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Extracellular Release of a Disintegrin and Metalloproteinase Correlates With Periodontal Disease Severity

Ahmad Aljohmani¹  | Hakon Heinze¹ | Federico Guillermo Gharzia¹ | Bashar Reda^{2,3}  | Ahmed Mohamed Mostafa Abdrabou^{4,5} | Sören L. Becker⁴  | Markus Bischoff⁴  | Matthias Hannig² | Daniela Yildiz¹ 

¹Institute of Experimental and Clinical Pharmacology and Toxicology, PZMS, ZHMB, Saarland University, Homburg, Germany | ²Clinic of Operative Dentistry, Periodontology and Preventive Dentistry, University Hospital Saarland, Saarland University, Homburg, Germany | ³Department of Periodontology, School of Dentistry, Al-Shahbaa Private University, Aleppo, Syria | ⁴Institute of Medical Microbiology and Hygiene, Saarland University, Homburg, Germany | ⁵Medical Microbiology and Immunology Department, Faculty of Medicine, Mansoura University, Mansoura, Egypt

Correspondence: Daniela Yildiz (daniela.yildiz@uks.eu)

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ABSTRACT

Aim: Periodontal disease is driven by oral pathogens, including *Porphyromonas gingivalis*, and the release of inflammatory cytokines. These cytokines (e.g., TNF) or their receptors (e.g., IL-1R) are substrates of a disintegrin and metalloproteinases (ADAMs). In this study, we aimed to determine the effects of ADAMs on periodontal disease phenotypes.

Materials and Methods: Western blot and FRET-based activity measurements of the gingival crevicular fluid (GCF) of patients were compared with those of infected (*P. gingivalis*) or cytokine-stimulated oral keratinocytes and primary human neutrophils, respectively. This was accompanied by an analysis of the released extracellular vesicles and MMP9 activity.

Results: In the GCF of patients, ADAM8 protein expression and activity were correlated with disease stage, whereas ADAM10 protein expression was inversely correlated with disease stage. Infection and the resulting cytokine release orchestrated the release of soluble ADAM8 by oral keratinocytes and primary neutrophils as soluble ectodomain and on exosomes, respectively. Furthermore, ADAM8 regulated the release of ADAM10 and MMP9.

Conclusion: Dysregulation of cell-associated and extracellular ADAM proteolytic activity may be an essential regulatory element in the progression of periodontal disease driven by ADAM8. The influence of ADAM8 on disease onset and the evaluation of targeting ADAM8 as a potential and novel local treatment option should be addressed in future translational in vivo studies.

1 | Introduction

Periodontal diseases are among the most common pathologies with an estimated global prevalence of 19% and more than 1

billion cases worldwide (Jain et al. 2024). Chronic periodontitis may develop into systemic responses based on hyperinflammation and disruption of the immune response, with the risk of endomyocarditis or changes in the gut microbiome (Martinez-Garcia

Abbreviations: ADAM, a disintegrin and metalloproteinase; BHIB, brain heart infusion broth; ELISA, enzyme-linked immunosorbent assay; EVs, extracellular vesicles; FCS, foetal calf serum; FRET, fluorescence resonance energy transfer; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IFN- γ , interferon gamma; IL-1 β , interleukin 1 beta; MCF, Macfarland; MMP-9, matrix metalloproteinase 9; *P. gingivalis*, *Porphyromonas gingivalis*; RT, room temperature; TNF- α , tumour necrosis factor alpha.

The first three authors share first authorship.

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and Hernandez-Lemus 2021). Periodontitis is initiated and driven by dysbiosis of the oral microbiome, resulting in overactivation of the host immune response and changes in the cytokine network (Pan, Wang, and Chen 2019). Poor oral hygiene and a lack of frequent biofilm disruption lead to the accumulation of bacteria, such as *Fusobacterium nucleatum*, eliciting gingival inflammation and the proliferation of *Porphyromonas gingivalis* (*P. gingivalis*) (Meyle and Chapple 2015). These oral bacteria manipulate innate and adaptive immune responses, specifically targeting the antimicrobial mechanisms of neutrophils to evade killing and promote inflammation (Miralda and Uriarte 2021). Excess of reactive oxygen species, released matrix metalloproteinases (MMPs) and cytokines lead to collateral periodontal tissue loss and osteoclast activation, resulting in bone loss (Meyle and Chapple 2015; Pan, Wang, and Chen 2019). Cytokines central to periodontitis are interleukin-1 β (IL-1 β), IL-6 and tumour necrosis factor alpha (TNF- α). Upon secretion by periodontal cells and innate immune cells, they elicit the release of other cytokines from T cells, such as interferon gamma (IFN- γ) (Pan, Wang, and Chen 2019). These cytokines (TNF- α) or their corresponding receptors (IL-1R and IL-6R) are either substrates of a disintegrin and metalloproteinases (ADAMs) or induce their expression or activation (for review, see Drey Mueller, Uhlig, and Ludwig 2015). ADAMs are multidomain transmembrane proteins that are activated upon cleavage of the inhibitory prodomain. In addition to their proteolytic activity, ADAMs perform several non-proteolytic functions, such as interaction with and regulation of integrins or the extracellular matrix. In various infectious diseases, ADAMs are involved in each step of the infection process, starting with initial pathogen recognition and entrance, phagocytosis and leukocyte recruitment, up to regeneration (for review, see Aljohmani and Yildiz 2020). ADAM proteases may not only act on the expressing cells themselves but may also be released as soluble variants or on exosomes, performing proteolytic cleavage in the extracellular matrix and at distinct cellular sites. Earlier studies had shown that ADAM8 is up-regulated in the saliva of patients with periodontitis, displaying proteolytic activity (Feng et al. 2019; Hiyoshi et al. 2022), and its expression is enhanced by stimulation with *Fusobacterium nucleatum* in epithelial cells (Aung et al. 2017). To date, few studies have addressed the relevance of ADAM10 and ADAM17 in periodontal disease. For ADAM17, the up-regulation of mRNA expression in gingival tissue has been observed (Tomita et al. 2013), and an increase in receptor activator of nuclear factor- κ B ligand (RANKL) expression in osteoblast has been linked to ADAM17 protein levels (Lee et al. 2011). Inhibition experiments have revealed that ADAM10 and ADAM17 contribute to the release of chemokines from human gingival fibroblasts, thereby controlling the migration of leukocytes into inflamed tissues (Hosokawa et al. 2007). However, comparative and mechanistic studies on ADAM-based proteolytic activity and its functional impact are lacking.

We hypothesised that the proteolytic activity of ADAMs released into the extracellular space plays an essential role in the initiation, progression and severity of periodontitis. In this study, we observed an increase in ADAM8 protein expression and proteolytic activity in the gingival crevicular fluid (GCF) of patients suffering from periodontitis, which was correlated with MMP9 release and the disease stage. Especially, the release of ADAM8 on exosomes disturbed the oral keratinocyte regeneration, further fostered by its influence on MMP9 and ADAM10. Our observation suggests that ADAM8 and its interaction partners

might represent novel treatment targets for the prevention of periodontal disease progression, which should be—together with a potential impact on disease initiation—addressed in future translational in vivo studies.

2 | Materials and Methods

2.1 | Patient Study

This study was approved by the ethics committee of the Landesärztekammer des Saarlandes (345/20 by D.Y. and M.H.) and was performed in accordance with the Declaration of Helsinki. Written informed consent was obtained from all individuals visiting either the Clinic for Dental Preservation, Periodontology, and Preventive Dentistry of Saarland University Hospital, or the dental office of Reinstädtler and Hektor. The samples were pseudonymized during their transfer from the clinic to the research area. The exclusion criteria, patient characteristics (Table S1) and classifications are given in the [Supporting Information](#).

2.2 | Sample Preparation

Paper points were inserted into the periodontal pockets and left for 30 s. The same procedure was followed for up to three further sampling sites on the same tooth, and all paper points were transferred together into a reaction vessel. The samples were stored at 4°C for a maximum of 7 days until further processing. Detailed sample preparation methods for western blotting and activity assays are given in the [Supporting Information](#).

2.3 | Buffers and Solutions

Buffers and solutions used in this study are shown in Table S2.

2.3.1 | Anaerobic Bacteria Preparation

P. gingivalis strain DSM 20709 (syn. ATCC 33277), originally isolated from human gingival sulcus, was obtained from the German Collection of Microorganisms and Cell Cultures GmbH (DSMZ) and used throughout the study. Bacterial culture and cell preparation are described in the [Supporting Information](#).

2.3.2 | Cell Culture and Cell Stimulation

Human keratinocytes (K2; a gift from Dr. Miosge, University of Göttingen) were cultured in Keratinocyte Growth Medium 2 (PromoCell, Heidelberg, Germany). Primary human neutrophils were cultured in RPMI1640 (with 0.2% foetal calf serum [FCS]). Neutrophil isolation, culture conditions, inhibitors and stimulant treatments are described in the [Supporting Information](#).

2.3.3 | Western Blot Analysis

K2 cells or human neutrophils were lysed with 150–200 μ L of lysis buffer (Table S2), and proteins were separated according

to their molecular weight by SDS-PAGE as described in the [Supporting Information](#) (Aljohmani, Andres, and Yildiz 2022; Aljohmani, Opitz, et al. 2022).

2.3.4 | Activity Measurement

To assess the activity of soluble ADAM8 in patient samples, as well as its release from K2 cells and human neutrophils, fluorescence resonance energy transfer (FRET)-based substrates containing specific substrate cleavage sites were used, as described in the [Supporting Information](#).

2.3.5 | Gelatin Zymography

Equal protein amounts isolated from the GCF were loaded onto an SDS-polyacrylamide (5%) gel supplemented with 0.1% gelatin (from porcine skin, Sigma-Aldrich, Taufkirchen, Germany). Enzyme activity was evaluated as described in the [Supporting Information](#).

2.3.6 | Enzyme-Linked Immunosorbent Assay

Cell lysates, cell culture supernatants and sulcus fluid from patients at different disease stages were evaluated for MMP-9 content using the ELISA Duo Set (R&D System, Wiesbaden, Germany, Cat No: DY911) following the manufacturer's protocol.

2.3.7 | Exosome Preparation and Wound Closure (Scratch) Assay

Isolation of exosomes from the cell culture medium of K2 cells or human neutrophils and the scratch assay, an *in vitro* wound closure and tissue layer regeneration assay, are described in detail in the [Supporting Information](#).

2.4 | Statistical Analysis

Quantitative data are shown as mean \pm SD. Statistical analyses were performed using GraphPad PRISM 9.0 (GraphPad Software, La Jolla, CA, USA). The numbers (*n*) and statistical tests are provided in the figure legends. Statistical significance was set at $p < 0.05$.

3 | Results

3.1 | Presence of ADAM8 and Its Proteolytic Activity in Gingival Fluid Increases With Disease Stage

To determine the potential contribution of ADAMs to the severity of periodontal disease, the protein expression levels of ADAM8, 10 and 17 were investigated in the sulcus fluid of healthy patients and those with periodontitis. We observed a stage-dependent increase in ADAM8 total protein expression,

shown by an increase in both the pro-form (120 kDa, only seen in 72% of the patients) and mature form (90 kDa), as well as an increase in maturation (the transformation of the pro-form to the mature form; Figures 1A–C and S1A). The remnant form (the part of ADAM8 remaining in the membrane after the autocatalytic release of the extracellular domain) was slightly, but not significantly, increased at all disease stages (Figure S1B). Interestingly, the protein expression of ADAM10, a substrate of ADAM8, was strongly increased upon disease onset (Stages I–II) but reduced at later stages of the disease (Figure 1D,E and Figure S1C; pro-form 100 kDa, mature form 70 kDa). This was further reflected in the maturation level (Figure 1F), which was measured as the ratio of the mature form to the pro-form. In contrast, the expression of ADAM17 did not change (Figure S1D–G; pro-form 130 kDa, mature form 100 kDa). Notably, the healthy patients did not show the presence of these forms of ADAM8, 10 or 17. Since maturation does not directly indicate activation, we analysed the activity of ADAM8 using a specific FRET substrate, including the cleavage site of CD23. At the early onset of disease (Stages I–II), no changes were observed in patients with periodontal disease compared with healthy patients, and activity massively increased in highly diseased patients (Figure 1G,H).

3.2 | *P. gingivalis* Induces ADAM8 Gene Expression and Release of ADAM8 to the Extracellular Matrix

The development of periodontitis is driven by initial dysbiosis at the local site, which includes *P. gingivalis* as a central player in the red complex causing gingivitis. Therefore, we analysed ADAM8 and ADAM10 expression in primary human keratinocytes infected with *P. gingivalis*. We observed an increase in ADAM8 pro-form and mature form, and a decrease in the pro-form of ADAM10 (Figures 2A–D and S2A,B). Since the intracellular marker protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH) can be detected only in the sulcus fluid of a few patients, we hypothesised that the observed expression and activity of ADAMs in patient samples may be mostly derived from cell-free structures. As mentioned previously, part of ADAM8 is released as a soluble ectodomain. We observed increased levels of the remaining ADAM8 remnant form upon infection (Figure 2E). The second major source of external activity is the ADAM8-carrying exosomes. However, during the period monitored in our study, only higher amounts of the remnant form, not the mature form, were released on exosomes, (Figure 2F). However, infection with *P. gingivalis* induced a clear release of mature ADAM10 on exosomes (Figure 2G). Furthermore, FRET-based activity measurements revealed a strong increase in ADAM8 activity in *P. gingivalis*-challenged cells, which was significantly inhibited by the ADAM8-specific inhibitor BK-1361 (Schlomann et al. 2015) (Figure 2H). Furthermore, we observed an increase in ADAM10 activity, which was significantly inhibited by the ADAM10-specific inhibitor GI254023X but only slightly affected by BK-1361 (Figure 2I). Thus, the release of ADAM8 as a soluble ectodomain by oral keratinocytes may contribute to the proteolytic pool observed in patient samples, whereas the membrane-associated forms may be derived from different cellular sources.

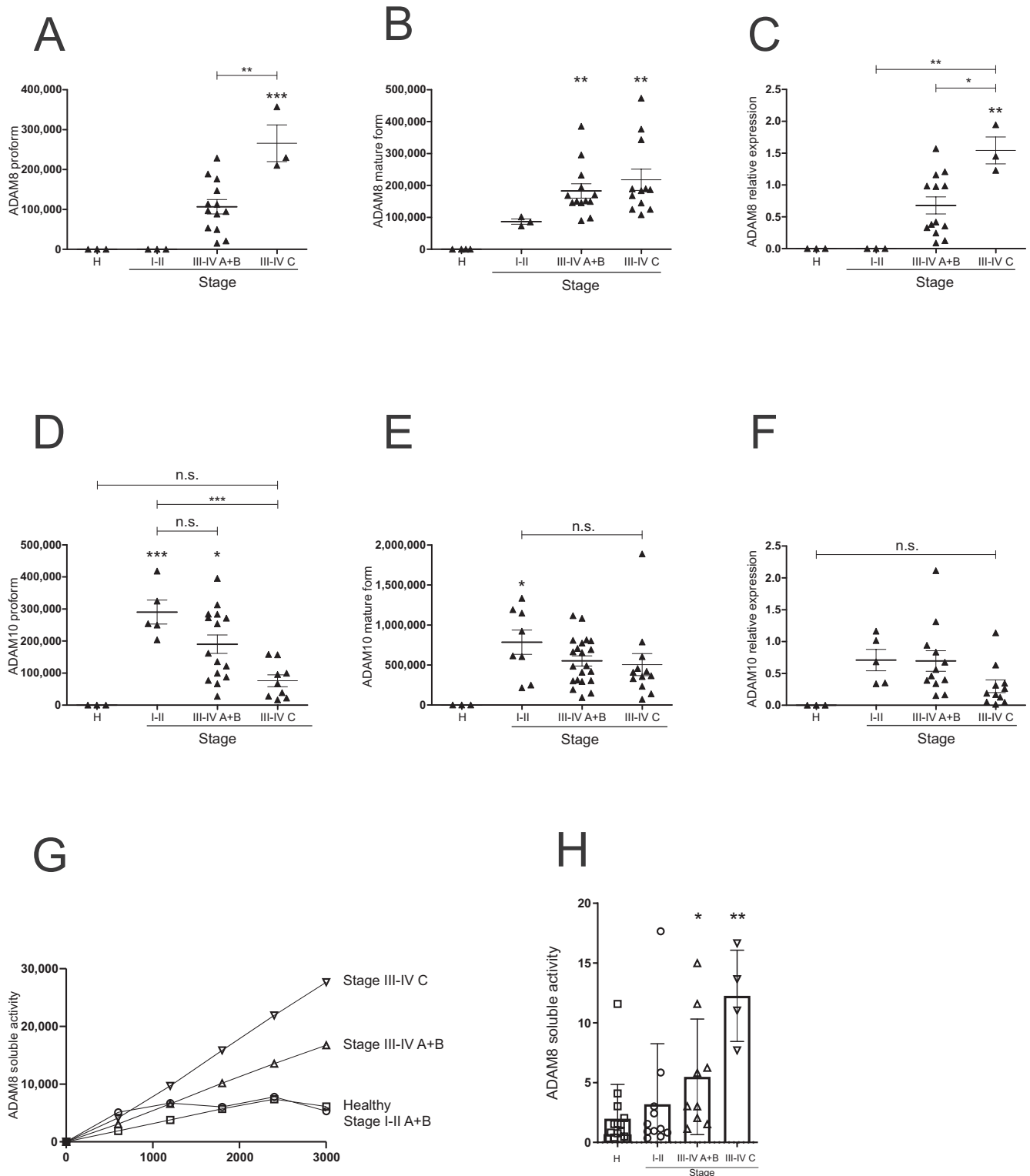


FIGURE 1 | ADAM8 proteolytic activity in gingival fluid is associated with disease severity. (A–F) Sulcus fluid from patients with different disease stages were investigated by Western blotting to analyse the protein expression and maturation of ADAM8 and ADAM10 using antibodies against the C-terminal domains. Band intensity was quantified by densitometry (A, pro-form ADAM8; 120 kDa; B, mature form ADAM8, 90 kDa; D, pro-form ADAM10; 100 kDa; E, mature form ADAM10, 70 kDa). Maturation was determined as ratio of the mature form and pro-form of the respective protease (C and F). (G, H) Sulcus fluid was resuspended in activity buffer and subjected to ADAM8 activity measurement using a FRET-based substrate ($n = 3–12$ per group). Activity over time is shown in (G) and was used for the calculation of the slope shown in (H). Data are shown as mean \pm SD in (A–F) and (H), and activity over time in (G). Statistical analysis was performed with one-way ANOVA followed by Tukey's post hoc test. Asterisks without line represent statistically significant differences to healthy patients; asterisks with line between groups ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$).

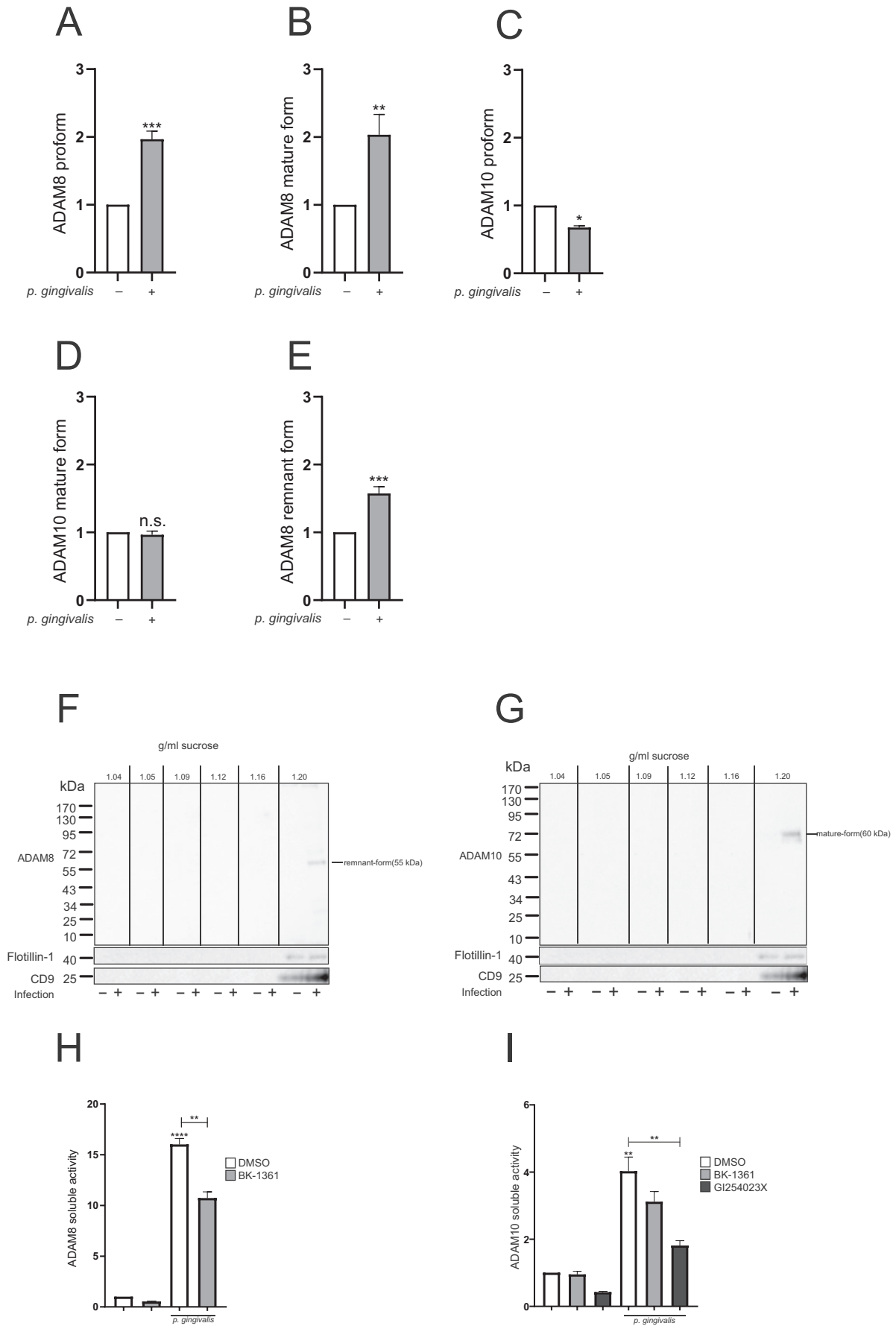


FIGURE 2 | Legend on next page

3.3 | *P. gingivalis*-Induced ADAM8 Activation Correlates With Tissue Destructive Activity

One characteristic of periodontal disease development is over-activation of the immune response, in which neutrophils dominate the activated lesion, bridging both the innate and acquired immune systems (Meyle and Chapple 2015). Infection of primary human neutrophils with *P. gingivalis* resulted in the release of exosomes carrying both the pro-form and the remnant form of ADAM8 (Figure 3A). In contrast, neutrophils showed a moderate constitutive release of ADAM10 in exosomes under these culture conditions, which did not increase upon infection (Figure 3B). Key factors for periodontal connective tissue destruction are MMPs such as MMP9, which have been shown to be regulated by ADAM8 (Cook et al. 2022). The zymography of patient samples revealed an increase in gelatinase activity at higher disease stages (Figure S3A), as confirmed by the MMP9 ELISA (Figure 3C). Infection of primary human neutrophils with *P. gingivalis* resulted in an increase in cell-associated MMP9 expression (Figure 3D), whereas the released content decreased (Figure 3E). However, in both cases, MMP9 expression was significantly inhibited by BK-1361, whereas no effect on ADAM10 inhibition was observed. In contrast to neutrophils, MMP9 expression or release by keratinocytes was unaffected by infection and inhibition, respectively (Figure S3B,C). To address the tissue-destructive action of the extracellular vesicles (EVs) and soluble factors (vesicle-free supernatant) in more detail, wound healing assays (scratch assays) were performed. Exosomes released by *P. gingivalis*-infected neutrophils strongly reduced keratinocyte wound closure, which was reversed by co-treatment with BK-1361. Inhibition of ADAM10 by GI254023X had no beneficial effect on the migration capacity of exosome-challenged keratinocytes but displayed general migration inhibition even in control-treated cells (Figures 4A and S4A), as observed earlier (Pruessmeyer et al. 2014). Similar to exosomes, soluble factors released by neutrophils inhibited scratch closure, which was not affected by BK-1361 because this compound does not inhibit the soluble form of ADAM8 (Figure 4B) (Conrad et al. 2022). We next investigated whether the release of damaging exosomes and soluble factors could be prevented by ADAM8 inhibition. Indeed, pre-incubation of neutrophils with BK-1361 before *P. gingivalis* challenge reduced the tissue-damaging effects of the released exosomes (Figure 4C) and soluble factors (Figure 4D). Thus, ADAM8 in neutrophils may regulate tissue destruction in periodontal disease.

3.4 | Cytokines May Orchestrate the ADAM Response in Periodontal Disease

Pathogens in the oral cavity lead to the release of cytokines that not only regulate the host immune response and osteolytic activity but also affect the tissue damage (Pan, Wang, and Chen 2019). The cytokine pool consists of the IL-1 and TNF families, which are released by periodontal cells, myeloid cells and lymphocytes. To mimic this process, we stimulated oral keratinocytes with a combination of TNF α , IL-1 β and IFN γ . After 24 h, stimulated keratinocytes showed an increase in ADAM8 pro-form, mature form and remnant form (Figure 5A–C). Interestingly, upon long-term stimulation (48 h), only the pro-form remained elevated, whereas the other forms returned to their unstimulated levels. For ADAM10, we observed a similar time-dependent increase in protein expression; however, this did not reach significance due to the high standard deviations (Figure 5D,E). In contrast to the expression of ADAM8, that of the pro-form of ADAM10 was reduced after 48 h of stimulation. Stimulated keratinocytes released an increased number of exosomes 48 h post stimulation, as indicated by an increase in flotillin-1 and CD9 intensity (Figure 6A,B). This was accompanied by moderate expression of ADAM8 on these exosomes but a strong increase in mature ADAM10 and a potential cleavage fragment (50 kDa). Notably, almost no ADAM8 signal was detected in the exosome fraction of unstimulated K2 cells. Activity measurements of ADAM8 and ADAM10 released from human primary keratinocytes and neutrophils indicated a strong increase in ADAM8 activity in stimulated cells, which was inhibited by BK-1361 (Figure 6C,E). The increase in ADAM10 activity caused by stimulation with the cytokine mixture was inhibited by GI254023X in both keratinocytes and neutrophils. However, ADAM8 inhibition by BK-1361 affected ADAM10 activity in cytokine-stimulated neutrophils but not in K2 cells (Figure 6D,F).

Thus, bacterial infection and induced cytokine release stimulate a cell-specific release of ADAM8, orchestrating tissue-destructive activity and resulting in novel proteolytic effector molecules such as ADAM10.

4 | Discussion

In the present study, we observed an increase in ADAM8 protein expression and activity in the sulcus fluid, correlating with the periodontal disease stage. Our data suggest that both *P.*

FIGURE 2 | *P. gingivalis* induces ADAM8 expression and activity in human oral keratinocytes. Human primary keratinocytes (K2 cells) were grown until confluency and infected with *P. gingivalis* (2 h, 0.1 McF). (A–E) The cells were lysed, and the samples were analysed by Western blotting using antibodies against the C-terminal domains of ADAM8 and ADAM10, respectively. GAPDH served as loading control. Band intensity was quantified by densitometry and normalized to uninfected cells. (F, G) The medium of the infected and non-infected cells was subjected to differential centrifugation and density fractionation for exosome purification. The resulting pellets of different densities were analysed by Western blotting for the expression of ADAM8 and ADAM10 as well as Flotillin-1 and CD9 as exosomal markers. Representative blots of three independent experiments are shown. (H, I) K2 cells were pre-incubated with 10 μ M BK-1361, 10 μ M GI254023X, or 0.01% DMSO (vehicle control) for 30 min and infected with *P. gingivalis* (2 h, 0.1 McF). The medium was analysed for ADAM8 or ADAM10 activity using a FRET-based assay, and the slope of the activity over time was calculated. Quantitative data are shown as mean + SD of three independent experiments. Statistical analysis was performed with one-sample *t*-test (A–E) and one-way ANOVA (H, I). Asterisks represent statistical differences (* p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001, n.s. not significant).

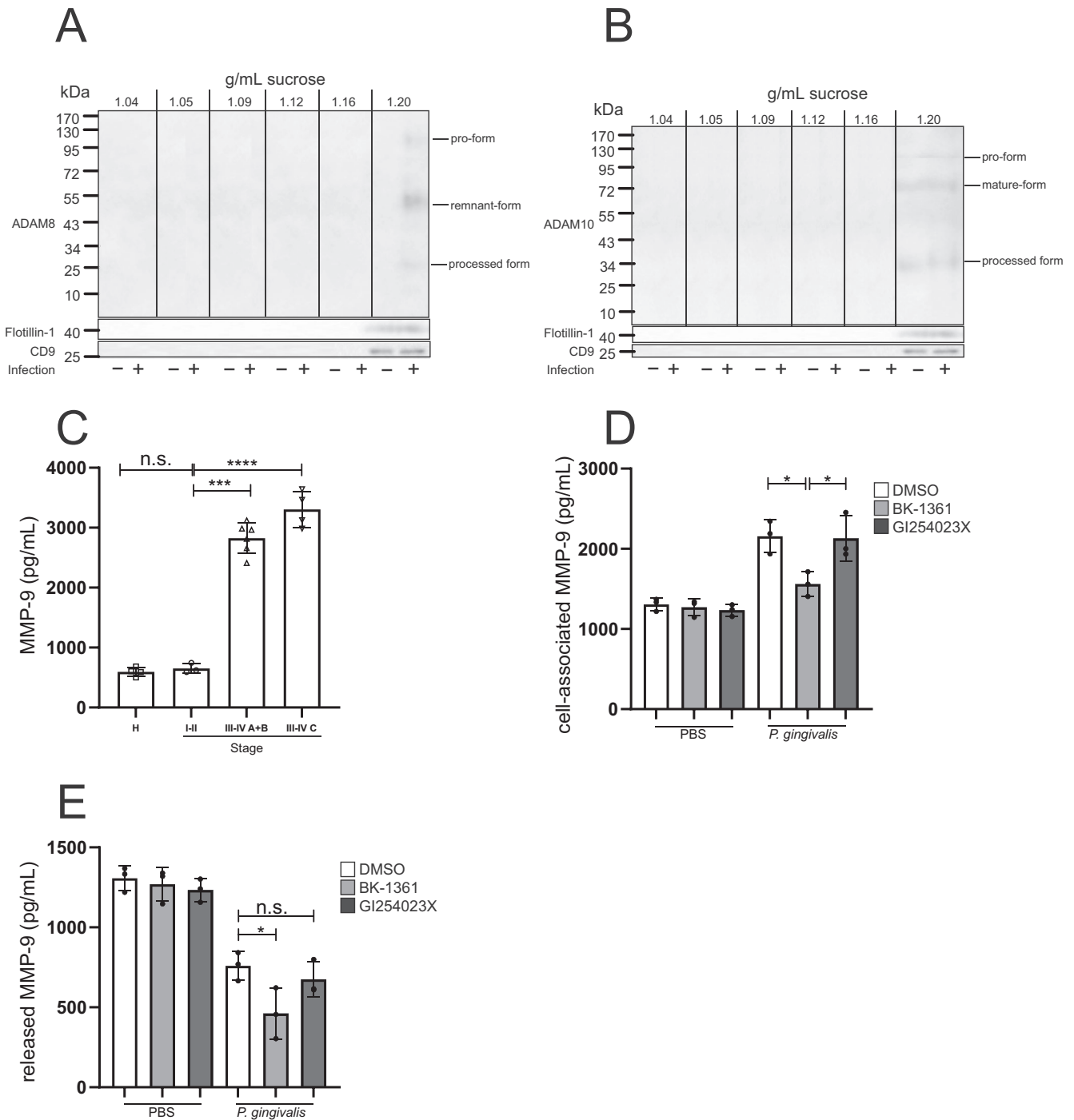


FIGURE 3 | *P. gingivalis*-induced ADAM8 activation correlates with proteolytic disbalance. (A, B) Primary human neutrophils were infected with *P. gingivalis* (2 h, 0.1 McF) or left uninfected. The medium of the infected and non-infected cells was subjected to differential centrifugation and density fractionation for exosome purification. The resulting pellets of different density were analysed by Western blotting for the expression of ADAM8 and ADAM10 as well as Flotillin-1 and CD9 as exosomal markers. Representative images of three independent experiments are shown. (C) Sulcus fluid from patients with different disease stages was analysed for MMP-9 expression by ELISA. (D, E) Primary human neutrophils were pre-incubated with 10 μ M BK-1361, 10 μ M GI254023X or 0.01% DMSO (vehicle control) for 30 min and infected with *P. gingivalis* (2 h, 0.1 McF). Subsequently, the cells were lysed, and the cell lysate and the medium were analysed for MMP-9 expression and release by ELISA. Neutrophil data are shown as mean \pm SD of three independent experiments. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. Asterisks without line represent statistically significant differences with healthy patients and asterisks with line between groups ($*p < 0.05$, $***p < 0.001$, $****p < 0.0001$, n.s. not significant).

gingivalis infection and the resulting cytokine release orchestrate the release of soluble ADAM8 by keratinocytes and neutrophils as soluble ectodomains or on exosomes, respectively.

Furthermore, we observed ADAM8 regulated the release of ADAM10 and MMP9, which influenced wound healing and tissue destruction at least *in vitro*.

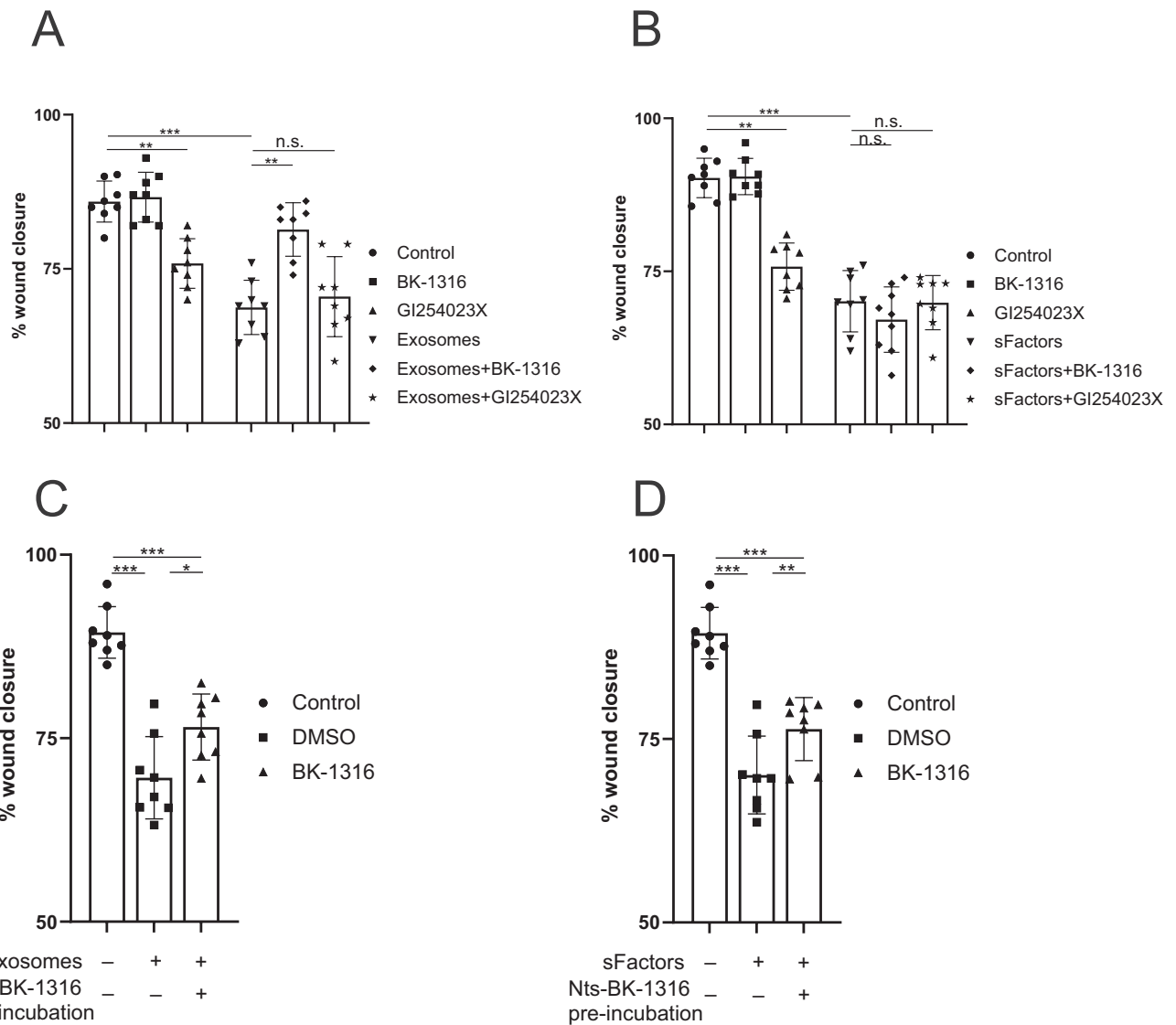


FIGURE 4 | *P. gingivalis*-induced ADAM8 activation correlates with tissue destruction. (A–D) K2 cells were grown until forming a monolayer, incubated with mitomycin ($1 \mu\text{g}/\text{mL}$) for 2 h followed by wound induction using an automatic scratcher. (A, B) Primary human neutrophils were infected with *P. gingivalis* (2 h, 0.1 McF). The medium of the infected cells was subjected to differential centrifugation and density fractionation for exosome purification and collection of soluble factors (supernatant). Subsequently, K2 cells were incubated with (A) exosomes or (B) soluble factors in the presence or absence of $10 \mu\text{M}$ BK-1361 or $10 \mu\text{M}$ GI254023X and evaluated for wound closure. (C, D) Primary human neutrophils were pre-incubated with $10 \mu\text{M}$ BK-1361 or 0.01% DMSO (vehicle control) for 30 min and infected with *P. gingivalis* (2 h, 0.1 McF) followed by exosomes purification and collection of soluble factors from the medium. Subsequently, K2 cells were incubated with (C) exosomes or (D) soluble factors and evaluated for wound closure. Data are shown as a percentage of wound closure relative to closed wound. Quantitative data are shown as means \pm SD of eight independent experiments. Asterisks indicate significance among treated cells calculated using one-way ANOVA and Tukey's post-test ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$).

In previous studies, only total ADAM8 protein expression was examined (Aung et al. 2017; Feng et al. 2019), which was similarly increased in our patient cohort and correlated with the disease stage. ADAMs are strongly regulated at the post-transcriptional level, resulting in the activation and generation of different forms of the protein. In our study, an increase in ADAM8 maturation and proteolytic activity was observed for the first time, again correlating with disease stage. It has been recently reported that ADAM8 exerts proteolytic activity on ADAM10 and ADAM17, thereby generating soluble ectodomains (Scharfenberg et al. 2020). Following this, we observed a decrease in the expression of membrane-associated

forms of ADAM10 protein in the sulcus fluid. In vitro, we confirmed that ADAM8 activation was responsible for the increase in ADAM10 proteolytic activity in both oral keratinocytes and neutrophils.

The observed soluble activity may result from the release of ADAM-carrying EVs (Cook et al. 2022; Groth et al. 2016) or soluble ectodomains that still exert proteolytic activity. ADAM8 requires trimerization for activation, resulting in the autocatalytic removal of the inactivating pro domain and the release of its soluble ectodomain (Naus et al. 2006). This trimerization is inhibited by the cyclic peptide inhibitor BK-1361

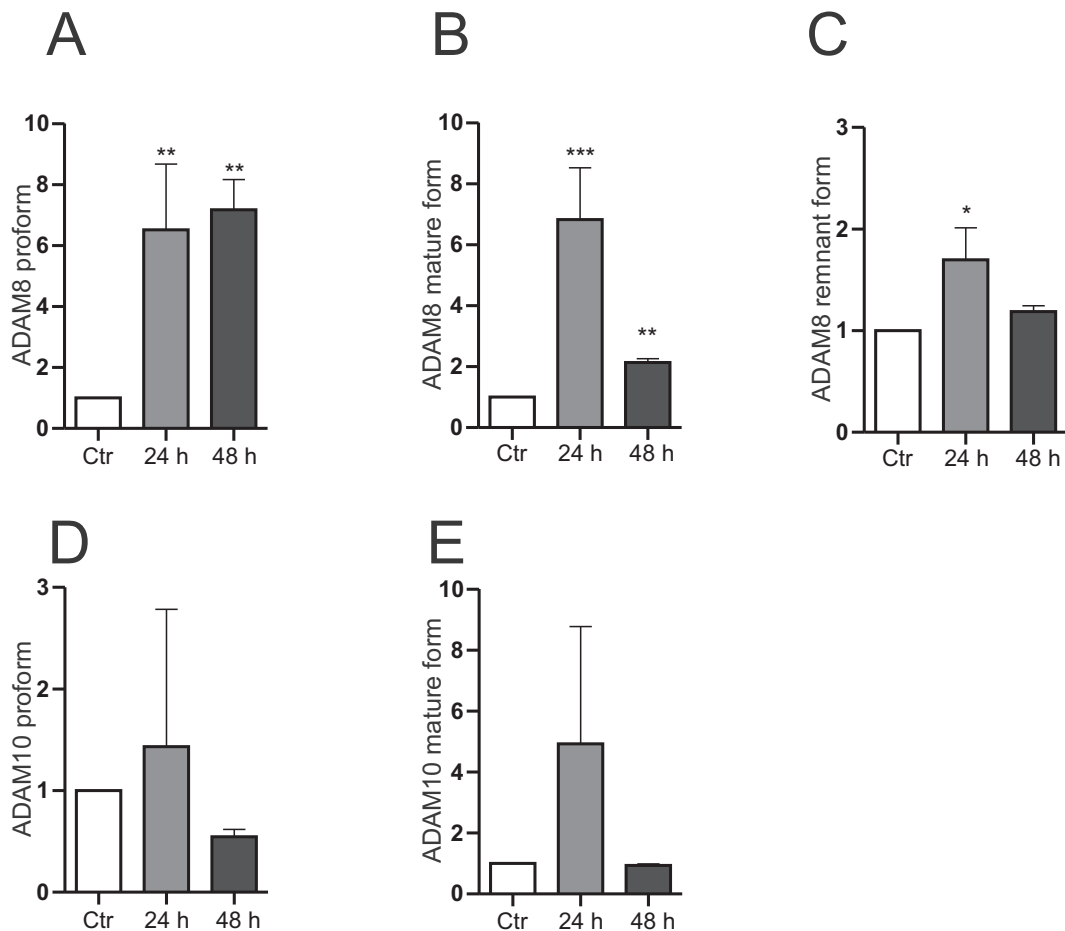


FIGURE 5 | Cytokines regulate ADAM8 expression in human oral keratinocytes. (A–E) K2 cells were grown until confluency and stimulated with a combination of TNF α , INF- γ and IL-1 β (10 μ M each) or sterile water (vehicle control) for 24 and 48 h. Subsequently, the cells were lysed, and the samples were analysed by Western blotting using antibodies against the C-terminus of ADAM8 and ADAM10, respectively, with β -actin as loading control. Band intensity was quantified by densitometry and normalized to unstimulated cells. Data are shown as mean + SD of three independent experiments. Statistical analysis was performed by one-sample *t*-test. Asterisks represent statistical differences (* p < 0.05, ** p < 0.01, *** p < 0.001).

(Schlomann et al. 2015). While we observed strong inhibition of ADAM8 activity by BK-1361 in cytokine-stimulated oral keratinocytes, the inhibition of ADAM8 activity was weaker in *P. gingivalis*-infected cells. This was further reflected in the membrane-associated release of the mature form of ADAM8 in EVs, which was absent upon infection; only the remnant form was observed. Furthermore, ADAM8 inhibition did not affect, or only slightly affected, the increase of ADAM10 activity in cytokine-stimulated keratinocytes. In contrast, the neutrophil-derived ADAM8 activity was strongly inhibited by BK-1361, which further inhibited ADAM8 release. Thus, keratinocytes appear to release ADAM8 as a soluble ectodomain, whereas neutrophils are the primary cellular source of ADAM8-carrying exosomes.

Periodontal pathogens have developed strategies to evade the immune defences of the host (Miralda and Uriarte 2021). It was recently shown that ADAM8-deficient mice are protected against bacterial lung infections, accompanied by a higher clearance capacity (Conrad et al. 2022). Similarly, increased ADAM8 protein expression in periodontal disease may be associated with reduced bacterial clearance and increased bacterial burden. Furthermore, ADAM8 is important for MMP-9 release in the inflammatory

tumour environment (Cook et al. 2022). Another study demonstrated that MMP-9 is released during infection with *P. gingivalis*, thereby affecting the breakdown of inflamed human pulp tissue (Ding et al. 1997), and MMP inhibition protects against alveolar bone loss (Ramamurthy et al. 2002). In our study, neutrophils were identified as the major source of MMP9 release, which was inhibited by ADAM8. However, ADAM8 may modulate tissue destruction and progression not only through MMP9 but also through the release of ADAM10. The soluble form of ADAM10 can cleave fibronectin (Scharfenberg et al. 2020), and fibronectin fragments have been associated with periodontal disease status (Huynh et al. 2002). In addition to the release of soluble ADAM10 variants, we detected a decrease in membrane-associated expression with increasing disease stage, which was also observed in our cell culture-based experiments. Cell-associated ADAM10 is required for the cleavage of growth factors such as epidermal growth factor, thereby ensuring proper wound healing (Shin et al. 2023). Cell-associated ADAM10 also contributes to keratinocyte adhesion and migration (Maretzky et al. 2005). Thus, ADAM8-dependent modulation of ADAM10 expression and activity may promote tissue degradation while inhibiting repair processes. Which factors and mechanisms drive the transition from gingivitis as inflammatory lesion to periodontitis is still poorly understood. Dysbiosis

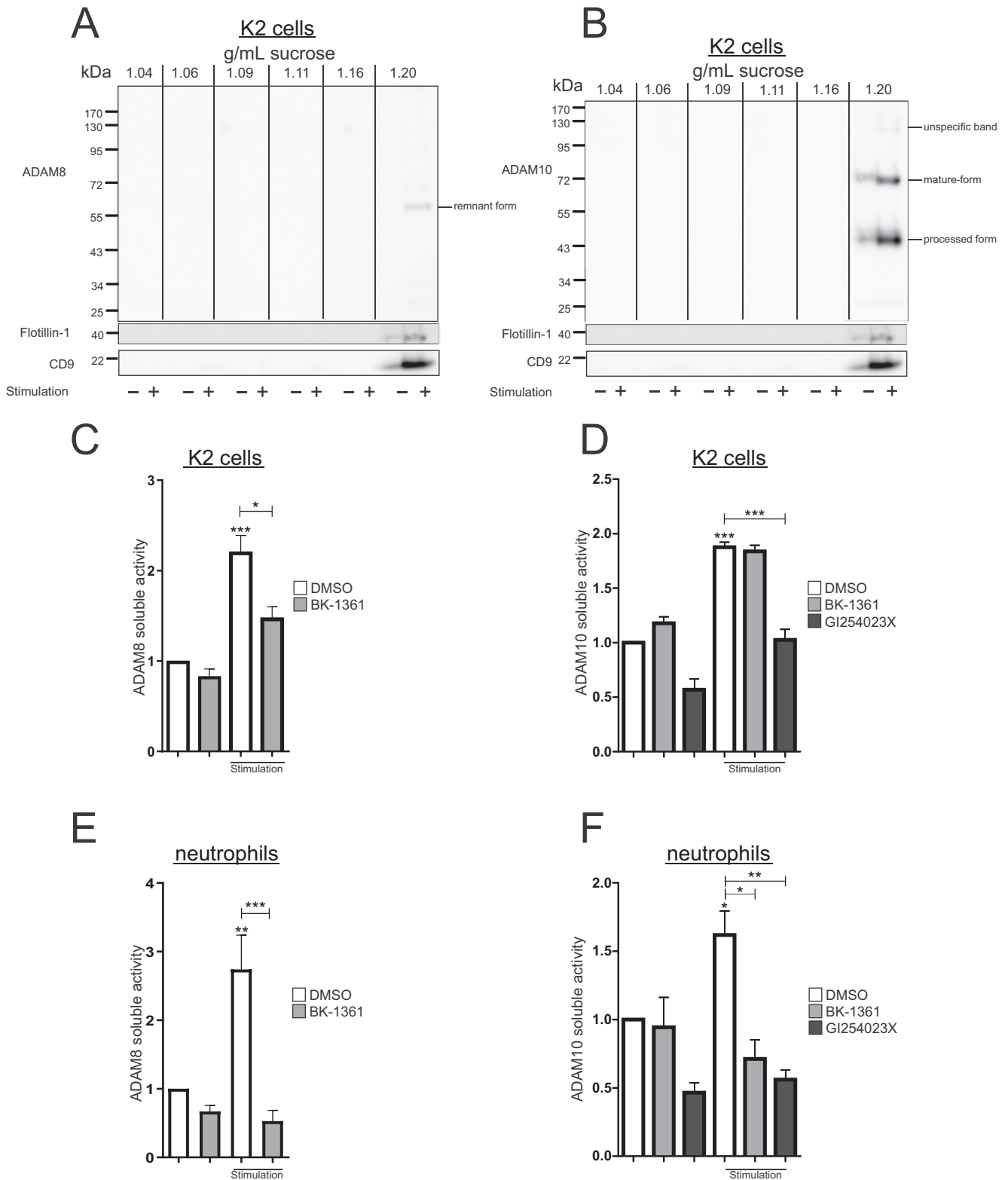


FIGURE 6 | Regulation of extracellular release of ADAM8 and ADAM10 by cytokines. (A, B) K2 cells were grown until confluency and stimulated with a combination of TNF α , INF- γ and IL-1 β (10 μ M each) or sterile water (vehicle control) for 48 h. The medium of the stimulated and non-stimulated cells was subjected to differential centrifugation and density fractionation for exosome purification. The resulting pellets of different density were analysed by Western blotting for the expression of ADAM8 and ADAM10 as well as Flotillin-1 and CD9 as exosomal markers. Representative blots of three independent experiments are shown. (C–F) K2 cells (C, D) or primary human neutrophils (E, F) were pre-incubated with 10 μ M BK-1361, 10 μ M GI254023X or 0.01% DMSO (vehicle control) for 30 min followed by stimulation with a combination of TNF α , INF- γ and IL-1 β (10 μ M each) or sterile water (vehicle control) for 48 h. The medium was analysed for ADAM8 (C, E) or ADAM10 (D, F) activity using a FRET-based activity assay. Data are shown as mean + SD of three independent experiments. Statistical analysis was performed using one-sample *t*-test. Asterisks without line represent statistically significant differences to healthy patients, and asterisks with line between groups (**p* < 0.05, ***p* < 0.01, ****p* < 0.001).

and inflammation are involved in both the onset and progression of periodontitis, which led to the ‘inflammation-mediated polymicrobial emergence and disease exacerbation’ (IMPEDE) model (Abdulkareem et al. 2023). Our recent observations indicate an ADAM8-dependent hyperactivity of neutrophils, leading to reduced regeneration and increased tissue destruction. Thus, it seems feasible that the proteolytic imbalance driven by ADAM8 may influence the shift from controlled inflammation in gingivitis to dysbiosis, increased recruitment of neutrophils and the transition to chronic periodontitis. However, further translational studies are required to identify the relevance of ADAM8 and its interaction with other proteases such as ADAM10 and MMP9 in the transition to periodontitis.

5 | Conclusion

The dysregulation of cell-associated and extracellular ADAM proteolytic activity, mainly driven by ADAM8, may be an essential regulatory element in periodontal disease severity, potentially fostering disease progression. In particular, ADAM8, which seems to play a minor role under physiological conditions and is mostly up-regulated under pathophysiological conditions (Chen et al. 2016; Conrad et al. 2022; Cook et al. 2022; Drey Mueller et al. 2017; Schломann et al. 2015), may be a potential target for local treatment or used as a diagnostic/prognostic marker, which should be addressed in future translational studies.

Author Contributions

Ahmad Aljohmani contributed to the conception, data analysis and interpretation; drafted the original manuscript; and critically revised the manuscript. Hakon Heinze contributed to the conception, data analysis, and interpretation and drafted the original manuscript. Federico Guillermo Gharzia contributed to data analysis and interpretation, drafted the original manuscript and critically revised the manuscript. Ahmed Mohamed Mostafa Abdrabou and Bashar Reda contributed to the acquisition and data analysis and critically revised the manuscript. Sören L. Becker and Markus Bischoff contributed to the conception, acquisition of data, and critically revised the manuscript. Matthias Hannig contributed to the conception and design of the study, acquisition and data analysis and critically revised the manuscript. Daniela Yildiz contributed to the conception and design of the study; acquisition, data analysis and interpretation; drafted the original manuscript and critically revised it. All authors provided their final approval and agreed to be accountable for all aspects of this study.

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Consent

Written informed consent was obtained from all participants.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

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

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