. Then, PBS was used to wash the slides three times, and 4′,6-diamidino-2-phenylindole (BioLegend) was added to stain the nuclei. Fluoromount Aqueous Mounting Medium (Sigma-Aldrich) was used to mount the microscopy tissue slides. Fluorescence microscopy and quantitative analysis were done using the Operetta CLS and Harmony software as well.

2.4 | Multiplex cytokine and chemokine analysis

Multiplex cytokine, growth factor, and chemokine analyses were done by employing a bead-based methodology (LEGENDplex; BioLegend) as per manufacturer's instructions. In brief, tissue biopsy supernatants were collected and later incubated with specific beads, with subsequent extraction of mean fluorescence intensities of each bead population (representing a single analyte) as carried out using flow cytometry(CytoFLEX S; Beckman-Coulter). The 10 analytes investigated were C-X-C motif chemokine 10 (CXCL10), granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon-gamma (IFNγ), interleukin-1β (IL-1β), IL-2, IL-6, IL-12p70, IL-17a, tumor growth factor-β (TGF-β), and tumor necrosis factor-α. The absolute concentrations of the analytes were retrieved in pg/mL using a standard curve that was plotted 5-log and fitted using dedicated software (VigeneTech).

2.5 | Statistical analysis

Statistical analysis as well as graphing were done by employing Prism 9.5.1 software (GraphPad Software). The Mann–Whitney *U* test (also the Wilcoxon rank-sum test) was used to compare the OL with the OL + CAP. Because of the small cohort and sample size, nonparametric testing was applied. The degree of significance is shown as outlined: $^*\alpha = 0.05$, $^{**}\alpha = 0.01$, $^{***}\alpha = 0.001$. Principal component analysis (PCA) was done using absolute secretion data based on the center methods and selecting principal components (PCs) based on eigenvalues greater than 1.0.

3 | RESULTS

3.1 | Cold plasma reduced lymphocytes in leukoplakia

To analyze immunohistochemically stained tissue sections from healthy mucosa, plasma-treated healthy mucosa, leukoplakia, and plasma-treated leukoplakia with the help of algorithm-driven object segmentation, the tissue sections were scanned using high-content imaging and analyzed using proprietary software (Figure 2a). Specifically, the appropriate image region was identified, the signal-to-noise was optimized, and the objects (T-cells) were quantified. Compared to untreated OL biopsies, cold plasma treatment led to significantly fewer $CD3^+$ T-cells in OL tissues (Figure 2b), while activation profiles of $CD8^+$ cytotoxic T-cells did not change significantly (Figure 2c). Subsequently, T-cell subpopulations were based on their phenotype being either naïve ($CD45R0^+$) or memory ($CD45RA^+$) or T-helper ($CD4^+$) or cytotoxic ($CD8^+$) origin. Cold plasma exposure did not significantly change the expression levels of CD8 in OL tissue. Similarly, plasma exposure did not reduce substantially any of the T-cell subpopulations, including $CD4^+$ T-helper cells (Figure 2d), $CD8^+$ cytotoxic T-cells (Figure 2e), $CD45RA^+$ naïve T-cells (Figure 2f), and $CD45R0^+$ memory T-cells (Figure 2g) in plasma-treated compared to untreated OL biopsies, albeit all of them showed a decrease in trend that cumulated to the overall

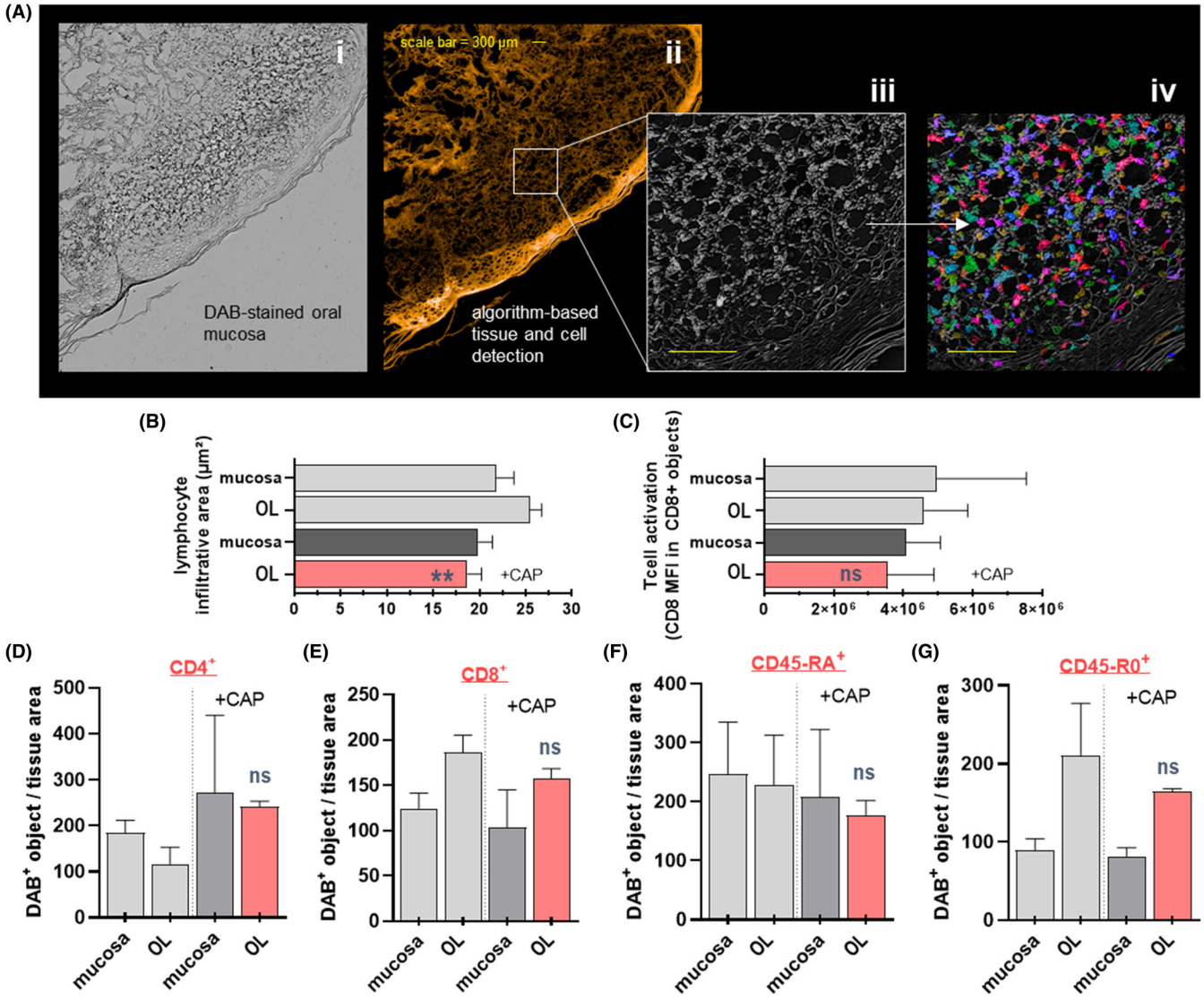

FIGURE 2 Immunohistochemistry of tissue sections from healthy and leukoplakia (OL) oral mucosa biopsies after incubation being left untreated or exposed to cold plasma (CAP) ex vivo. (A) Representative example whole tissue-section brightfield (i) and absorption (ii) imaging, sliding parabola-enhanced contrast leveling (iii), and algorithm-driven image quantification (iv) of lymphocytes or their T-cell subpopulations; (B) lymphocyte quantification; (C) $CD8^+$ T-cell receptor expression intensity per cytotoxic T-cell; (D) $CD4^+$ T-cell quantification; (E) $CD8^+$ T-cell quantification; (F) naïve ($CD45RA^+$) T-cell quantification; (G) memory($CD45R0^+$) T-cell quantification. In B–G, data of healthy mucosa did not differ significantly from those of plasma-treated mucosa. Data are from tissue sections of at least three patients and show mean + SE; statistical analysis was performed using the Mann–Whitney *U* test comparing OL and OL + CAP; ns = not significant, $^{**}p < 0.01$. Scale bar = 300 µm.

reduction of T-cells (Figure 2b). These results suggested that the plasma exposure potentially induced cytotoxic effects in T-cells within leukoplakia tissues and may have also rendered other cell populations, putatively leading to a change in the secretion of inflammatory secreted molecules such as cytokines, chemokines, and growth factors.

3.2 | Cold plasma leukoplakia treatment modulated the secretion of inflammatory mediators

Ten different inflammation-related analytes, which can be associated with an inflammatory tissue phenotype, were quantified in the supernatants of healthy untreated as well as cold plasma-treated mucosa, and untreated and plasma-treated OL tissues, and shown as absolute (Figure 3a) and normalized (Figure 3b–k) values. The highest absolute levels were identified for IL-6, while moderate levels were observed for CXCL10, GM-CSF, and IL-1β. The other analytes' concentrations were low. Supernatants of OL tissue differed from that of healthy mucosa by significantly increased levels of CXCL10, GM-CSF, IL-1β, and IL-6 (Figure 3a, OL orange). Plasma treatment of healthy mucosa led to significantly decreased levels of CXCL10 and IL-12p70 compared to untreated healthy mucosa. Plasma treatment of OL tissue led to significantly reduced levels of CXCL10, GM-CSF, IL-1β, and TGF-β1 compared to untreated OL tissue. Significantly increased

(A)

cytokines (pg/nl)		CXCL10	GM-CSF	IFNγ	IL1β	IL2	IL6	IL12p70	IL17a	TGFβ1	TNFα
mucosa	mean	48.8	34.5	4.5	26.9	7.3	11429.6	5.6	4.2	5.6	6.3
	S.E.	7.6	14.0	0.9	8.3	1.9	1755.1	0.7	0.8	1.5	1.2
OL	mean	93.1	112.9	3.9	119.3	5.8	17703.1	4.2	17.6	8.2	7.2
	S.E.	19.5	33.1	0.7	38.8	1.9	759.7	0.5	7.3	2.1	1.4
mucosa +CAP	mean	26.5	8.4	3.1	24.4	3.6	8191.5	3.4	2.5	3.7	3.9
	S.E.	2.1	2.5	0.3	17.4	0.8	2794.6	0.1	0.4	0.3	0.4
OL+CAP	mean	26.9	4.3	2.9	8.3	3.5	14522.2	4.4	3.4	3.0	5.4
	S.E.	1.4	0.4	0.5	2.1	1.2	1557.2	0.6	0.6	0.2	0.9

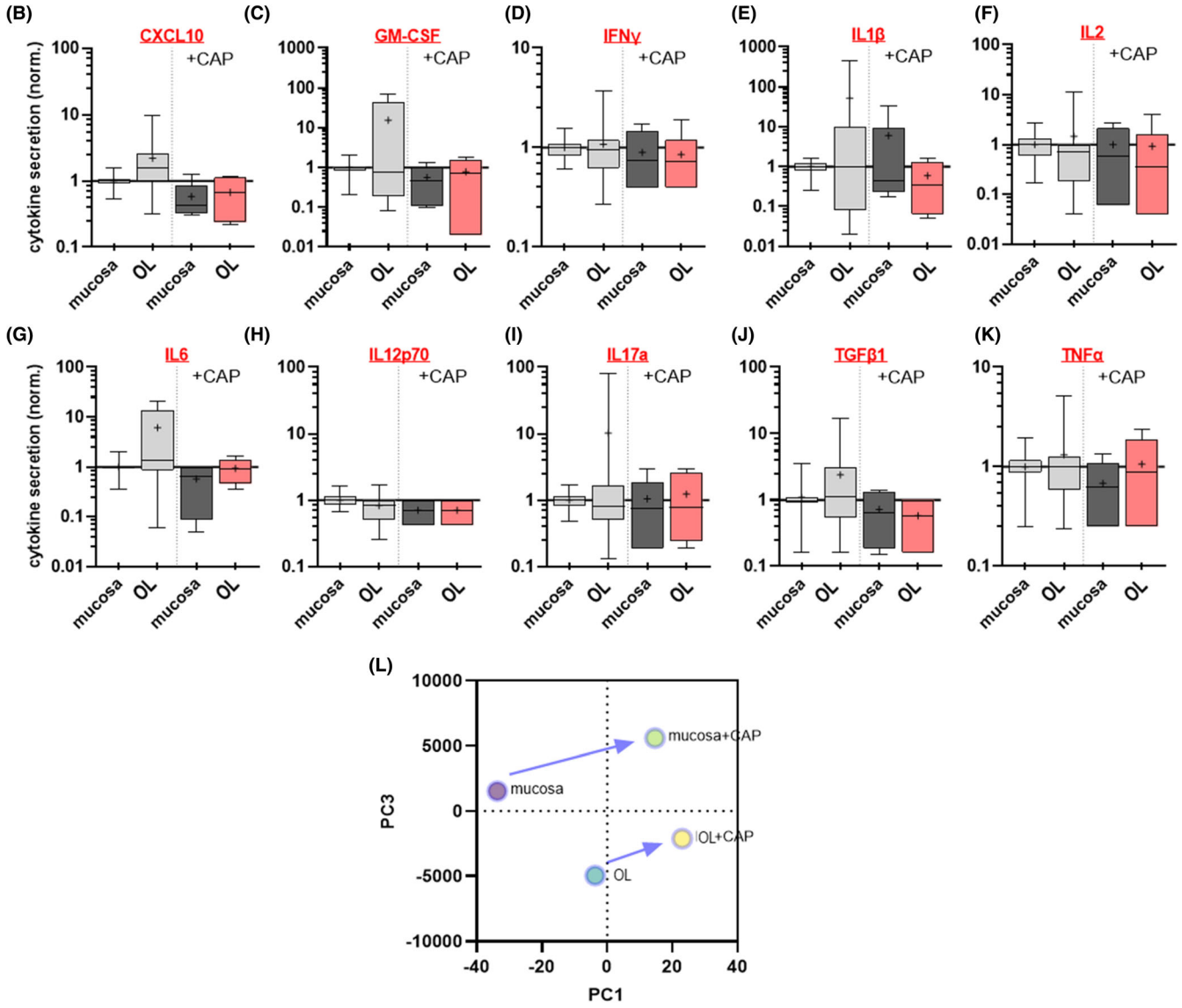

FIGURE 3 Pan-cytokine and chemokine analysis of supernatants from healthy and leukoplakia (OL) oral mucosa biopsies incubation after being left untreated or exposed to cold plasma (CAP) ex vivo. (A) Mean cytokine and chemokine concentrations in supernatants of the four different sample types with statistically significant differences of OL and mucosa + CAP to mucosa shown in orange and OL to OL + CAP shown in violet; (B–K) box plots normalized to mucosa for mucosa + CAP, OL, and OL + CAP for CXCL10 (B), granulocyte-macrophage colony-stimulating factor (GM-CSF) (C), interferon-gamma (IFNγ) (D), IL-1β (E), IL-2 (F), IL-6 (G), IL-12p70 (H), IL-17a (I), tumor growth factor-β1 (TGF-β1) (J), tumor necrosis factor-α (K); (L) principal component analysis (PCA) of secretion data. Data are from tissue biopsies from at least three patients.

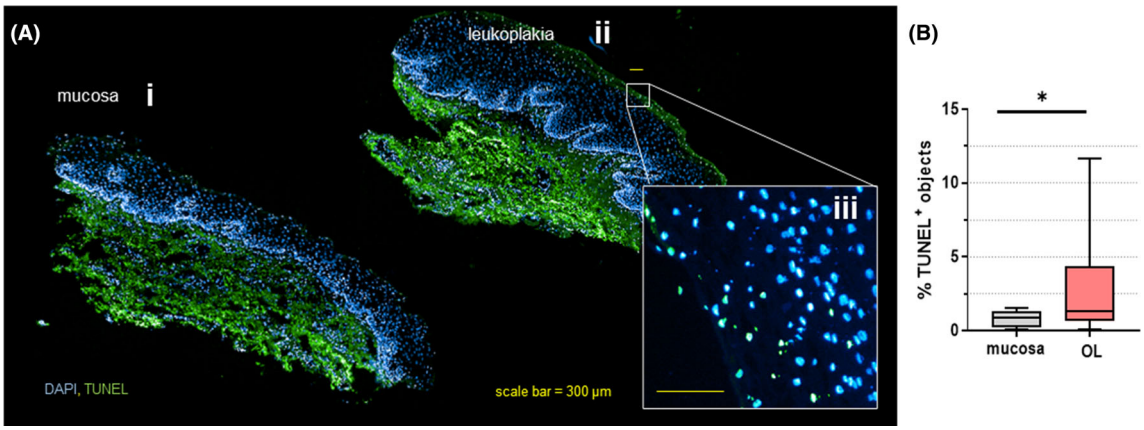

FIGURE 4 Apoptosis analysis of tissue sections from healthy and leukoplakia (OL) oral mucosa biopsies after incubation being left untreated or exposed to cold plasma (CAP) ex vivo. (A) Representative whole tissue section immunofluorescence image digitally stitched from multiple individual images of healthy mucosa (i) and OL (ii) as well as an OL apical close-up (iii) with blue showing DAPI-stained nuclei and green showing TUNEL-stained nuclei; (B) quantification of TUNEL data. Data are from tissue sections of at least three patients and show mean + SE; statistical analysis was performed using the Mann–Whitney *U* test; ns = not significant, $^*p < 0.05$. The scale bar in the insert is 300 µm.

levels with plasma treatment were not observed for any of the analytes investigated. One method to analyze different methods on a meta-data level is employing PCA. By feeding the PCA with the secretion data, it became apparent that healthy mucosa and OL tissue differed in both PC1 and PC2 (Figure 3l). Interestingly, plasma treatment led to similar shift patterns for both types of tissues to the left in PC1, reflecting the decreased levels of analytes being secreted. One possibility of why the baseline secretion of OL and healthy mucosa in the absence of plasma was already different to a greater degree was that sustained inflammation is often associated with increased cell death. To this end, immunofluorescence staining of apoptotic cells was done using the TUNEL technique (Figure 4a). Analysis of the number of apoptotic cells revealed a significant but modest increase of apoptotic cells in OL over healthy mucosa (Figure 4b). Hence, cell death might be one but less likely the most critical factor in OL.

4 | DISCUSSION

Leukoplakia is a potentially malignant lesion with an exceeding inflammatory profile demanding therapy.[6,7,17] To exploit the putative beneficial consequences of cold plasma jet treatment, an approved therapy for several indications in dermatology, toward modulating and potentially resolving the disease, leukoplakia tissue biopsies were plasma-treated ex vivo, and effects were not only compared against untreated leukoplakia but also untreated and plasma-treated healthy mucosa.

The most consistent change observed in mucosal tissue was the decreased release of the chemokine CXCL10, also known as interferon-inducing protein 10. This decrease was not only observed in leukoplakia biopsies plasma-treated ex vivo but also in those of healthy and chronically inflamed mucosa. Hence, reduced CXCL10 might be a general mechanism of the ROS exposure produced by the plasma jet. IFNγ induces CXCL10 release in human keratinocytes, which in turn attracts T-cells into the tissues, especially $CD4^+$ T-helper and $CD45R0^+$ memory T-cells.[18] The reason why especially basal pathological keratinocytes are such a potent source of the chemokines CXCL10 and CXCL11 is only partly unraveled.[19] While there is ample literature on the immune regulation in lichen planus, leukoplakia has been less explored so far. However, partly similar mechanisms might be at work regarding immuno-pathogenesis. For instance, it is known that IFNγ-producing T-helper cells are present in oral mucosal inflammatory disorders, such as Lichen planus.[20] Since we observed decreased T-cell levels in plasma-treated leukoplakia samples, a likely consequence of ROS-induced apoptosis, these cells might have been less capable of producing IFNγ. Consequently, the exacerbated CXCL10 production of keratinocytes was not promoted anymore. Another cytokine that strongly declined in plasma-treated leukoplakia tissue was IL-1β, albeit it increased in plasma-treated-chronically inflamed tissue. IL-1β release is part of the inflammasome response upon priming (e.g., via microbial products such as lipopolysaccharide, abundantly present in the oral cavity and micro-lesions) and activation (e.g., via ROS).[21] IL-1β plays pleiotropic roles in health and pathophysiology, including cancer,[22] but little is reported on its role in leukoplakia.

The second main finding of our study was the tendency of the decline of T-cells in plasma-treated oral healthy or diseased (leukoplakia, chronic inflammation) human mucosa. Although this trend was insignificant in all subpopulation analyses, there was an apparent trend of decreasing T-cells in all calculations (except $CD4^+$ in leukoplakia). The opposite, an increase, was not expected as the tissue biopsies are disconnected from the circulation for new lymphocytes to migrate into the tissue. Memory T-cells are known to persist in diseased and inflamed tissue of the oral cavity, waiting for their antigen to pass by and become activated.[23] It is known that T-cells are principally susceptible to oxidative stress and ROS-induced cellular demise.[24] Regulatory T-cells are somewhat less sensitive but, at the same time, not expected in great numbers in chronically inflamed tissue. Especially $CD8^+$ $CD45R0^+$ memory cytotoxic T-cells succumb to

apoptosis in response to low concentrations of long-lived reactive oxygen species such as hydrogen peroxide (H_2O_2).[25] We have previously identified a similar sensitivity of T-cells in general and memory T-cells, in particular, following cold plasma treatment in vitro.[26]

A correlation between inflammation and transformation from potentially malignant to cancerous lesions has long been speculated.[7] As a result, the cellular immune response strongly correlates to the severity of oral dysplasia.[6,27,28] Using immune cells such as macrophages and lymphocytes as potential biomarkers was also suggested, as their numbers correlate with the oral carcinogenesis stage.[29] Gannot et al. found that differences in the pathology epithelial tissues correlated to stronger degrees of inflammation cells in comparison to lesions with fewer changes.[17] Hereby, extensive inflammation elevated the probability of more detrimental lesions fourfold compared to lesions with lower inflammation.[6] It was also found that T-cell motility is affected by the secretion of inflammatory molecules released by cells of precancerous origin.[30] However, in order to gain more detailed information and because of large differences between different patients, investigations with larger cohorts are needed. All in all, the immune system plays an important role in the microenvironment of this potentially malignant lesion. The information on their role and the suggestibility of this microenvironment could open further therapy and prevention possibilities.

5 | CONCLUSION

Since inflammation plays a crucial role in tumor transformation, its modulation represents a target for targeted therapies. Our findings suggest CAP to have immuno-modulatory properties, which could be of preventive and therapeutic significance in the treatment of OL. Future studies should investigate the efficacy of CAP therapy in a larger cohort of OL patients in vivo.

AUTHOR CONTRIBUTIONS

Christian Seebauer: Conceptualization; methodology; data curation; investigation; writing—original draft; visualization; validation; writing—review and editing; project administration. **Eric Freund:** Investigation; formal analysis; visualization; writing—original draft; writing—review and editing. **Tobias Dieke:** Data curation; investigation. **Sybille Hasse:** Data curation; investigation; resources; validation. **Maria Segebarth:** Data curation; investigation. **Christoph Rautenberg:** Data curation; investigation; resources. **Hans-Robert Metelmann:** Project administration; supervision; validation; writing—review and editing. **Sander Bekeschus:** Funding acquisition; conceptualization; methodology; data curation; investigation; writing—original draft; visualization; validation; writing—review and editing; project administration.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

Tissue sampling for ex vivo cold plasma treatment and their investigation was approved by the local ethics committee of the University Medical Center Greifswald (approval number: BB61/11b).

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