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The receptor for advanced glycation end products is dispensable in a mouse model of oral and esophageal carcinogenesis

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Summary. Aberrant expression of the receptor for advanced glycation end products (RAGE) and its ligands, such as S100/Calgranulins, has been demonstrated in squamous cell carcinomas of the upper aerodigestive tract. However, the question whether RAGE signaling is causally linked with neoplastic transformation of keratinocytes in mucosal epithelia has not been addressed so far. We used the well-established mouse model of 4-nitroquinoline-1-oxide (4-NQO) induced tumorigenesis to investigate tumor development in control and RAGE-deficient (*Rage*$^{-/-}$) animals. Although 4-NQO induced lesions of the tongue and the esophagus showed strong induction of the RAGE ligands S100a8 and S100a9, we did not observe any significant difference in tumor incidence or multiplicity between control and *Rage*$^{-/-}$ mice. Furthermore, detailed analysis of tumor sections by histological and immunohistochemical staining revealed no difference in either the size or histological architecture of dysplastic lesions, tumor cell proliferation, or the number of inflammatory immune cells in the tumor micro-environment. Finally, we detected induced transcript and protein levels of the Toll-like receptor 4 (Tlr4) in 4-NQO induced lesions, suggesting that signaling via the S100-Tlr4 axis may compensate for the lack of RAGE in early stages of tumor development.

Our data demonstrate that RAGE is dispensable in the onset of genotoxic induced oral and esophageal squamous cell carcinoma and provide evidence for an alternative pathway of S100-Calgranulin signaling via Tlr4.

Key words: Oral cancer, RAGE, Calgranulins, 4-nitroquinoline-1-oxide, Tlr4

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Introduction

The receptor for advanced glycation end products (RAGE) is a multifunctional pattern recognition receptor that belongs to the immunoglobulin super-family (Schmidt et al., 2001; Fritz, 2011). Multiple ligands, such as advanced glycation end products (AGEs), S100/Calgranulins, especially the functional dimer of S100a8 and S100a9, amyloid beta proteins, high mobility group box 1 (HMGB1), and fibrillar protein aggregates trigger intracellular signaling pathways via RAGE and thereby regulate tissue homeostasis and regeneration, as well as pro-inflammatory responses in endothelial and epithelial cells, smooth muscle cells, and mononuclear phagocytes (Riehl et al., 2009). Under physiological conditions most tissues and cell types display no or very low RAGE expression. By contrast, elevated levels of RAGE and/or its ligands have been reported under pathological conditions of late diabetic complications, acute and chronic inflammation and

neurodegenerative disorders, as well as cancer (Bierhaus and Nawroth, 2009; Riehl et al., 2009; Leuner et al., 2012).

In the past, the analysis of several genetically-modified mouse models of intestinal and pancreatic cancer provided experimental evidence that ablation of RAGE inhibits tumor development and progression (DiNorcia et al., 2012, Heijmans et al., 2012; Kang et al., 2012). Moreover, RAGE-deficient (*Rage*$^{-/-}$) mice showed impaired tumorigenesis in chemically induced models of inflammation-driven skin and colon carcinogenesis (Gebhardt et al., 2008; Turovskaya et al., 2008). In these settings *Rage*$^{-/-}$ mice displayed reduced leukocyte recruitment and cytokine production during the initial phase of tumor promotion, supporting the assumption that RAGE is a key player in the establishment and maintenance of a pro-inflammatory tumor microenvironment (Riehl et al. 2010).

Similar to most other solid human malignancies, tumorigenesis in the epithelial tissue lining of the upper aerodigestive tract represents a multistage process that is characterized by the accumulation of genetic and epigenetic aberrations. The majority of tumors represent squamous cell carcinoma. Important risk factors are smoking and alcohol abuse, leading to neoplastic transformation of mucosal keratinocytes. In addition, squamous epithelia of the upper aerodigestive tract are prone to the exposure of factors causing irritation and inflammation, and it is worth assuming that chronic inflammation promotes tumor development and progression (Pries et al., 2006; Kross et al., 2010). Several studies reported aberrant expression of RAGE and its ligands in squamous cell carcinomas of the head and neck as well as the esophagus; however, the results are controversial concerning the impact on histo-pathological and clinical parameters (Kong et al., 2004; Roesch Ely et al., 2005; Sasahira et al., 2007a,b; Landesberg et al., 2008; Tateno et al., 2009; Chuangui et al., 2012; Wild et al., 2012). Until now, the question whether RAGE has a direct impact on the neoplastic transformation of mucosal keratinocytes under settings of genotoxic stress or promotes tumorigenesis mainly due to its impact on the tumor microenvironment has not been addressed *in vivo*. Therefore, we analyzed tumor development in control and *Rage*$^{-/-}$ mice using a well-established mouse model of 4-nitroquinoline-1-oxide (4-NQO) induced oral and esophageal cancer (Tang et al., 2004; Kanoija and Vaidya, 2006). This tumor model reliably produces pre-neoplastic and malignant lesions, which morphologically and histologically resemble human squamous cell carcinoma. Importantly, these lesions develop in the absence of a strong inflammatory response (Hawkins et al., 1994).

Material and methods

Animal experiments and sample preparation

Rage$^{-/-}$ animals were described previously (Liliensiek et al., 2004) and were housed in individually ventilated cages under specific pathogen-free conditions. All experiments were performed with C57Bl6 wild type, heterozygous (*Rage*$^{+/-}$) (control group) and homozygous Rage knockout (*Rage*$^{-/-}$) females. We applied a protocol of 4-nitroquinoline-1-oxide (4-NQO) administration via drinking water starting at 6 weeks of age. 4-NQO treatment was maintained for 4 months, followed by 2-3 months of observation. The 4-NQO group was treated with 100 μg/ml 4-NQO in 2% polypropylene glycol in drinking water ad libitum, while the control group received 2% polypropylene glycol (PPG) only. The investigated sample contained ten animals per treatment per genotype, totaling twenty 4-NQO treated and twenty controls. In particular, ten *Rage*$^{-/-}$ mice were 4-NQO treated, and then *Rage*$^{-/-}$ mice received PPG for the comparison of tumor incidence and multiplicity. 4-NQO treated mice were sacrificed at the end of the observation period or at the sign of severe weight loss and PPG treated mice were monitored for 3 months after withdrawl of PPG in the drinking water. With the exception of one animal with heterozygous genotype, and one wild type animal (both were analyzed for tumor development), which died shortly after 4-NQO treatment, all mice survived the 4-NQO treatment.

For histological analysis, tissues were fixed and prepared as described previously (Gebhardt et al., 2008). The procedures for performing animal experiments were in accordance with the principles and guidelines of the "Arbeitsgemeinschaft der Tierschutzbeauftragten in Baden-Württemberg" and were approved by the German Regional Council, Karlsruhe, Germany.

In vivo BrdU incorporation

Mice were injected intraperitoneally with 100 μl (1 μg/μl) BrdU labeling agent per gram body weight according to the manufacturer's protocol four hours before sacrificing (cell proliferation kit GE healthcare, Little Chalfornt, UK). BrdU incorporation was quantified on tissue sections by immunohistochemical staining using an anti-BrdU antibody (GE healthcare, cell proliferation kit). The relative number of BrdU positive nuclei (brown signal) in basal cells was assessed to quantify the proliferation index in PPG treated versus 4-NQO treated hyperplastic tongue and esophageal tissue, counting different areas from sections of three independent animals. For dysplastic lesions the proliferation index was determined by quantification of the relative number of BrdU positive cells per defined quadrant in sections of three independent animals per group.

Immunohistochemistry and HE staining

5 μm sections from formalin fixed and paraffin-embedded tissues were deparaffinized and rehydrated. 5 μm cryosections were dried over night and fixed in aceton at -20°C. Hematoxylin and eosin (HE) staining was performed as described elsewhere (Gebhardt et al., 2008).

Endogenous peroxidase was blocked with 3% H_2O_2 for 10 min and antigen retrieval was performed in citrate buffer (pH 6) for 30 min in a steam-cooker. Sections were blocked in horse serum (ImmPress, Vector Laboratories,Burlingame, CA, USA) for 20 min and subsequently incubated with the primary antibody (Table 1) according to the manufacturer's instructions, either 2 hours at room temperature or overnight at 4°C. After incubation for 30 min at room temperature with the corresponding, peroxidase coupled secondary antibody (ImmPress), sections were incubated (1-5 min) with the DAB peroxidase substrate (Vector Laboratries Inc., CA 94010, USA) or AEC substrate (Vector Laboratories). Samples were counterstained with hematoxylin and mounted in Evanol or Eukitt (Kindler GmbH, Freiburg, Germany), respectively.

RNA extraction, cDNA synthetis and semi-quantitative RT- PCR

RNA was extracted from cryopreserved tissue specimens using the RNAeasy Kit (Qiagen, Hilden, Germany). 15 μm tissue sections were cut with a cryostat (Leika, Nussloch, Germany) and subsequently homogenized with mortar and pestle in liquid nitrogen. The homogenized tissue was lysed in lysis buffer and total RNA was extracted according to the manufacturer's instructions with an additional on column DNase I (Qiagen, Hilden, Germany) digest to ensure sample quality. For cDNA synthesis, 2.5 μg of total RNA were reversely transcribed as described elsewhere (Gebhardt et al., 2008). Semi-quantitative RT-PCR was performed with primers as listed in Table 2.

Statistical analysis

Differences in the incidence of tumors were investigated by Pearson's chi-squared tests with probability values computed by Monte Carlo simulation with 2000 replicates. Differences in tumor multiplicity were evaluated by unpaired two-tailed Student's t-test. Although the present study has an explorative character, a power calculation may help to quantify the size of detectable effects. Assuming a normal distribution of the number of tumors with mean three and standard deviation 2.8, a study with ten 4-NQO treated and ten control mice is able to detect a true mean difference of 3.7 tumors with 80% statistical power (Type I error rate 5%).

Results

Expression of RAGE ligands S100a8 and S100a9 in oral mucosa after 4-NQO treatment

In order to determine the expression of S100a8 and S100a9 proteins in chemically induced mucosal neoplasia, wild type mice were treated with 4-NQO in drinking water for four months, followed by an observation period of two months. IHC staining revealed induced S100a8 and S100a9 protein expression in tissue sections of the tongue and the esophagus from 4-NQO treated animals (Fig. 1C,D and data not shown), whereas no staining was detectable in normal mucosa or PPG treated controls (Fig. 1A,B and data not shown). Interestingly, we observed a prominent nuclear staining for both calgranulins in epithelial cells of the tongue and the esophagus after 4-NQO treatment. Moreover, S100a8 and S100a9 positive stromal cells that most likely represent inflammatory immune cells were present in the stroma of 4-NQO induced hyperplasia and neoplastic lesions (Fig. 1E,F).

RAGE signaling is dispensable for tumor formation and multiplicity

To address the implication of S100a8 and S100a9 in neoplastic transformation of mucosal keratinocytes by the activation of RAGE-dependent signaling, we treated control and *Rage*$^{-/-}$ mice with 4-NQO. A similar weight loss during and after 4-NQO administration was observed in both groups as compared to PPG treated control animals (data not shown). Total RNA was prepared and semi-quantitative RT-PCR analysis confirmed RAGE expression in the tongue of control and 4-NQO treated mice (Fig. 2A).

Control and *Rage*$^{-/-}$ mice were sacrificed (mean follow-up of 25 weeks after initiation of 4-NQO treatment), and the presence of neoplastic lesions was determined by macroscopic inspection of the upper

Table 1. Primary antibodies.

Name	Species	#	Company
primary antibodies			
Myeloperoxidase Ab-1	rabbit	RB-373	Thermo Scientific
S100a8	goat	sc-8113	Santa Cruz
S100a9	goat	sc-8115	Santa Cruz
TLR4	goat	sc-16240	Santa Cruz
secondary antibodies			
ImmPress® rabbit	horse		Vector
ImmPress® goat	horse		Vector
anti rabbit (biotinylated)	goat		Vector

Table 2. Primers for RT-PCR.

Primer		Sequence	company
Rage	forward	acaggcgagggaaggaggtc	
	reverse	tttgccatcgggaatcagaag	Sigma
Tlr4	forward	tcagcaaagtccctgatg	Aldrich,
	reverse	ttgagaggtgtgtaagc	Steinheim,
Hprt	forward	ctggttaagcagtacagcccc	Germany
	reverse	caaaagtctggggcgcagc	

aerodigestive tract. 70% (N=7) of *Rage*$^{-/-}$ mice developed tongue tumors compared to 90% (N=18) of control mice (P-val=0.29). 90% (N=9) of *Rage*$^{-/-}$ mice developed esophageal tumors compared to 80% (N=16) of control mice (P-val=0.64). In line with previous publications (Tang et al., 2004; Schoop et al., 2009), 4-NQO treated control mice developed papillary lesions on the tongue and/or the esophagus. Only a few tumors were observed on the soft palate and the gingiva (data not shown). The median multiplicity of lesions at the tongue (size between 1-3 mm in diameter) was two per animal, whereas the median multiplicity of lesions at the esophagus (size between 0.5-1 mm in diameter) was 2-3 per animal (Fig. 2B,D). The differences in tumor multiplicity between 4-NQO treated control and *Rage*$^{-/-}$ animals did not reach statistical significance (P-val=0.66 for the multiplicity of tongue tumors, P-val=0.60 for the multiplicity of esophageal tumors, Fig. 2C,D), suggesting that RAGE expression is dispensable for neoplastic transformation of mucosal keratinocytes in a mouse tumor model that is driven by genotoxic stress.

In the past, we found impaired epidermal hyperplasia and accelerated differentiation in papilloma of *Rage*$^{-/-}$ mice as compared to control animals in a chemically induced mouse model of skin carcinogenesis (Gebhardt et al., 2008). However, histological inspection

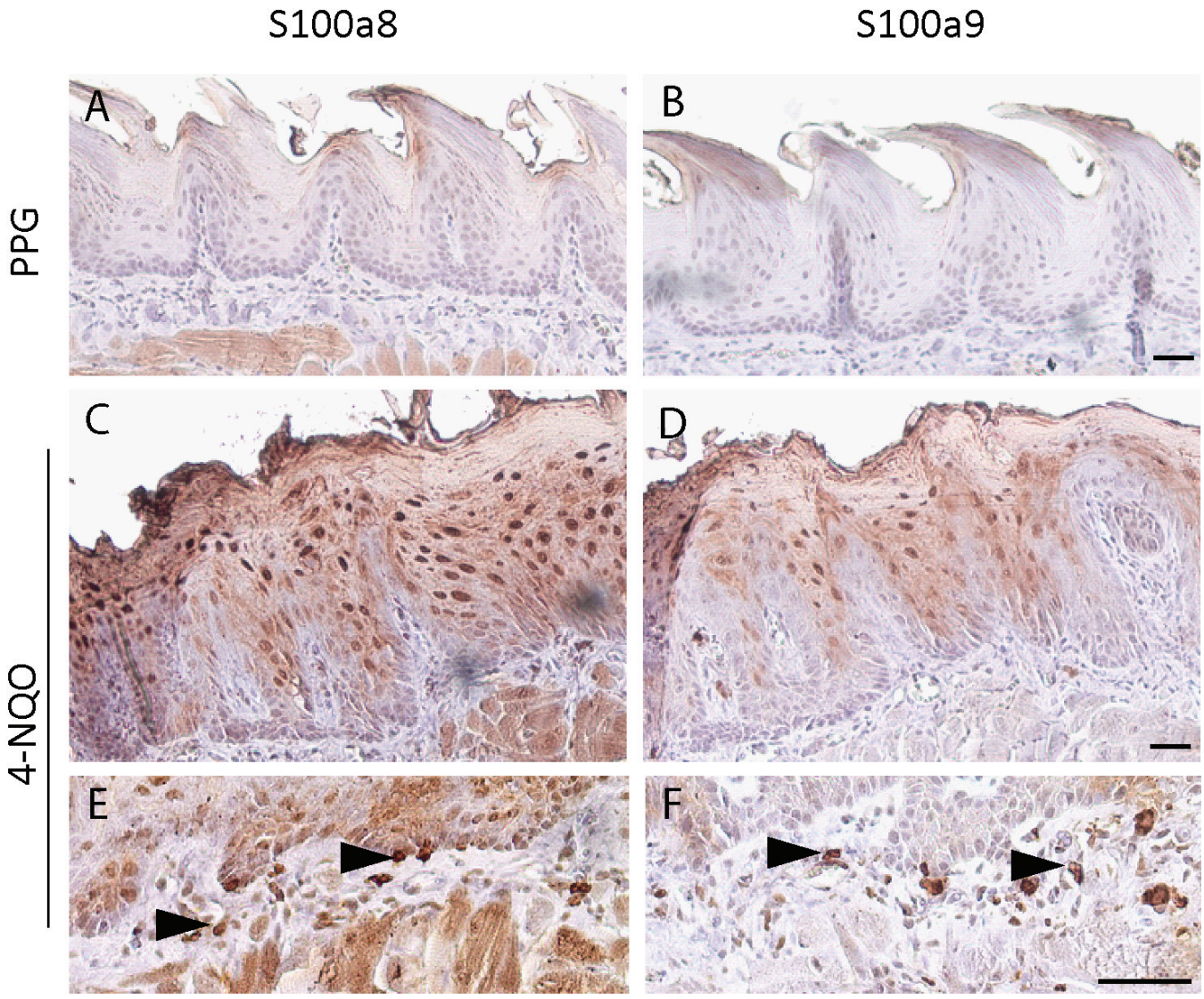


Fig. 1. S100a8 and S100a9 expression in PPG and 4-NQO treated tongue of wild type mice. Representative pictures of an immunohistochemical staining show no expression of S100a8 and S100a9 in PPG treated tongue **(A, B)**, and induced expression (brown signal) in 4-NQO treated tongue epithelium **(C, D)** as well as stromal S100a8 and S100a9 positive immune cells **(E, F)**. Counterstaining was performed with hematoxylin. Scale bars: 50 μm.

of tongue (Fig. 3A-F) and esophageal (data not shown) tissue sections derived from 4-NQO treated control and *Rage*$^{-/-}$ mice revealed similar epithelial hyperplasia and histological architecture of dysplastic lesions in both groups. Accordingly, we did not observe any obvious difference in keratinocyte proliferation between 4-NQO treated control and *Rage*$^{-/-}$ mice as determined by a BrdU incorporation assay (Fig. 3G-M).

Lack of RAGE showed no impact on the number of inflammatory immune cells in the tumor microenvironment and epithelial expression of RAGE ligands S100a8 and S100a9

Several studies confirmed an important role of RAGE signaling in the establishment and maintenance of an inflammatory reaction, specifically in mouse models of inflammation-associated carcinogenesis (Gebhardt et al., 2008; Turovskaya et al., 2008; Riehl et al., 2010). Hence, we monitored the number of myeloperoxidase (MPO) positive immune cells on tissue sections of the tongue and the esophagus from PPG or 4-NQO treated control and *Rage*$^{-/-}$ mice. Notably, we observed only a mild increase in the overall number of MPO-positive immune cells in the stroma of 4-NQO induced tumors as compared to PPG treated controls, and we found no major difference between control and *Rage*$^{-/-}$ animals (Fig. 4C,D). In line with this finding, IHC staining of tongue and esophagus tissue (data not shown) sections revealed similar expression of the pro-

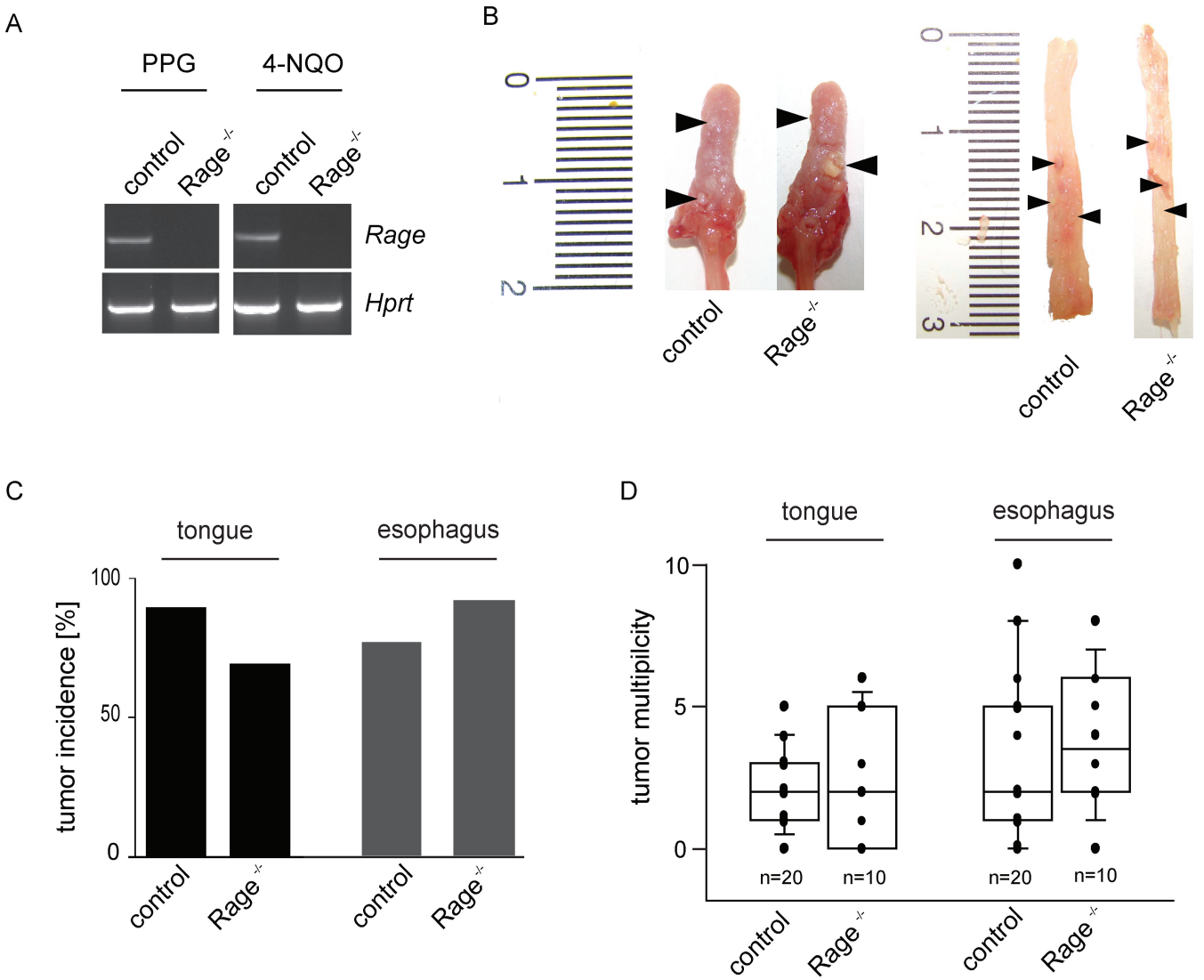
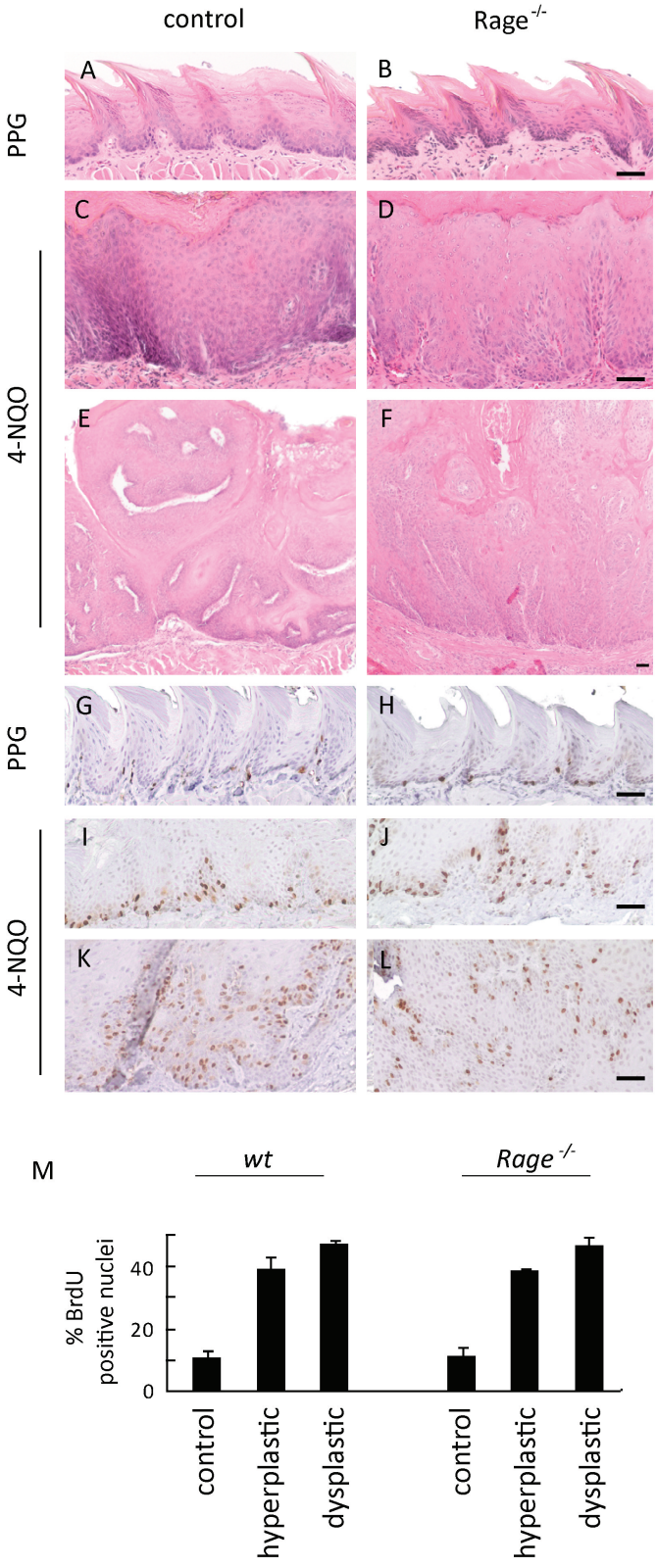


Fig. 2. 4-NQO induced oral and esophageal tumorigenesis in control and *Rage*$^{-/-}$ mice. Rage expression was determined by semi-quantitative RT-PCR with cDNA from whole tongue **(A)**. Detection of the Hprt amplicon served as control for cDNA quantity and quality. Macroscopic inspection of tongues and esophagi shows similar levels of tumor formation **(B)**. Tumor incidence **(C)** and multiplicity **(D)** is comparable between control and *Rage*$^{-/-}$ mice.



inflammatory mediators S100a8 and S100a9 in epithelial cells of 4-NQO induced dysplastic lesions and tumors, and comparable amounts of S100a8- or S100a9-positive stromal immune cells (Fig. 4 E-H,I-L)

Expression of the alternative S100a8/S100a9 receptor Tlr4 in 4-NQO induced tumors

Finally, we addressed the possibility that alternative receptors for calgranulins might compensate for the lack of RAGE in the neoplastic transformation of mucosal keratinocytes during 4-NQO induced tumorigenesis. One well-known receptor for S100a8 and S100a9 is the Toll-like receptor 4 (Tlr4), which has been shown to promote tumor growth by S100a9 (Ehrchen et al., 2009). Semi-quantitative RT-PCR analysis demonstrated induced *Tlr4* transcript levels in 4-NQO induced tumors of the tongue in both genotypes as compared to tongue tissue of PPG treated controls (Fig. 5A). Moreover, we confirmed induced expression of Tlr4 protein in both groups using IHC staining of tongue tissue sections from PPG and 4-NQO treated control and *Rage*$^{-/-}$ animals (Fig. 5B-G), suggesting that Tlr4 signaling may compensate for the lack of RAGE in the mouse model of 4-NQO induced carcinogenesis.

Discussion

So far, genetically driven as well as chemically induced mouse tumor models, which depend on a strong inflammatory response have been used to address tumor growth and progression with regard to the involvement of the S100-RAGE signaling axis. In contrast to the mouse model of inflammation-driven skin tumorigenesis, in which *Rage*$^{-/-}$ mice were almost protected from tumor development (Gebhardt et al., 2008), expression of RAGE was dispensable for tumor incidence and multiplicity in the 4-NQO model of mucosal carcinogenesis. A major difference between the DMBA/TPA and the 4-NQO model is the strength of stromal inflammation. TPA is a potent pro-inflammatory stimulus that promotes tumor formation of DMBA-initiated skin, neoplastic transformation and malignant progression. In contrast, the 4-NQO model mainly depends on genotoxic stress. In line with these data, *Rage*$^{-/-}$ mice develop less and smaller tumors in a mouse model of inflammation associated hepatocellular

Fig. 3. Histological staining and determination of keratinocyte proliferation. Representative pictures for HE staining of PPG treated normal tongue **(A, B)** and 4-NQO induced hyperplastic **(E, D)** and dysplastic **(E, F)** tongue epithelium. Keratinocyte proliferation was detected by immunohistochemical staining for BrdU (brown signal), revealing induced proliferation in 4-NQO treated hyperplastic **(I, J)** and dysplastic **(K, L)** tongue compared to PPG treated controls **(G, H)**. The relative amount of BrdU-positive cells was quantified (M) as described in the Material and Methods section. Counterstaining with hematoxylin. Scale bars: 50 μm.

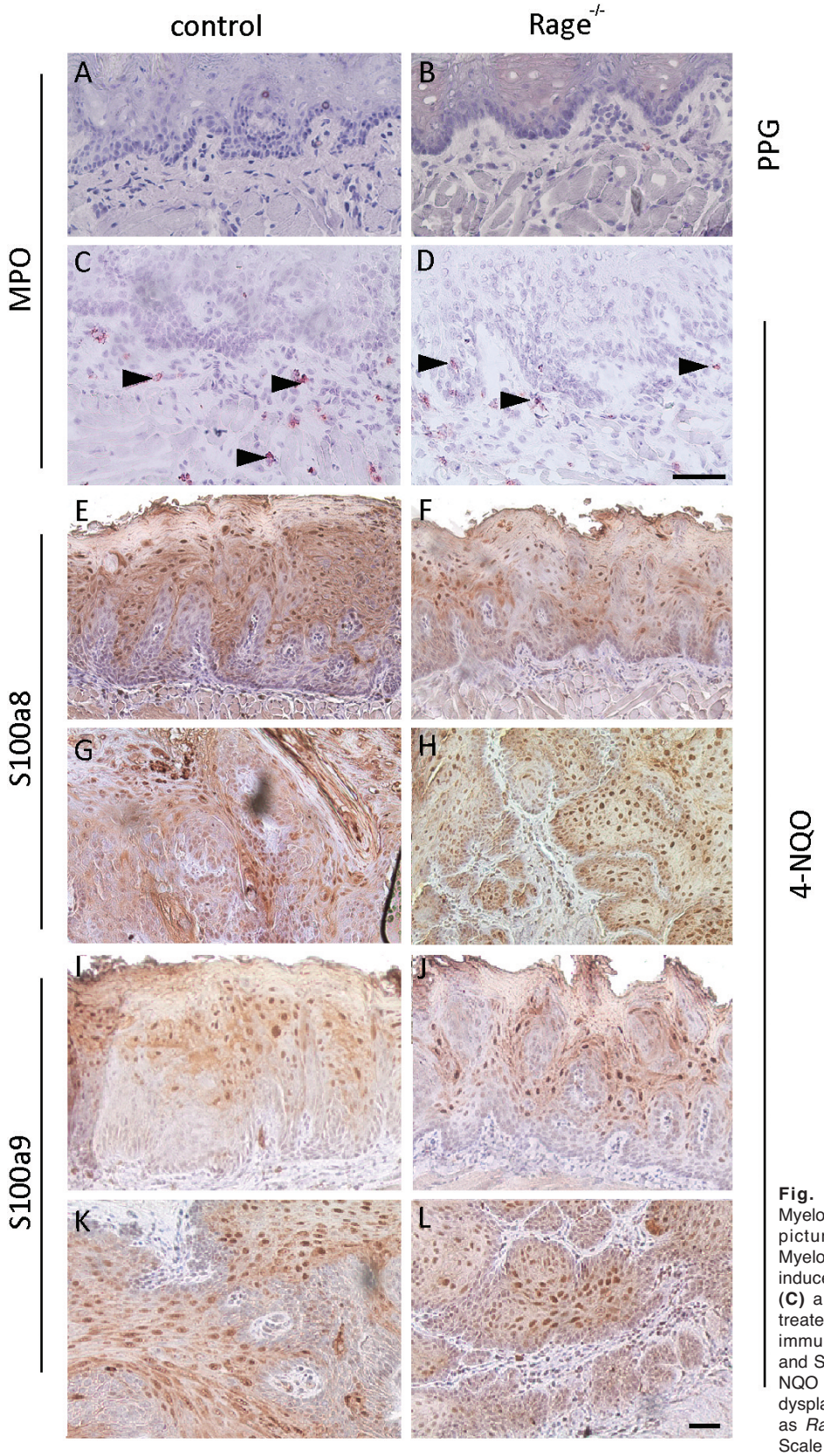


Fig. 4. Immunohistochemical staining for Myeloperoxidase and Calgranulins. Representative pictures for immunohistochemical staining for Myeloperoxidase (MPO, red signal) shows 4-NQO induced infiltration of inflammatory cells in control **(C)** and *Rage*$^{-/-}$ **(D)** mice as compared to PPG treated tongues **(A, B)**. Representative picture for immunohistochemical staining of S100a8 **(G, H)** and S100a9 **(I-L)** expression (brown signal) after 4-NQO shows induced expression in hyperplastic and dysplastic tongues in both wild type **(E, F)** as well as *Rage*$^{-/-}$ mice Counterstaining with hematoxylin. Scale bars: 50 μm.

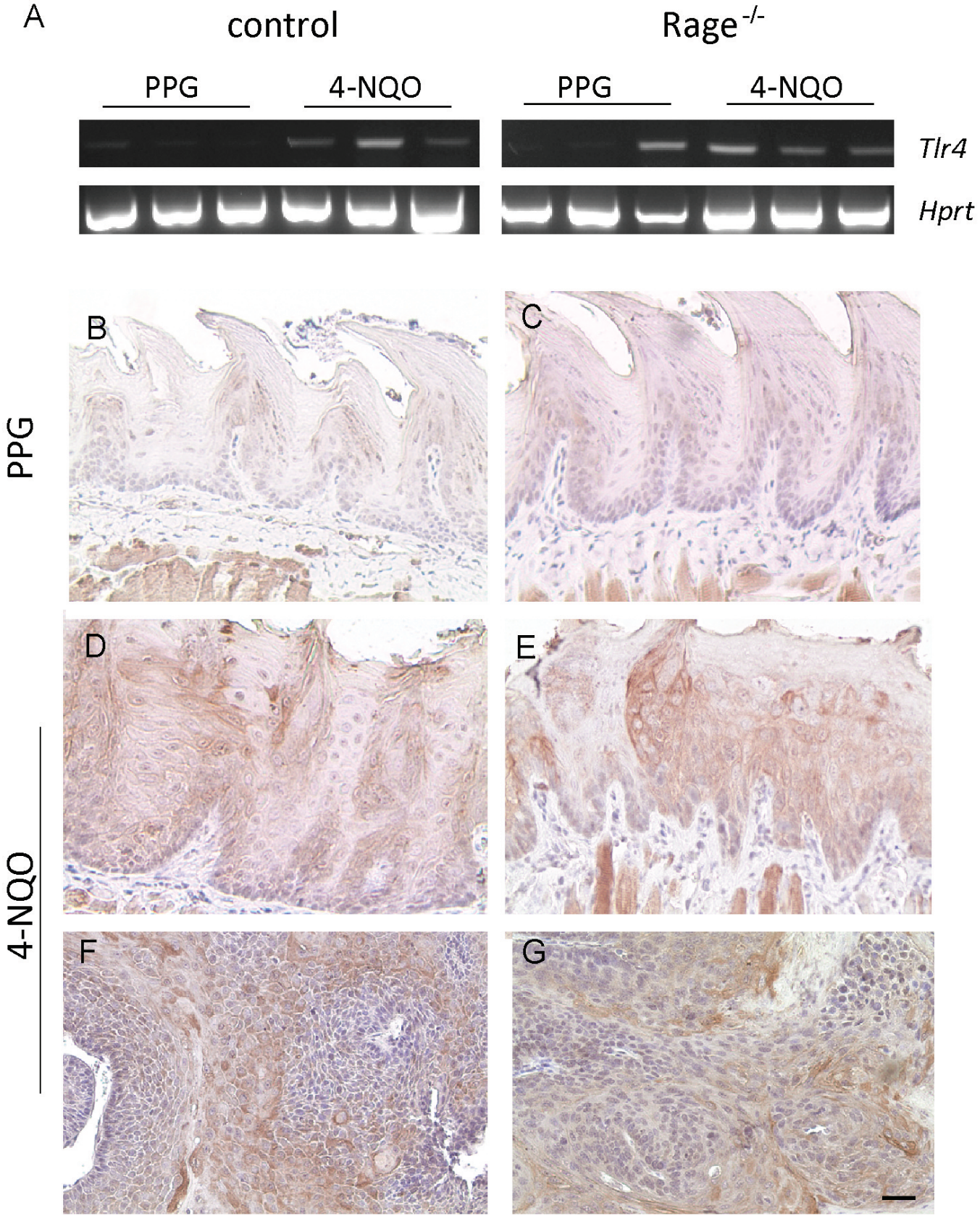


Fig. 5. Tlr4 expression in 4-NQO induced tumors of control and *Rage*$^{-/-}$ mice. Tlr4 expression was determined by semi-quantitative RT-PCR with cDNA from whole tongue in three independent animals per group **(A)**. Detection of the Hprt amplicon served as control for cDNA quality and quantity. **(B-G)** Representative pictures of an immunohistochemical staining for Tlr4 protein (brown signal). Comparison between PPG treated tongue **(B, C)** and 4-NQO treated hyperplastic **(D, E)** and dysplastic tongue **(F, G)** revealed an increased staining for Tlr4 protein after 4-NQO treatment. Counterstaining with hematoxylin. Scale bars: 50 μm.

carcinogenesis, while no significant difference was found in a mouse model of diethylnitrosamine (DEN) induced liver cancer, which is characterized by tissue damage and compensatory proliferation, but only minor infiltration of inflammatory immune cells (Pusterla et al., 2013).

Independent of the presence of RAGE, 4-NQO treatment induced expression of its ligands S100a8 and S100a9 in mucosal keratinocytes and resulted in the recruitment of S100a8 and S100a9-positive immune cells. This raised the question whether extracellular S100a8 and S100a9 may trigger alternative receptors, such as Tlr4 (Ehrchen et al., 2009) and thereby compensate for the lack of Rage in early stages of neoplastic transformation and tumor growth. Interaction of S100a9 with Tlr4 has recently been shown to promote tumor growth in a model of prostate cancer (Kallberg et al., 2012). Interestingly, impaired tumor growth was found for both *Tlr4* and *S100a9* knockout mice, but not in *Rage* deficient mice. As a perspective, S100a9 and Tlr4 single as well as double knockout mice could shed light on the role of S100/Calgranulins and Tlr4 signaling in genotoxic induced neoplastic transformation and oral carcinogenesis. However, these studies should be complemented by the analysis of S100/Calgranulin and TLR4 protein expression in larger patient cohorts in order to unravel their significance in the pathogenesis and clinical outcome of oral cancer patients.

Furthermore, while S100a8 and S100a9 are supposed to act in an extracellular fashion, we observed a prominent nuclear staining of both calgranulins after 4-NQO treatment, which were also visible, yet never described in more detail in previous studies on human HNSCC patients (Roesch Ely et al., 2005). So far, little is known about the regulation and function of nuclear S100A8 and S100A9. Even though in prostate cancer cells, S100A8 and S100A9 were previously described to be located in the nucleus and nuclear localization in immunohistochemical staining is indeed observed frequently (Hermani et al., 2006; Uhlen et al., 2010). Another yet unsolved question is the mechanism, by which S100A8 and S100a9 enter the nucleus. Neither protein has a nuclear localization signal, leaving space for speculation on possible shuttling partners and import or export mechanisms. This may argue for an intracellular mode of action, which is independent of RAGE expression. Indeed several publications describe the intracellular role of S0100a8 and S100a9 as inducers of cellular reactive oxygen species (Benedyk et al., 2007; Bøyum et al., 2010).

In summary, we employed a loss-of-function approach in genetically modified mice to demonstrate that RAGE signaling is dispensable for early onset and tumor growth in a chemically induced mouse model of mucosal carcinogenesis lacking signs of a strong inflammatory tumor microenvironment. Our data highlight the need for careful stratification of patients with regard to tumor etiology and the presence of a supportive inflammatory microenvironment as a prerequisite for efficient prevention and therapy of human malignancies, including HNSCC via therapeutic targeting of receptor RAGE (Riehl et al., 2009; Arumugam et al., 2012). This aspect needs to be taken into account, especially with regard to the current trend of Rage-targeted cancer therapy (Taguchi et al., 2000; Hudson et al., 2003; Struchler et al., 2008).

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