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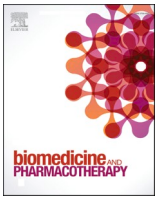

Effects of Vitamin D on tumor cell proliferation and migration, tumor initiation and anti-tumor immune response in head and neck squamous cell carcinomas

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ABSTRACT

Background: Head and neck squamous cell carcinomas (HNSCCs) are among the six most common cancers, with a constantly poor prognosis. Vitamin D has been found to have antineoplastic and immunomodulatory properties in various cancers. This study investigated the impact of Vitamin D on the initiation and progression as well as antitumor immune response in HNSCCs, both *in vitro* and *in vivo*.

Methods: An immunocompetent, orthotopic oral carcinogenesis mouse model was used to examine the influence of Vitamin D_3 substitution on HNSCC initiation and progression *in vivo*. Tumor immune infiltration was analyzed by immunohistochemistry targeting CD3, CD8, NKR-P1C, FOXP3, and CD163. Two HPV- and two HPV+ HNSCC cell lines were treated with 1,25-dihydroxyvitamin D_3 to analyze effects on tumor cell proliferation, migration and transcriptomic changes using RNA-sequencing, differential gene expression and gene set enrichment analysis.

Results: Vitamin D_3 treatment led to a significant suppression of HNSCC initiation and progression, while also stimulating tumor immune infiltration with CD3+, CD8+ and NKR-P1C+ cells and lowering levels of M2 macrophages and T_{reg} cells *in vivo*. *In vitro* experiments showed an inhibition of HNSCC cell proliferation and migration in HPV+ and HPV- cell lines. RNA-sequencing showed significant regulations in IL6 JAK STAT3, hypoxia signaling and immunomodulatory pathways upon Vitamin D_3 treatment.

Conclusion: The findings of our study highlight the promising potential of Vitamin D in the therapeutic repertoire for HNSCC patients given its immune modulating, anti-proliferative and anti-migratory properties. Clinical transferability of those *in vitro* and *in vivo* effects should be further validated in clinical trials.

1. Introduction

Head and neck squamous cell carcinoma (HNSCC) represents the 6th most prevalent cancer type worldwide with 892,000 new cases and 458,000 deaths in 2022 [1]. The incidence is expected to increase by up to 30 % by 2030 [1], underscoring the public health importance of this disease. Major risk factors for developing HNSCC include smoking and alcohol consumption [2] with an increasing influence of high-risk HPV infection, especially in oropharyngeal cancers [3]. Although there has been observed a slight trend towards improved overall survival over the past 30 years, mainly due to the increasing proportion of HPV-associated cases. However, locally advanced and metastatic stages are still associated with a poor prognosis and 5-year survival rates that have plateaued at around 60 % over the past few decades [4]. Most patients diagnosed with HNSCC require multimodal treatment regimens including surgery, radiation and chemotherapy often resulting in severe functional and

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aesthetic impairments [5]. Despite the provision of standard of care treatment, many tumors recur. Recently, anti-PD1 immune checkpoint inhibitors (ICI), such as pembrolizumab and nivolumab have been approved for treatment of recurrent and metastatic (RM) HNSCC patients [6]. Nevertheless, response rates remain below 25 %, highlighting the need for further investigation in this field, especially as HNSCC is one of the most immune-infiltrated human cancer types [7]. Numerous ongoing clinical trials investigating new therapeutic combinations as well as therapeutic sequences show promising results and are expected to dynamically change the standards of head and neck oncology therapy in the coming next years [8]. Vitamin D_3 (cholecalciferol) is a steroid hormone with pleiotropic effects on human physiology [9] including well characterized effects on bone metabolism, calcium and phosphate homeostasis [10]. In addition, there is also increasing evidence that Vitamin D_3 exerts immunomodulatory effects with the potential to stimulate or suppress the immune system, contingent on the specific disease context [11]. In addition, an expanding body of evidence supports the antineoplastic properties of Vitamin D in various human cancer entities [12] predominantly based on *in vitro* and epidemiological data. Here, improvement in Vitamin D status was linked to a reduced risk for developing a variety of cancers and reducing associated morbidity and mortality [13]. It has been demonstrated that low serum levels of 25 hydroxyvitamin D [25(OH)VitaminD_3] are associated with an increased risk of developing colon carcinoma, tobacco-related cancers including lung cancer, kidney cancer, bladder cancer and HNSCC. Furthermore, sufficient serum levels of 25(OH)VitaminD_3 have been linked to an overall survival benefit across all cancer types [14–17]. Importantly, synergistic effects of cancer immunotherapy and Vitamin D supplementation were recently reported for melanoma and non-small cell lung cancer [18,19]. However, the molecular mechanisms underlying the antineoplastic effects of Vitamin D remain unclear. Furthermore, there is only limited evidence regarding the antineoplastic modes of action and immunomodulatory properties of Vitamin D in head and neck cancer. Only two published *in vivo* studies have addressed this knowledge gap. Both studies are limited by their small sample size and have shown partly contradictory results [20,21].

In light of the aforementioned background, our study investigated the antineoplastic modes of action and tumor-immunological properties of 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2$VitaminD_3] in head and neck cancer, both *in vitro* and *in vivo*, using cell proliferation and migration assays, RNA-sequencing and an immunocompetent murine oral carcinogenesis model.

2. Materials and methods

2.1. Immunocompetent murine 4NQO oral carcinogenesis model

To examining the induction and progression of oral tumors, as well as the anti-tumor immune response *in vivo*, 8-week-old male C57BL/6NRj mice (Janvier Labs, Le Genest-Saint-Isle, France) were treated with the carcinogen 4-nitroquinolinline-1-oxide (4NQO). The oral carcinogenesis model using 4NQO is a well-established mouse model that has been shown to reliable induce HNSCCs with histological changes and mutational signatures that are similar to those observed in tobacco and alcohol-induced HNSCCs [22]. 4NQO was administered to animals at a concentration of 50 μg/ml (stock solution with propylene glycol as solvent) via their drinking water and offered to the animals *ad libitum*. The mice were treated with 4NQO-containing water for 16 weeks followed by an additional 6-week observation period with 4NQO-free drinking water (Fig. 1 (A)). Depending on the experimental group of mice, animals were fed with a standard Vitamin D_3 free diet or a special diet containing different doses of Vitamin D_3 (7500 IU/kg or 50,000 IU/kg; C101; Altromin, Lage, Germany) *ad libitum*. Each experimental group and control group consisted of 12 animals. Over the experimental course of 22 weeks, the development of oral epithelial lesions was monitored on a weekly basis via examination of the animals' oral cavities under isoflurane anesthesia. Every 5 weeks, blood samples were analyzed for 25(OH)VitaminD_3 serum levels using the Mouse/Rat 25 (OH) Vitamin D ELISA Assay Kit (Eagle Biosciences Inc., Amherst, NH, USA). After 22 weeks, the animals were sacrificed, and the tongue was resected via mandibular split procedure after further examination of the oral cavity. The number, localization, and dimension of the tumors were documented, and the tumor tissue was obtained for preparation of formalin-fixed and paraffine-embedded (FFPE) samples. Tumor volume was calculated by the formula (LxWxH)/2 (L – length, W – width, H – height [mm]; resulting volume [μl]). The larynx, trachea, lung, esophagus, liver, spleen, and stomach were carefully examined in all animals during necropsy, and any suspicious findings were processed for histological analysis. All animal experiments were previously approved by the local animal welfare authority (index number 12–2021).

2.2. Animal tissue samples and immunohistochemistry (IHC)

Macroscopically visible oral tumors were resected with a safety margin of 3 mm at the end of each animal experiment as described above. All macroscopically identified lesions were subsequently characterized by H&E staining and histopathological analysis by 3 independent investigators. The generally accepted histologically defining malignancy criteria were used to identify malignant lesions [23]. Tissue samples were placed in PBS-buffered 4 % formalin for 24 h and embedded in paraffin. FFPE sections were prepared to perform immunohistochemical staining targeting CD3+, CD8+, CD163+, NKR-P1C+ and FOXP3+ immune cells. 3 μm sections were deparaffinized followed by heat-induced epitope unmasking in a 10 mM citrate buffer (pH 6.0). Nonspecific binding was blocked by incubation of the slides with 3 % BSA (Sigma Aldrich, St. Louis, MO, USA) in PBS (Sigma Aldrich) at pH 7.2 for 30 min. Sections were then incubated with primary antibodies targeting CD3 (1:5000 in 1 % BSA/PBS, ab215212), CD8 (1:2000, ab209775), CD163 (1:750, ab182422), NKR-P1C (1:100, ab289553) or FOXP3 (1:75, ab215206) for 1 h at room temperature. Visualization was performed using the Dako EnVision+ System- HRP Labelled Polymer Anti-Rabbit staining kit (Dako Agilent Technologies, Glostrup, Denmark) according to the manufacturer's instructions. Subsequently, counterstaining was performed using hematoxylin (Sigma Aldrich). A semiquantitative analysis of oral tumor samples using a self-established counting protocol for intra- and peritumoral immune cell infiltration was performed. Therefore, histologically validated squamous cell carcinomas that measured more than 3 visual fields at 40X magnification were considered. The total number of immune cells per 40X visual field of tumor was determined and compared between the different Vitamin D_3 treatment groups. IHC stainings were analyzed by 3 independent investigators including one board-certified pathologist.

2.3. Cell culture and in vitro 1,25$(OH)_2$VitaminD_3 treatment

Primary HNSCC cell lines SCC1 (CVCL_7707; HPV-), HN30 (CVCL_5525; HPV-), SCC47 (CVCL_7759; HPV+) and 93VU147 (CVCL_L895; HPV+) were cultured in DMEM GlutaMAX™ media (GIBCO® Life Technologies, Darmstadt) containing 10 % (v/v) fetal calf serum and 1 % (v/v) penicillin/streptomycin. Cell cultivation was performed in an incubator at 37°C and 5 % CO_2 air content. For *in vitro* 1,25$(OH)_2$VitaminD_3 treatment, 2.0×10^5 SCC1, 2.5×10^5 SCC47, 2.5×10^5 HN30 and 2.5×10^5 93VU147T cells were seeded per well of a 6 well cell culture dish, respectively. Prior to the functional and transcriptomic experiments, an internal treatment validation was performed via Western blot analysis of VDR (Vitamin D receptor) for all utilized cell lines. Depending on the experimental group, DMSO as a control or predefined 1,25$(OH)_2$VitaminD_3 (Sigma Aldrich, D1530–1MG, HPLC-validated purity >99 %) concentrations were added to the cell culture medium to obtain the corresponding concentrations of 0.1 % DMSO as a solvent control or 0.048 nmol/L, 0.144 nmol/L, 0.48 nmol/L, 4.8 nmol/L, and 120 nmol/L 1,25$(OH)_2$VitaminD_3. During the experiment, the

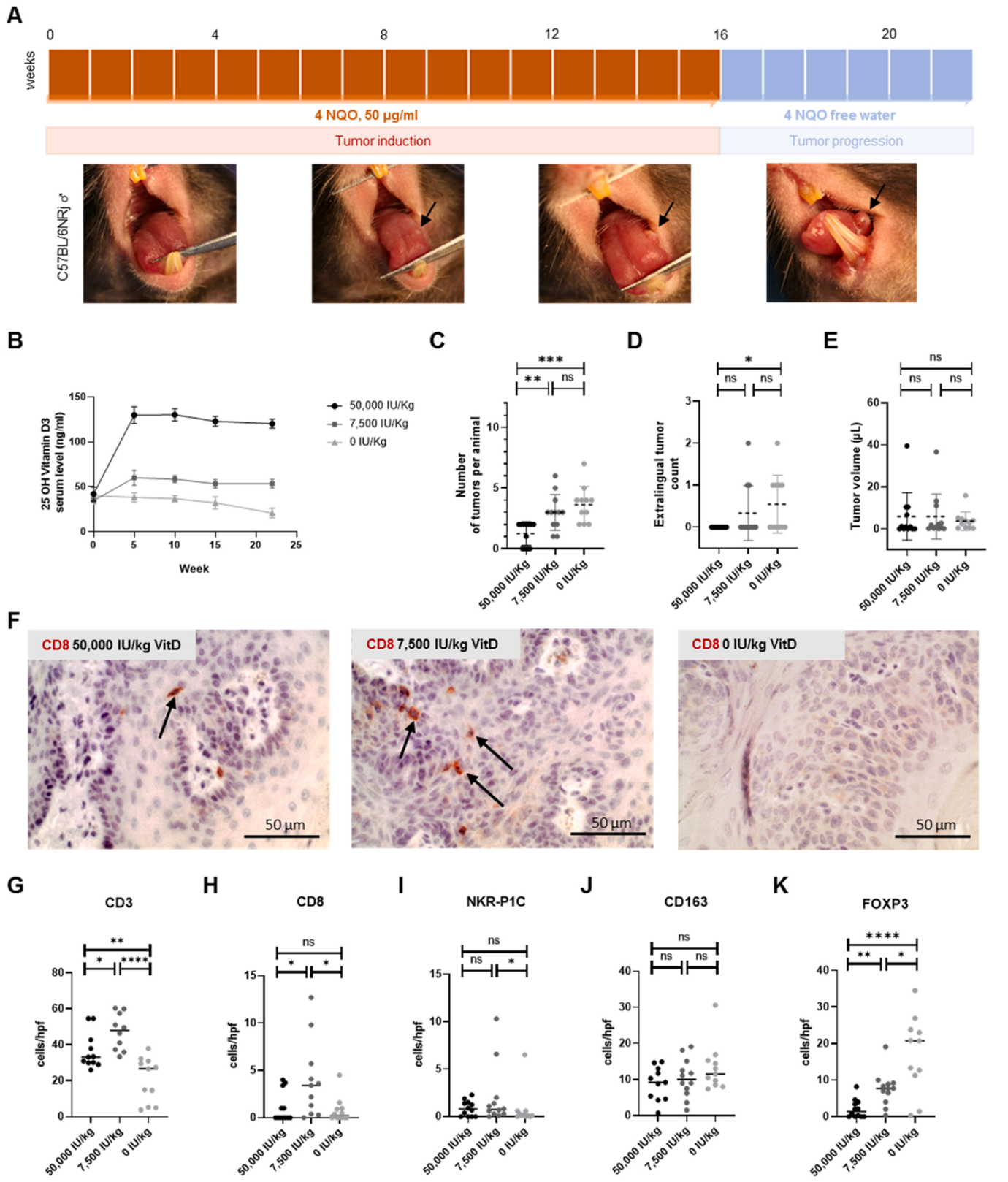

Fig. 1. 4NQO *in vivo* tumor induction and progression model of HNSCCs and immunohistochemical analysis of the immunological TME in three Vitamin D_3 treatment groups: (A) The carcinogen 4NQO (4-Nitroquinoline 1-oxide) was added to the drinking water of 8-week-old immunocompetent C57BL/6NRj mice at a concentration of 50 µg/ml until week 16. First visible lesions on the animals' tongue and cheek were detected at week 12 followed by tumor progression. Black arrows indicate the location of premalignant and malignant epithelial lesions. (B-E) Time course of 25(OH) Vitamin D serum levels, number of tumors, and cumulative tumor volume for the three treatment groups over a 22-week observation period. (F) Immunohistochemical staining of the tumor microenvironment with CD8 showed the strongest immune enrichment in the 7500 IU/kg treatment group. The black arrows indicate CD8+ immune cells. (G-K) Comparison of the total number of CD3+ (G), CD8+ (H), NKR-P1C+ (I), CD163+ (J), and FOXP3+ (K) immune cells in the TME between animals of the three treatment groups.

medium was replenished- with either DMSO or 1,25(OH)$_2$VitaminD$_3$ every 24 h. After 96 h of treatment, the cells were harvested for further migration and proliferation analyses, as well as RNA-sequencing. All cell culture experiments were performed in triplicates. All experiments were performed with mycoplasma-free cells that have been authenticated using STR profiling within the previous three years.

2.4. Cell proliferation and migration analysis

To analyze the proliferation and migration behavior of the various HNSCC cell lines, Incucyte-based (Satorius AG, Göttingen, Germany) proliferation assays and Boyden chamber migration assays (FluoroBlok™ migration system; Corning, NY, USA) were used. For proliferation analysis, cells were seeded in a total volume of 150 µl of media already containing the specific concentration of 1,25(OH)$_2$VitaminD$_3$ in a 96-well plate. The number of cells seeded per well was dependent on the cell line. For SCC1 0.75×10^4 cells were seeded, while all other cell lines were seeded at a density of 1.5×10^4 cells per well in sextuplicates. The plate was placed into the Incucyte S3 device and cells were incubated for at least 96 h. During incubation the device took images of each well at 10X magnification every 4 h. The subsequent analysis used Incucyte 2021 A software (version 20211.2.0) to calculate confluence based on the previously captured images. For migration analysis, 24-well companion plates and FluoroBlok™ inserts were used. The bottom of the insert is covered with a fluorescence blocking membrane with 8 µm pores allowing cells to migrate through these pores. The inserts were loaded with 5×10^4 cells in 500 µL DMEM media suspension containing 0.1 % FCS and 0.048 nmol/L, 0.144 nmol/L, 0.48 nmol/L, 4.8 nmol/L, and 120 nmol/L 1,25(OH)$_2$VitaminD$_3$. The lower chamber was loaded with the same media containing 10 % FCS with an FCS gradient as chemoattractant. During the following 72 h, cells migrated through the pores and adhered to the underside of the membrane, where the cells were fixed with MeOH at −20 °C and stained with DAPI (1:1000). The migrated cell monolayer was visualized by bottom-reading fluorescence microscopy using a Nikon Eclipse Ti2 microscope (Nikon Corporation, Minato, Japan). For semi-automated analysis, 3 representative high-power fields were manually selected and DAPI-stained nuclei were counted using NIS elements HC software (version 5.21.03, Nikon Corporation, Minato, Japan).

2.5. RNA-sequencing and analysis

As previously described, cells were cultured for RNA sequencing (RNAseq). To investigate the influence of 1,25(OH)$_2$VitaminD$_3$ on gene expression profiles in HNSCC cells, a range of HPV+ (93VU147 and SCC47) as well as HPV- (SCC1 and HN30) HNSCC cell lines were treated with 120 nmol/L 1,25(OH)$_2$VitaminD$_3$ over 96 h. RNA extraction was performed from frozen cell pellets as described by Wang et al. in 2023 [24]. All cell lines (SCC1, HN30, SCC47, and 93VU147T) were analyzed in triplicates. Library preparation and quality control were performed as previously described by Lindner et al. in 2024 [25]. Samples were barcoded and sequenced on a NovaSeq X (Illumina, CA, USA) with a PE150 configuration. An average of 28 million paired reads was generated per sample. The proportion of ribosomal reads among the total number of reads generated ranged from 0.03 % to 0.12 %, while the percentage of mRNA bases averaged 93 %. RNAseq pipeline was performed as previously described [26]. The PCA (principal component analysis) and DEG (differentially expressed gene) analyses were performed using the R package DESeq2 [27]. Given the raw count data and gene model used, DESeq2 normalized the raw count data by sample-specific size factor and took covariates, for example cell line, into account while testing for significant differences in gene expression between 1,25(OH)$_2$ VitaminD$_3$-treated and DMSO-control sample groups with multiple test correction through FDR. DEG analysis results were used as input for GSEA [28] (gene set enrichment analysis) against with a number of gene sets, including Hallmark, GO:BP (C5), and Canonical Pathway (C2) sets from MSigDB [29], through the R package clusterProfiler [30].

2.6. Statistical analysis

For statistical analysis, Prism 9 software (GraphPad Software, Boston, MA, USA) was used. The Anderson-Darling test, D'Agostino & Pearson test, Shapiro-Wilk test, and Kolmogorov-Smirnov test were used to test the acquired data for Gaussian distribution. If data passed ≥2 of the normality tests, parametric tests were used for statistical testing (unpaired t test with Welch's correction, one-way ANOVA test). If the data failed ≥2 of the above normality tests, non-parametric tests were used (Mann-Whitney-U test, Kruskal-Wallis test). Fisher's exact test was used to assess the association of gene expression signatures with 1,25(OH)$_2$VitaminD$_3$ treatment in HPV+ and HPV- HNSCC cell lines. P values <0.05 were considered statistically significant ($\alpha=0.05$). Statistically significant results are indicated with asterisks: (*) $p<0.05$, (**) $p<0.01$, (***) $p<0.005$. Non-significant results are indicated as n.s. The tests used for statistical testing are indicated in the figure legends or in the text.

3. Results

3.1. Vitamin D_3 suppresses tumor induction and progression in an immunocompetent oral carcinogenesis mouse model

To evaluate the effects of Vitamin D$_3$ on HNSCC induction and progression *in vivo* we used an immunocompetent orthotopic HNSCC mouse model (Fig. 1 A). Oral squamous cell carcinomas were induced in male C57BL/6NRj mice by adding 50 µg/ml 4NQO to the animals' drinking water over 16 weeks followed by a subsequent 6-week observation period. After 22 weeks, number and size of oral squamous cell carcinomas were analyzed and the tumors were resected for further analysis. Using this *in vivo* model, 36 mice were fed with either 50,000 IU/Kg, 7500 IU/Kg, or 0 IU/Kg Vitamin D$_3$ containing special diet, respectively, with n=12 animals per treatment group. The Vitamin D$_3$ doses in this experiment were based on published literature on Vitamin D$_3$ supplementation in C57BL/6NRj mice [20,21], as well as a previous toxicity study carried out by our group in this mouse strain (supplementary Figure 1). Blood samples taken at week 0, 5, 10, 15, and 22 revealed statistically significant differences in serum levels of 25(OH)VitaminD$_3$. The highest level was observed in the 50,000 IU/kg group [120.2 ng/ml ± 5 ng/ml vs. 53.5 ng/ml ± 4.8 ng/ml (7500 IU/kg) vs. 20.9 ng/ml ± 5.4 ng/ml (0 IU/kg) at week 22; ordinary one-way ANOVA, p<0,0001; Fig. 1 B]. Vitamin D$_3$ diet did not affect the animals' general condition but it did result in slight effects on their body weight [31.5 g ± 3.2 g (50,000 IU/Kg) vs. 31.5 g ± 3.2 g (7500 IU/kg) vs. 29.2 g ± 2.8 g (0 IU/kg) at week 22; ordinary one-way ANOVA p=0.01; supplementary Figure 2].

The number and location of macroscopically visible intraoral tumors was determined during necropsy of the animals at week 22 following mandibular split procedure. A highly significant difference in tumor counts was observed between the three treatment groups (p=0.0006, Kruskal-Wallis test). The lowest number of tumors was observed in the 50,000 IU/Kg group (0–2 tumors per animal; mean 1.25), followed by the 7500 IU/kg group (1–6 tumors per animal; mean 3) and the 0 IU/kg group (2–7 tumors per animal; mean 3.63; Fig. 1 C). Only 3 animals showed no intraoral tumor and all belonged to the 50,000 IU/kg treatment group. When looking at extralingual tumor manifestations significant differences were once again observed between the three groups (p=0.0413, Kruskal-Wallis test). The highest number of extralingual tumors was observed in the 0 IU/Kg group (mean 0.55) followed by the 7500 IU/kg group (mean 0.33) and the 50,000 IU/kg group (mean 0; Fig. 1 D). The total tumor burden, as quantified by cumulative tumor volume per animal, did not differ significantly between the three treatment groups (p=0507, Kruskal-Wallis test; Fig. 1 E).

3.2. Effects of Vitamin D_3 treatment on antitumor immune response in vivo

In order to evaluate the impact of Vitamin D_3 on the antitumor immune response, as previously described in our *in vivo* experiments, the resected tumors from the three treatment groups were analyzed by immunohistochemistry targeting a specific subset of immune cell populations within the tumor microenvironment: T cells (CD3), cytotoxic T cells (CD8), natural killer (NK) cells (NKR-P1C), M2 macrophages (CD163), and Treg cells (FOXP3). Significant differences were observed between the treatment groups for CD3 and CD8 ($p<0.0001$ for CD3, $p=0.0248$ for CD8, Kruskal-Wallis test). The highest number of positive immune cells was noted in the 7500 IU/kg group (Fig. 1 G+H). A comparable trend was observed for the NK cells (Fig. 1 I). Conversely, a significant inverse correlation of immune infiltration with FOXP3-positive cells was observed in response to increasing Vitamin D_3 concentrations ($p=0.0003$, Kruskal-Wallis test; Fig. 1 K). The mean number of tumor-infiltrating CD163-positive M2 macrophages was highest in the 50,000 IU/Kg treatment group, followed by the 7500 IU/Kg treatment group and the 0 IU/Kg control group. However, no significant difference was observed between the groups ($p=0.187$; Kruskal-Wallis test; Fig. 1 J). Taken together, Vitamin D_3 substitution induced an immune-stimulating TME *in vivo* with higher levels of CD3, CD8, and NKR-P1C positive cells and lower levels of FOXP3 positive cells, especially in the 7500 IU/kg treatment group. Fig. 1 F illustrates exemplary images of CD8-IHC of the three treatment groups.

3.3. 1,25(OH)$_2$VitaminD$_3$ induces a dose-dependent inhibition of HNSCC cell proliferation and migration in vitro

In order to investigate the impact of 1,25(OH)$_2$VitaminD$_3$ on the proliferation and migration of HNSCC cells *in vitro*, both HPV+ and HPV- HNSCC cells were treated with either 0.1 % DMSO as an internal control or 0.048 nmol/L, 0.144 nmol/L, and 0.48 nmol/L, 4.8 nmol/L, and 120 nmol/L 1,25(OH)$_2$VitaminD$_3$, respectively. Proliferation rates were assessed in real time via the Incucyte S3 system. A dose-dependent inhibition of cancer cell proliferation was observed. All examined cell lines showed a significant inhibition in their proliferative behavior when treated with 120 nmol/L, regardless of their HPV status (Fig. 2 A-D). For HN30 (HPV-) and 93VU147 (HPV+) no effect compared to solvent control was observed under low-dose concentrations of 1,25(OH)$_2$VitaminD$_3$ up to 4.8 nmol/L. Similarly, a trend but no significant effect was observed with regard to the migratory potential of the cancer cells when low concentrations were applied, up to 0.144 nmol/L. This was particularly evident in HN30 (HPV-), SCC1 (HPV-), and 93VU147 cells (HPV+) (Fig. 3). With regard to SCC47 cells (HPV+), an inhibition of cell migration was observed, which was reversed when concentrations of 0.48 nmol/L and 4.8 nmol/L were applied. The highest concentration tested resulted in a suppression of migratory potential across all cell lines. Overall, 120 nmol/L 1,25(OH)$_2$VitaminD$_3$ resulted in a significant inhibition of proliferation as well as migration of all tested cell lines. Lower doses of 1,25(OH)$_2$VitaminD$_3$ concentrations led to a stimulation or an inhibition of proliferation and/or migration depending on the respective cell line.

3.4. 1,25(OH)$_2$VitaminD$_3$ treatment significantly changes transcriptomic signatures in HPV+ and HPV- HNSCC cells

In a next step, we examined the impact of 1,25(OH)$_2$VitaminD$_3$ on gene expression in HPV+ and HPV- HNSCC cell lines. This was achieved by RNA sequencing and differential expression analysis. In total, the expression of 109 genes was significantly suppressed, including 12 genes that were congruently regulated in both HPV+ and HPV- HNSCC cells. Furthermore, the expression of 134 genes was significantly upregulated with an overlap of 41 genes between HPV+ and HPV- cells (Fig. 4). Among the most significantly regulated genes, several cancer- and immune-related genes were identified e.g. SEMA3B (tumor suppressor gene, upregulated), CD14 (innate immunity, upregulated), CAMP (innate immunity, upregulated), and CXCL10 (immune regulation, downregulated). All significantly regulated genes for all tested cell lines are shown in supplementary table 1+2.

The RNAseq data from HNSCC cells treated with 1,25(OH)$_2$VitaminD$_3$ were subsequently utilized in GSEA. Hallmark gene data sets were assembled from significantly regulated single genes and analyzed for significance and regulation (induction vs. suppression). Here, we observed a concurrent and significant suppression of the following pathways in both HPV+ and HPV- HNSCC cell lines: HYPOXIA, COMPLEMENT, INFLAMMATORY RESPONSE, ALLOGRAFT REJECTION, and TNFA SIGNALING VIA NFKB. Additionally, we found a significant repression of IL6 JAK STAT3 SIGNALING in HPV- cells. MYC TARGETS V1 and MYC TARGETS V2 were found to be significantly induced by 1,25(OH)$_2$D$_3$VitaminD$_3$ treatment in HPV- cells and significantly repressed in HPV+ cells. Furthermore, 1,25(OH)$_2$VitaminD$_3$ treatment induced a highly significant and pronounced inhibition of INTERFERON ALPHA RESPONSE and INTERFERON GAMMA RESPONSE signaling pathways in HPV- cell lines (Fig. 5).

4. Discussion

Head and neck squamous cell carcinomas are globally prevalent cancers that impose a significant social and health-economic burden world-wide. Advanced-stage HNSCCs are associated with a limited range of treatment options and a poor prognosis [4]. Immune checkpoint inhibitors offer new therapeutic perspectives for recurrent or metastatic HNSCCs, though with modest response rates, which remain below 25 % [7]. While Vitamin D has demonstrated promising effects *in vitro* by inhibiting tumor growth, the lack of *in vivo* data limits- its clinical transferability. The present study investigated the antineoplastic and immunomodulatory potential of Vitamin D in HNSCCs in both *in vitro* and *in vivo* settings. The results demonstrate that Vitamin D exerts synergistic anticancer and immunomodulatory effects, suggesting a high potential for clinical transferability.

In general, Vitamin D deficiency is highly prevalent in HNSCC patients and thus requires attention from treating physicians [17,32]. Moreover, recent clinical trials have demonstrated that low serum levels of 25(OH)VitaminD$_3$ are associated with worse overall survival and advanced stages of HNSCCs [17]. In accordance with our *in vivo* findings, a meta-analysis conducted by Pu *et al.* demonstrated that elevated physiological 25(OH)VitaminD$_3$ serum levels are associated with a reduced incidence of HNSCC and enhanced overall survival of HNSCC patients over a 5-year follow-up period [33]. These findings underscore the significance of Vitamin D in head and neck oncology, as it appears to exert a pivotal influence not only on tumor induction and progression but also on patient outcome [34]. Consequently, a more profound comprehension of the intrinsic molecular mechanisms that are affected by Vitamin D in HNSCC cells is of high importance.

Regarding the *in vivo* experiments of our study using an immunocompetent 4NQO HNSCC mouse model, we observed a notable inhibition of oral carcinogenesis by Vitamin D_3, with the most pronounced effects observed in the Vitamin D_3 high group (50,000 IU/kg). Additionally, a reduced incidence of extralingual tumors was noted in this cohort. These results are consistent with existing evidence from *in vitro*, *in vivo*, and epidemiological studies. A recent meta-analysis revealed an inverse correlation between serum levels of 25(OH)VitaminD$_3$ and the incidence of 12 distinct types of cancer. The analysis suggests that an increase in 25(OH)VitaminD$_3$ serum levels from 10 ng/ml to 80 ng/ml could potentially reduce the overall incidence of cancer by 70 ± 10 % [35]. In a separate study, the incidence of breast cancer was prospectively analyzed in a cohort of 5038 women over a 4-year period. The results demonstrated an 82 % reduction in the incidence of breast cancer in women with 25(OH)VitaminD$_3$ levels of ≥60 vs <20 ng/ml [36]. Similarly, a meta-analysis published in 2007 predicted a 50 ± 20 %

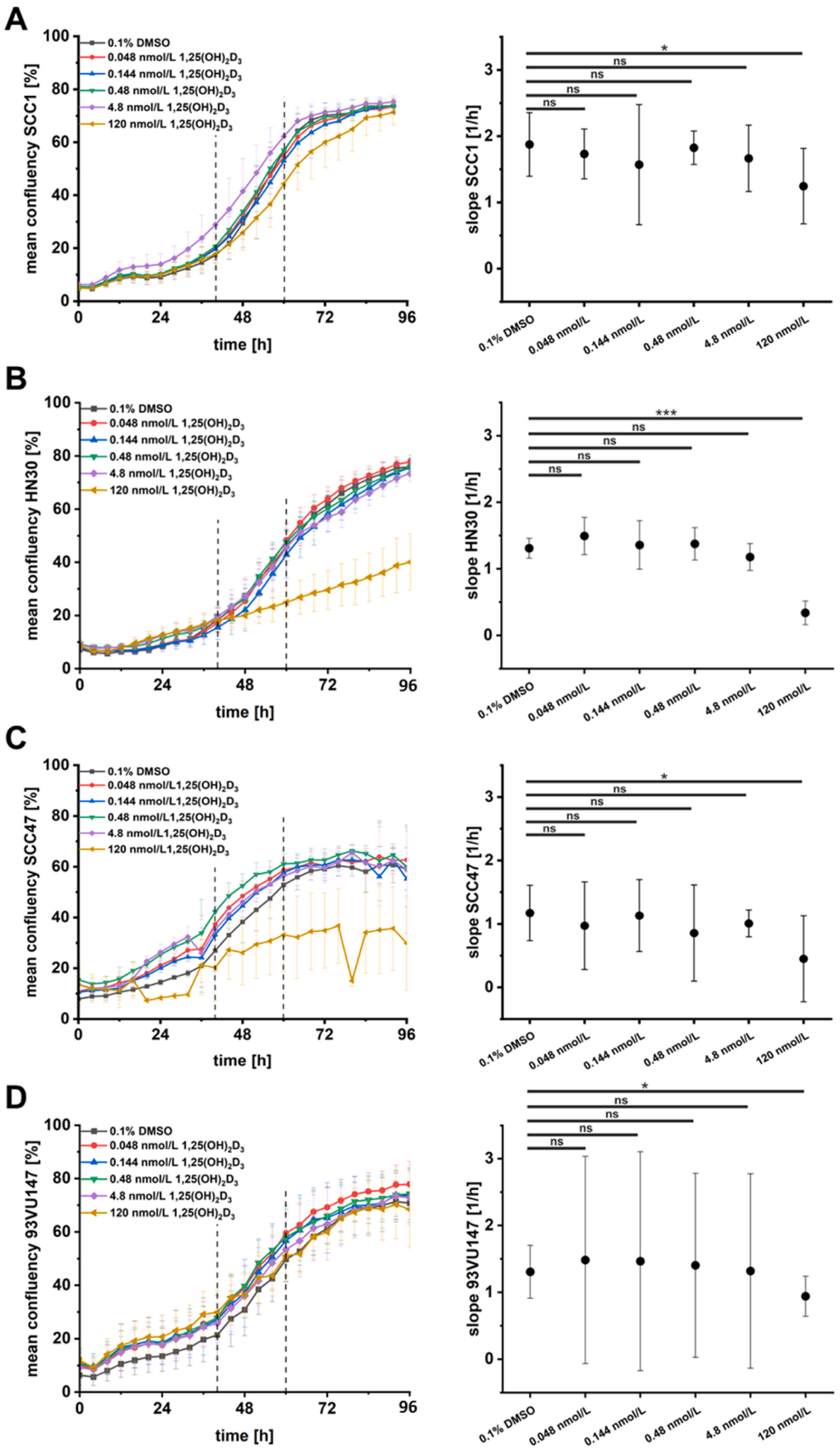

Fig. 2. : Impact of 1,25(OH)$_2$VitaminD$_3$ on HNSCC cell proliferation: (A-D) show dose-dependent inhibition of the proliferation of different HNSCC cells by 1,25 (OH)$_2$VitaminD$_3$ using the Incucyte S3 system real-time proliferation assay. All cell lines showed a significant reduction in proliferation at 120 nmol/L 1,25 (OH)$_2$VitaminD$_3$ regardless of HPV status. To analyze the slope of proliferation behavior, a time period with exponential cell growth between hour 38 to hour 60 was chosen. Unpaired t-tests with Welch correction were used for statistical analysis. DMSO – dimethyl sulfoxide.

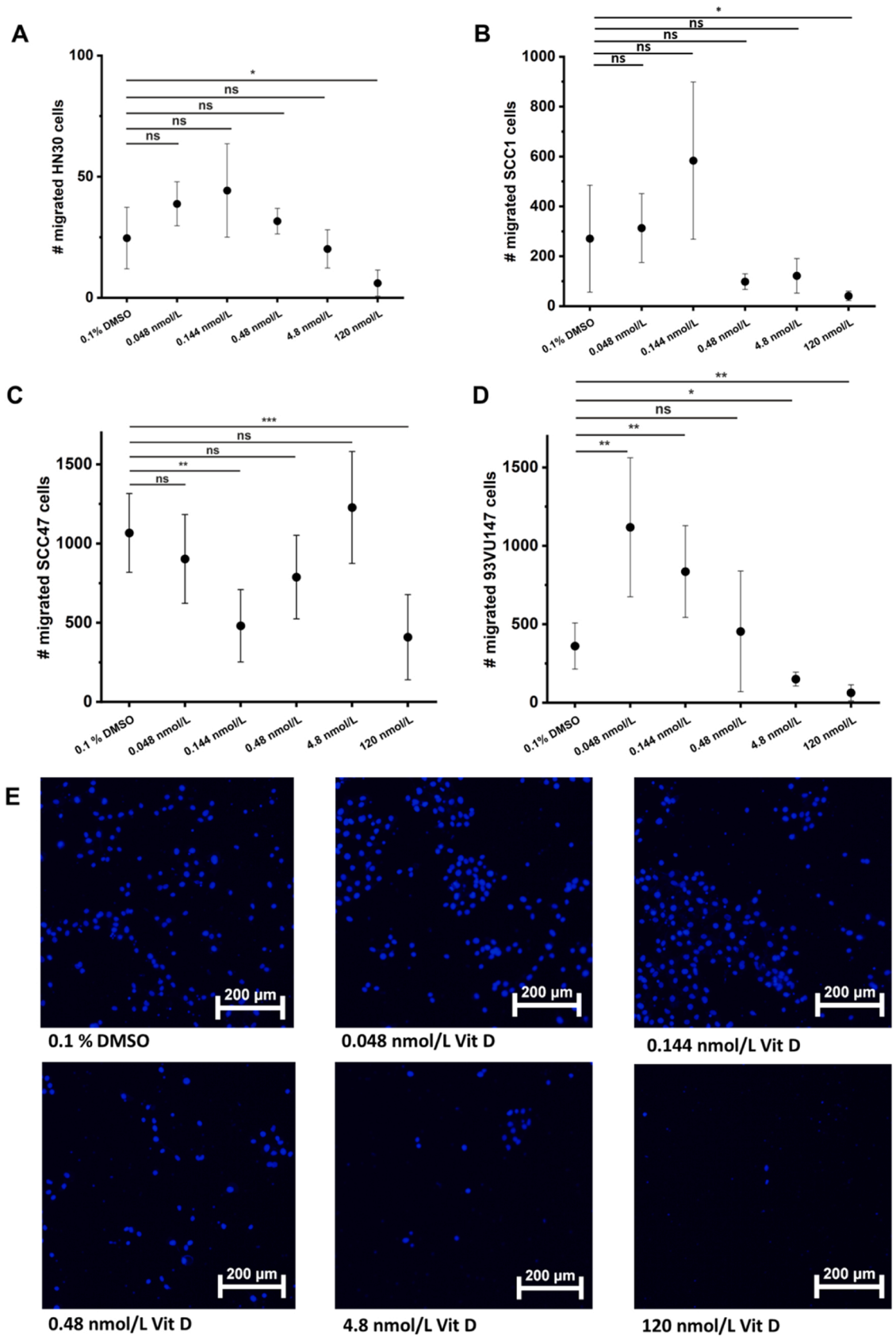

Fig. 3. : Impact of 1,25(OH)$_2$VitaminD$_3$ on HNSCC cell migration: (A-D) Dose-dependent modulation of cell migration by using the FluoroBlok™ Transwell Migration Assay. A tendecial stimulation of migration at low doses and a significant inhibition at high doses of 1,25(OH)$_2$VitaminD$_3$ independent of cell line and HPV status is visible. **(E)** Visualization of migrated cells in different 1,25(OH)$_2$VitaminD$_3$ treatment concentrations in the 93VU147 cell line (HPV+). The nuclei of the cells that migrated through the pores of a fluorescence-blocking membrane after a period of 72 h are stained blue. A clear reduction in cell migration is visible with increasing 1,25(OH)$_2$VitaminD$_3$ concentration. Unpaired t-tests with Welch correction were used for statistical analysis.

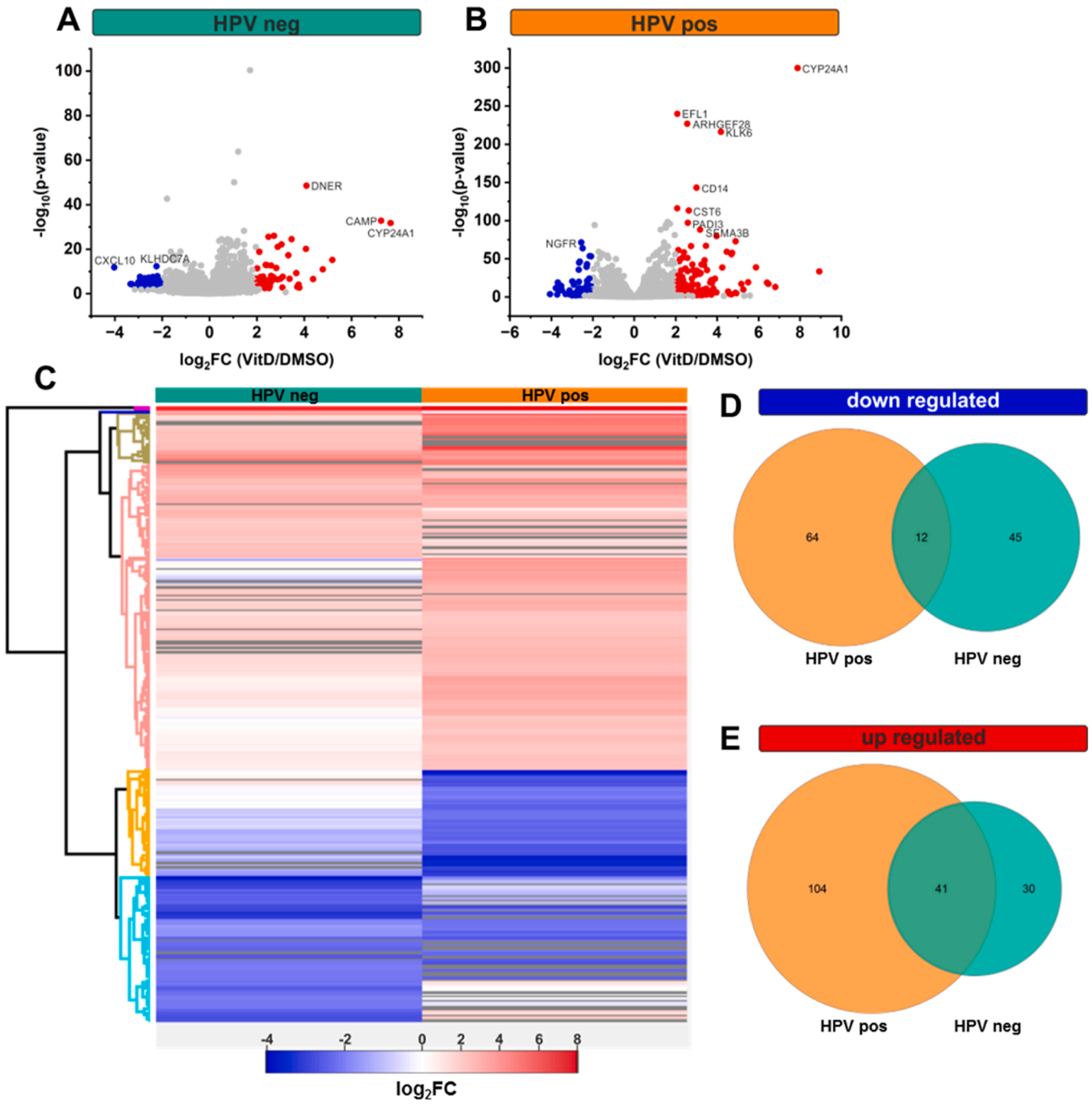

Fig. 4. : Differentially expressed genes in 1,25(OH)$_2$VitaminD$_3$ treated versus DMSO treated HPV+ and HPV- HNSCC cells: (A+B) Volcano plots illustrate the log2-fold change in the expression level of a gene under 1,25(OH)$_2$VitaminD$_3$ treatment. A highly upregulated gene in both cell lines is CYP24A1, which serves as a validity marker of 1,25(OH)$_2$VitaminD$_3$ treatment. **(C)** The Heatmap illustrates significant differentially expressed genes comparing HPV+ and HPV- cells treated with 120 nmol/L 1,25(OH)$_2$D$_3$ versus 0.1 % (v/v) DMSO. High gene expression is indicated by a red color, blue indicates gene suppression. **(D+E)** Representation of the number and the intersection of significantly regulated individual genes by the 1,25(OH)$_2$VitaminD$_3$ treatment. In the intersection of HPV+ and HPV- cells, 12 genes are suppressed while 41 are induced.

reduction in colorectal cancer incidence for 25(OH)VitaminD$_3$ serum levels of 34 ng/ml vs. 6 ng/ml [37]. A comparison of the target 25(OH) VitaminD$_3$ serum levels for achieving the optimal antineoplastic effects indicates that high physiological concentrations may be sufficient to suppress the development of head and neck cancer, as reported in the aforementioned studies. While we also observed in our *in vivo* experiments an inhibition of oral carcinogenesis at serum levels corresponding to moderate to high physiological levels in humans' supra-physiological levels showed an even stronger reduction of tumor incidence. Hence, the optimal 25(OH)VitaminD$_3$ target serum level for cancer prevention requires further discussion and evaluation in large-scale clinical trials. In contrast, there was no significant difference in the cumulative total tumor volume per animal between the treatment and control group. Vitamin D$_3$ could alter the TME in ways that prevent the initiation of new tumors, such as enhancing immune surveillance or reducing local inflammation. However, once tumors are established, these microenvironmental changes may not be sufficient to limit their growth, leading to similar volumes across both groups. Vitamin D$_3$ might affect the early

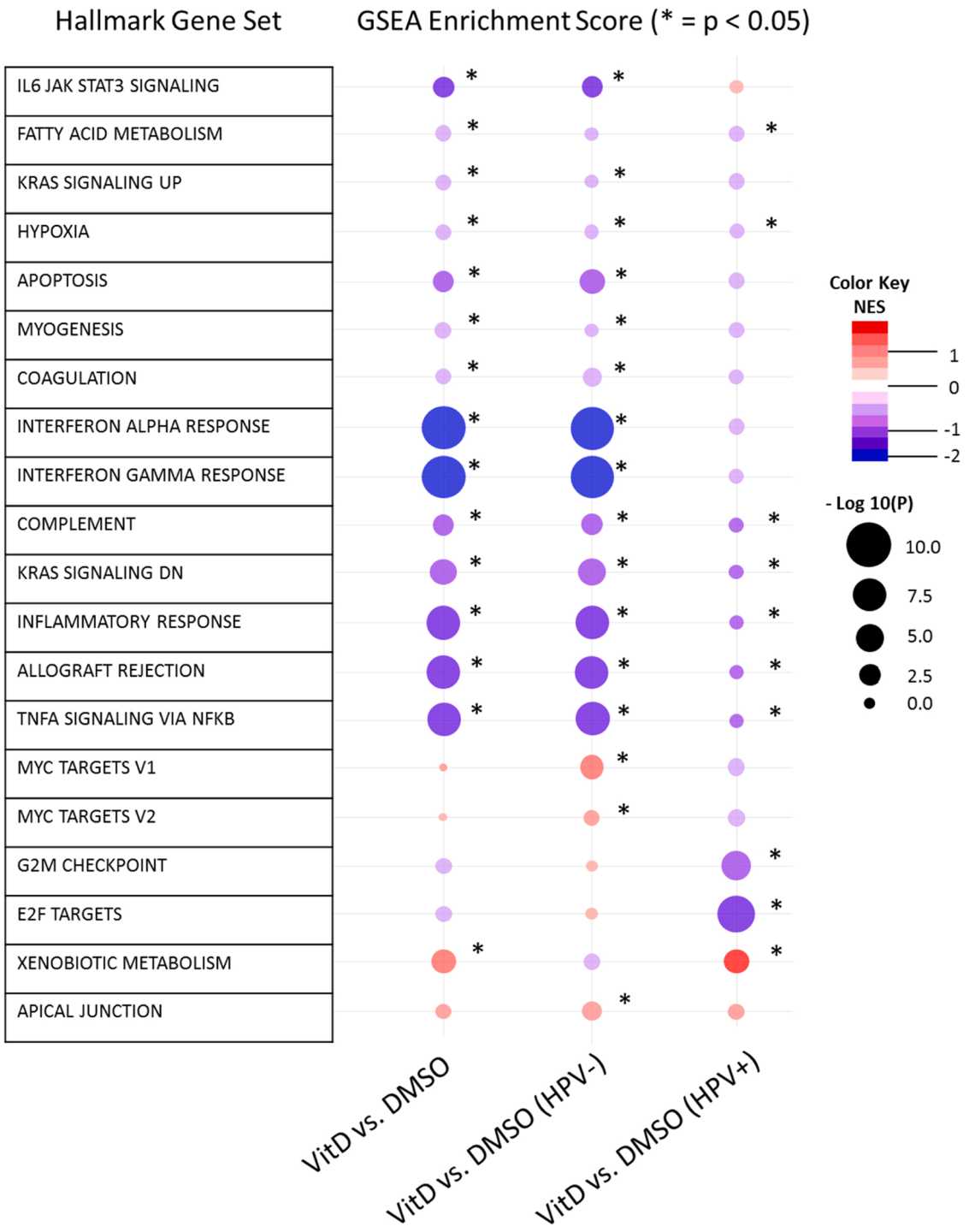

Fig. 5. : Impact of 1,25(OH)$_2$VitaminD$_3$ on Hallmark Gene Sets in HPV+ and HPV- HNSCC cells: The asterisk shows statistical significance ($p<0.05$), determined with GSEA enrichment score. The color key represents the NES (normalized enrichment score). Immunomodulatory signaling pathways are strongly and significantly affected by 1,25(OH)$_2$VitaminD$_3$ treatment. Pathways assessed include INTERFERON ALPHA RESPONSE, INTERFERON GAMMA RESPONSE, COMPLEMENT, INFLAMMATORY RESPONSE, ALLOGRAFT REJECTION, TNFA SIGNALING VIA NFKB. There are also differences within the regulation depending on HPV status. Myc signaling pathways are upregulated in HPV- cells and downregulated in HPV+ cells.

stages of tumor development (e.g., hyperplasia or dysplasia) by preventing the progression from normal cells to malignant ones, resulting in fewer tumors. Furthermore, Vitamin D_3 might inhibit the development of certain tumor clones that are more susceptible to its effects, allowing only those that are more resistant or have a faster growth rate to develop. While tumor initiation is significantly reduced by the Vitamin D_3 treatment, modulation of TME could cause a selection advantage of the grown tumors and thus lead to larger tumor volumes. The only comparable mouse models investigating Vitamin D_3 administration over a longer period of time in the oral carcinogenesis model by Verma et al. and Vincent-Chong et al. do not show tumor volumetry, so further clinical and preclinical trials are necessary [20,21].In addition to a significant reduction in the development of oral cancer, our *in vivo* experiments demonstrated that Vitamin D_3 can promote the infiltration of immune cells into HNSCCs, including those with favorable anti-tumor properties [17,38]. This was accompanied by a reduction in

immune-suppressive cells. The 7500 IU/kg treatment group exhibited the most pronounced effect, displaying a notable increase in CD3+, CD8+ and NKR-P1C+ cells and a concomitant reduction in FOXP3+ immune cells. These data indicate a strong immune-stimulating effect of Vitamin D_3 in head and neck cancer, which is of high importance in an entity that frequently develops mechanisms to evade immune surveillance [39] and shows low response rates to immune checkpoint inhibition [6,40]. Our results suggest that the best effects in terms of immune stimulation can be achieved with high-physiological 25(OH) VitaminD_3 serum levels.

As HNSCCs are among the most immune infiltrated cancer entities [7] immune modulation e.g. by immune checkpoint inhibition represents a highly promising therapeutic approach, although there are persisting challenges in terms of therapy resistance and low response rates that need to be addressed in future pre-clinical and clinical trials. Currently, numerous ongoing clinical trials are investigating the use of checkpoint inhibitor monotherapy or combination therapies in different therapeutic settings [41]. It is noteworthy that two recently published studies have demonstrated that Vitamin D supplementation can enhance the efficacy of immune checkpoint inhibition in patients with lung cancer [19] and melanoma [18]. Hence, Vitamin D represents a promising therapeutic option for the prevention of HNSCC. Furthermore, it may also enhance the efficacy of immunotherapeutic treatments through its immune-stimulating effects, which require investigation in future clinical trials.

The results of our *in vitro* experiments indicated a significant and dose-dependent inhibition of both the proliferative and migratory potential of HPV+ and HPV- HNSCC cells. Nevertheless, low concentrations of Vitamin D tended to have stimulatory effects on cell migration. This observation is consistent with the existing literature [34]. These results indicate that the antiproliferative and migration-inhibiting effects observed *in vitro* may also be achieved *in vivo* depending on systemic 25(OH)VitaminD_3 serum levels. Further *in vivo* investigations using metastasis models as e.g. established by Körner et al. [42] are necessary to determine whether the effects observed *in vitro* can also induce a relevant tumor phenotype change in a living organism.

In order to elucidate the underlying molecular mechanisms of the observed antineoplastic and immune modulatory effects *in vitro* and *in vivo*, RNAseq for DEG and GSEA analysis were performed. [43,44] Hypoxia signaling pathways were significantly downregulated in both HPV+ and HPV- samples. This may provide an explanation for the observation that mice developed fewer oral carcinomas following high-dose Vitamin D treatment *in vivo*. In general, the tumor microenvironment is characterized by low oxygen conditions. Hypoxia-inducible factor 1 (HIF-1) is a pivotal modulator of the metabolic reprogramming that occurs in cancer cells under hypoxic conditions [45]. It may represent a potential link between 25(OH)VitaminD_3 serum levels and tumor induction. Hypoxia in different tumor regions (center vs. periphery) also plays a significant role in the response to radiotherapy [46]. The combined administration of Vitamin D and radiotherapy could therefore represent a potential therapeutic strategy. Furthermore, our findings indicate that Vitamin D markedly inhibits the Hallmark signaling pathways COMPLEMENT, INFLAMMATORY RESPONSE, ALLOGRAFT REJECTION, and TNFA SIGNALING VIA NFKB independent of HPV status. All of these pathways exert regulatory effects within the context of immunological cascades. Given the evidence of gene-directed downregulation, it seems reasonable to conclude that an immunosuppressive mode of action is plausible. This is also consistent with the fact that low serum levels 25(OH)VitaminD_3 are associated with an increased incidence of autoimmune diseases [47]. On the other hand, IL6 JAK STAT3 SIGNALING was found to be significantly downregulated in HPV- cells. Numerous studies have shown that IL6 JAK STAT3 SIGNALING pathway is highly activated in a range of cancers, including gastric, breast, lung, and pancreatic cancer. This activation has been shown to significantly suppress the anti-tumor immune response [48]. This regulatory mechanism may account for the pronounced increase in antineoplastic immune response observed *in vivo*, which may also be dependent on HPV tumor status, as shown by our RNAseq data. However, one has to consider that our RNAseq experiments focused exclusively on the transcriptome of cultured tumor cells. Consequently, the potential effects of Vitamin D on the immunological tumor microenvironment remain largely uninvestigated and require further *in vivo* experiments.

Finally, there are also some limitations of our study that need to be mentioned. The demonstrated results of Vitamin D levels studied *in vitro* and *in vivo* using a mouse model allow only limited transferability to human physiology. To address these questions, further clinical studies are required to determine the optimal concentration to achieve the observed effects also in human organisms. It is also noteworthy that other studies found no effect of Vitamin D on the induction of HNSCCs *in vivo* [20,21]. Vincent-Chong *et al.* exposed C57BL/6NRj mice to 4NQO in their drinking water for 16 weeks, while administering different calcitriol regimens via intraperitoneal injections. This study demonstrated a significant reduction in the number of premalignant lesions in the group receiving concurrent calcitriol and 4NQO treatment. However, continuous administration of calcitriol appeared to increase the risk of developing invasive HNSCCs. Verma *et al.* treated exclusively female C57BL/6NRj mice with three different Vitamin D_3 diets (25 IU/kg, 100 IU/kg, 10,000 IU/kg) and exposed them to 4NQO in their drinking water for 16 weeks. The 100 IU/kg treatment group exhibited a lower incidence of HNSCCs and demonstrated an increased number of intratumoral CD3+ T-cells. The group that received the highest treatment dose of 10,000 IU/kg exhibited the highest incidence of invasive carcinomas. The interpretation of these findings is challenging due to partially contradictory results and the low number of animals investigated, as well as the use of different modes of Vitamin D supplementation. It should be noted that different application forms and treatment concentrations were used in these studies, which must be taken into account and clearly limit comparability.

5. Conclusion

Our study demonstrates that Vitamin D and its metabolites exert a dose-dependent inhibition of HNSCC cell migration and proliferation *in vitro* in both HPV+ and HPV- HNSCC cells. Potential pathways involved, as evidenced by DEG and GSEA analysis, include hypoxia and immune modulation. Additionally, Vitamin D suppressed oral carcinogenesis and stimulated an anti-tumor immune response in an immunocompetent orthotopic HNSCC mouse model *in vivo*. Given the high prevalence of Vitamin D deficiency in HNSCC cancer patients and the low response rates to immune checkpoint inhibition, our data indicate that Vitamin D supplementation may be a promising future strategy for HNSCC prevention as well as improvement of immunotherapeutic concepts in this hard-to-treat disease.

Additional information

Ethics approval and consent to participate

All animal experiments were previously approved by the State Office for Consumer Protection through an application for approval of an animal experiment project according to §8 paragraph 1 of the Animal Protection Act (license number 12–2021).

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Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Maximilian Linxweiler reports financial support was provided by Else Kroner-Fresenius Foundation. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author contributions

ML and SK conceived the project idea. The project represents an extension and continuation of ideas from the working group around LM. LB was the main person responsible for carrying out the *in vitro* and *in vivo* experiments. The experimental protocols of the cell experiments were carried out by LB and SK. The animal experiments were performed under the supervision of ML, SK, and LB. All animal experiments were performed at the institute of MM. Interpretation, evaluation, and analysis were performed by LB, ML, KF and SK. Corrections, ideas for experimental additions were added by MH. The writing of the manuscript and the preparation of the graphics were done by LB, ML, and SK. MH, LM, BS, MK, JK, JS, KF, SW and FB carefully reviewed and approved the final version of the manuscript. The work reported in the paper has been performed by the authors, unless clearly specified in the text.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2024.117497.

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