DENTAL MATERIALS 35 (2019) e235–e248

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.intl.elsevierhealth.com/journals/dema

Establishing a macrophage model with relevance for oral methacrylate monomer exposures: Attenuated *Staphylococcus aureus*-induced cytokine release from human macrophages

A.K. Bølling[a,b,*], B.P. Olderbø[a], J.T. Samuelsen[a], H.V. Rukke[a]

[a] Nordic Institute of Dental Materials, Sognsveien 70A, N-0855 Oslo, Norway
[b] Norwegian Institute of Public Health, Domain of Infection Control and Environmental Health, P.O. Box 4404, N-0403 Oslo, Norway

ARTICLE INFO

Keywords:
Dental materials
HEMA
Bacteria
Cytokine
Macrophage

ABSTRACT

Background. Leakage of unpolymerized methacrylate monomers after placement of methacrylate-containing polymeric dental materials leads to human exposure. Based on studies using murine macrophages and LPS from *Escherichia coli* (*E. coli*), dental monomers like 2-hydroxyethyl methacrylate (HEMA) are known to inhibit lipopolysaccharide (LPS) induced cytokine release. The aim of this study was to establish a model system with relevance for human oral monomer exposure using exposure to live gram-positive bacteria, and to confirm the HEMA-induced effects on cytokine release in this model.

Methods. The human THP-1 monocyte cell line was differentiated to macrophages using phorbol 12-myristate 13-acetate (PMA), before exposure to 0.5–2 mM HEMA and live *Staphylococcus aureus* (*S. aureus*) in various multiplicity of infections (MOI). Cytokine release and cytotoxicity were determined after (i) 2–24 h pre-exposure to HEMA followed by 2–4 h *S. aureus* exposure and (ii) 2–4 h simultaneous exposure. The 24 h pre-exposure regime was also tested in primary human airway macrophages and for phagocytosis of *S. aureus* in THP-1 macrophages.

Results. HEMA attenuated the cytokine release more strongly in the pre-exposure than combined exposure regime, with a maximal reduction of 95% in the *S. aureus*-induced cytokine release. A MOI of 0.1 (corresponding to a bacteria-macrophage ratio of 1:10) was determined to be optimal in the THP-1 macrophages as it induced sufficient cytokine release and negligible cytotoxicity. Attenuated release of *S. aureus*-induced interleukin (IL)-1β after HEMA exposure was confirmed in primary airway macrophages, while HEMA increased the phagocytosis of *S. aureus* in THP-1 cells.

Conclusion. The model was successfully established and attenuated bacteria-induced cytokine release after HEMA exposure confirmed.

* *Corresponding author.*
E-mail addresses: anette.kocbach@gmail.com, anette.kocbach@fhi.no (A.K. Bølling).
https://doi.org/10.1016/j.dental.2019.07.005

1. Introduction

Methacrylate-based polymeric composites, in combination with methacrylate-based adhesive systems, have become the most commonly used filling materials in many countries. The methacrylate monomer 2-hydroxyethyl methacrylate (HEMA) is a common constituent of adhesives and resin-inforced glass ionomer cements. Both these materials are cured by light after placement. Since the setting process is not complete, unpolymerized monomers leak, causing oral exposure to HEMA [1,2]. This is supported by *in vitro* leakage studies reporting that HEMA is released to various water based solutions [3–5].

In vitro exposure to HEMA induces DNA damage, apoptosis and necrosis in various cell lines [6–8]. Although methacrylate monomers appear to have a limited potential to induce pro-inflammatory cytokines [9], they attenuate LPS-induced release of interleukin (IL)-1β and tumor necrosis factor (TNF)-α from macrophages and monocytes in relatively low concentrations (0.2–1 mM) [10,11]. HEMA has been reported to bind directly to cellular glutathione (GSH), and form GS-HEMA adducts, due to its high thiol affinity [12,13]. This adduct formation causes a transient drop in cellular GSH levels that has been suggested as a possible mechanism for HEMA-induced effects [14,15]. More specifically, the initial cellular oxidative stress induced by HEMA has been demonstrated to cause activation of signal transduction and transcription factors resulting in apoptosis in murine macrophages [14,16].

Dental biofilms are complex ecosystems consisting of a diverse community of bacteria [17]. The first colonizers and the early biofilm is dominated by gram-positive aerobic bacteria [18]. In healthy individuals, the oral resident bacteria live in homeostasis with the host. However, environmental and genetic factors may cause a shift in the biofilm composition as well as the interaction between biofilm and host cells, causing development of caries or periodontal disease [17,19]. Inflammatory responses in oral epithelial cells and immune cells like polymorphonuclear leukocytes, neutrophils and macrophages play a role in initiation and development of disease [19,20].

Bacteria or their components present in the saliva or biofilm may trigger inflammatory responses, including release of pro-inflammatory cytokines [21,22]. During dental treatment, cells in the oral cavity may be exposed to HEMA and bacteria simultaneously. The effects of dental monomers on inflammatory responses to bacteria have generally been studied in an *in vitro* model using the bacterial component lipopolysaccharide (LPS) originating from the Gram negative Escherichia coli (*E. coli*) bacteria in combination with murine macrophages (RAW264.7 cells) [9,10,15,23]. Moreover, two studies assessed effects of methacrylate monomers in human gingival fibroblasts after co-exposure with LPS or *Streptococcus mitis (S. mitis)* bacteria [24,25]. In the studies in macrophages, both simultaneous exposure to LPS and monomers and pre-exposure to monomers followed by simultaneous LPS and monomer exposure attenuated the LPS-induced cytokine release.

HEMA may affect both pre- and post-translational processes [15,26,27]. Although the cellular mechanisms involved in the attenuated LPS-induced cytokine release due to HEMA exposure have not been fully elucidated, GSH-depletion and formation of HEMA-protein adducts have been suggested to play a role [15,27]. Recently, Schweikl et al. demonstrated that the decreased cytokine release from murine macrophages for 4–8 mM HEMA was due to inhibition of the nuclear transcription factor NFκB, possibly involving formation of oxidative stress and cross-talk with the transcription factor Nrf2 [27]. However, GSH depletion, indicative of oxidative stress, could not fully explain the attenuated LPS-induced IL-1β release due to HEMA concentrations ≤2 mM, i.e. concentrations that do not induce cell-death [15]. Instead, formation of HEMA-protein conjugates due to the thiol-reactivity of HEMA was suggested as a possible mechanism [15]. Accordingly, protein binding assays reveal that dental monomers bind to serum proteins [5,28], while conjugates of albumin and HEMA had an adjuvant effect on allergic responses to ovalbumin in a mouse model [29].

Although it is well documented that methacrylate monomers like HEMA attenuate cytokine release from murine RAW264.7 macrophages after exposure to LPS from *E. coli* [9,10,15,23,27], similar effects on exposure to live bacteria have not been reported for macrophages in the literature. Therefore, the aim of the current study was to establish a model with relevance for human monomer exposures, and to confirm the effects of "non-lethal" concentrations of HEMA on cytokine release in this model. Staphylococcus aureus (*S. aureus*) is frequently detected in the oral cavity, and it has recently been suggested that oral *S. aureus* infections originate from the oral microbiota [30–34]. This species is also known to induce a strong IL-1β signal [35,36], and was therefore chosen as a model to study the effects of HEMA on bacteria-induced cytokine release. Human THP-1 macrophages and human primary airway macrophages were applied as cellular models. The effects of HEMA were tested for different multiplicity of infections (MOI) in both pre-exposure and combined exposure regimes. In addition, the cytokine levels in THP-1 cell lysates were analyzed to assess whether HEMA-induced effects were due to pre- or post-translational mechanisms. Finally, effects of 24 h pre-exposure to HEMA on phagocytosis of *S. aureus* was studied as a second THP-1 endpoint reflecting another aspect of macrophage functionality.

2. Materials and methods

2.1. Reagents and chemicals

RPMI 1640 medium with 2 mM L-glutamine, sodium pyruvate and HEPES were purchased from Lonza BioWhittaker (VWR, PA, USA), while HEMA (CAS no. 868-77-9, purity > 99%) was from Fluka Chemie AG (Buchs, Switzerland). THP-1 cells were obtained from the European Collection of Cell Cultures (ECACC). Phorbol 12-myristate 13-acetate (PMA), LPS (from *E. coli* 026:B6), fetal bovine serum (FBS, heat inactivated and sterile filtered), Triton x-100, dithiothreitol (DTT), Penicillin/Streptomycin, Trypan blue (0.4%), Eukitt quick-hardening mounting medium and Roche LDH kit was purchased from Merck (Darmstadt, Germany). Gentamicin and Amphotericin B were purchased from Gibco while Brain Heart Infusion (BHI) was from Oxoid (both distributed by

Thermo Fisher Scientific, MA, USA), while Diff-Quik was from Medion Diagnostics (Duedingen, Switzerland). Enzyme-linked immunosorbent assay (ELISA) kits for human IL-1β and TNF-α and MCP-1 were obtained from R&D Systems Inc. (MN, USA), and for human IL-8 and IL-10 from Invitrogen (Thermo Fisher Scientific, MA, USA). *S. aureus* strains Newman was a kind gift from Tadashi Baba, Juntendo University School of Medicine, Tokyo, Japan.

2.2. Cell cultures

THP-1 monocytes were cultured in complete medium (c-RPMI), i.e. RPMI 1640 cell culture medium with 2 mM L-glutamine supplemented with 1 mM sodium pyruvate and 10 mM HEPES, with 10% FBS and 100 μg/ml Gentamicin. Cells were cultured at 37 °C under 5% CO_2 in a humidified incubator and kept in a logarithmic growth phase at 10^6–2×10^7 cells/75 cm^2 in 20 ml. This was achieved through routine subculturing three times a week by mixing and subsequent dilution of cells.

The THP-1 cells were differentiated to macrophage-like cells for exposures, achieved by 48 h exposure to PMA followed by 24 h rest in the absence of PMA (Fig. 1, upper panel). Cells were treated with 40 ng/ml PMA and seeded in 12-well plates at 0.5×10^6 cells/ml in 1 ml medium per well using c-RPMI with 10% FBS and 100 μg/ml Gentamicin. After 48 h, the cells were washed and replenished with c-RPMI with 10% FBS without Gentamicin to remove excess PMA, and allowed to rest for 24 h. After this differentiation, there was a 28 h exposure period for exposure to HEMA and *S. aureus* (Fig. 1, middle panel, see Section 2.3 for further description), resulting in a total culturing period of 100 h.

To prepare for bacteria exposure, cells were cultured without antibiotics the last 48 h prior to bacteria exposure. Thus, medium without Gentamicin was used for the 24 h resting period and the first 24 h of the exposure period (Fig. 1, lower panel). Then, immediately before exposure to *S. aureus*, the medium was changed to c-RPMI with 1% FBS (instead of 10%), to provide optimal conditions for the bacteria.

Primary airway macrophages were obtained from induced sputum [37] from 6 individuals. Informed consent was obtained from all subjects and ethical approval was obtained from the Norwegian Regional Committees for Medical and Health Research Ethics (REC; 2015/1322). In short, hypertonic saline was inhaled from an ultrasonic nebuliser (DeVilbiss, Sunrise Medicals, Longmont, CO, USA) for 7 min in 3 intervals. Expectorates were collected after each inhalation period and combined to one sample, which was dissolved in 0.1% DTT in PBS, filtered, centrifuged and re-suspended in c-RPMI with 10% FBS, penicillin (100 U/ml)/streptomycin (100 μg/ml) and 0.5 mg/ml Amphotericin B. Based on the relative cell viability (Trypan blue exclusion) and differential cell counts (cytospins stained with Diff-Quik and mounted), the macrophage concentration was calculated and cells were seeded in 48 well cell culture plates at 10^5 macrophages/well in 250 μl. The cells were incubated for 1 h in an atmosphere of 37 °C and 5% CO_2 to allow macrophages to attach. Then, the wells were washed twice with pre-heated c-RPMI with 10% FBS, antibiotics and Amphothericin B, before 250 μl of the same medium was added. The cells were allowed to rest for 24 h prior to exposure to HEMA. The HEMA exposure was performed in c-RPMI with 10% FBS, while c-RPMI with 1% FBS was used for *S. aureus* exposure (both without antibiotics and Amphothericin B). In this method, the primary airway macrophages were separated from the other cell types in the sputum sample by attachment to the cell culture. The macrophages were not characterized with regard to their phenotype, but the resulting culture purity was found to be 88% macrophages [37].

2.3. Exposure to HEMA and S. aureus

Stock solutions of 200 mM HEMA were freshly prepared in c-RPMI for each experiment, and added to cell culture wells to provide concentrations of 0.5–2 mM. Three different exposure scenarios were used to assess effects of HEMA on macrophage responses to *S. aureus*. These represent the exposure scenarios applied in the literature; (i) 24 h pre-exposure to HEMA followed by 2–4 h exposure to *S. aureus* (Fig. 2a), (ii) 2 h pre-exposure to HEMA followed by 2–4 h exposure to *S. aureus* (Fig. 2b) and (iii) 2–4 h combined exposure to HEMA and *S. aureus* (Fig. 2c).

For the macrophage infection assay, pre-cultures of *S. aureus* were made from overnight cultures of *S. aureus* in Brain Heart Infusion (BHI) at 37 °C in a 5% CO_2 supplemented atmosphere. These overnight cultures were diluted at 1:50 in pre-heated BHI and grown until an optical density of approximately 0.7 (measured at 600 nm). Then, cultures were aliquoted in 30% glycerol and stored at −80 °C. To determine the concentration of *S. aureus*, a serial dilution in PBS was performed, followed by plating on BHI agar, incubation overnight at 37 °C in a 5% CO_2 supplemented atmosphere, and finally enumeration of colony forming units (CFU).

For the cellular exposure, these frozen batches of *S. aureus* were thawed and centrifuged at 5000 × *g* for 5 min. The supernatant was discarded and the pellet re-suspended in c-RPMI at a concentration of approximately 6.67×10^7 CFU/μl. This *S. aureus* suspension was further diluted in c-RPMI to obtain stock solutions with concentrations suitable for macrophage infection at MOI between 0.02 and 10.

2.4. Other exposures

To facilitate comparison of the current model system with the data in the literature from LPS-induced cytokine release, the effect of HEMA on LPS-induced cytokine release was tested in THP-1 macrophages in the 24 h pre-exposure and combined exposure regimes. Frozen stocks of LPS from *E. coli* (1 mg/ml in PBS) were thawed and diluted further in c-RPMI for cellular exposure resulting in a final concentration of 0.05 ng/ml. This concentration was based on preliminary experiments (data not shown) and chosen to provide an IL-1β concentration in the same range as exposure to live *S. aureus*.

Since conjugation of HEMA with proteins has been suggested as a possible mechanism for HEMA-induced effects, the influence of interaction with serum proteins was tested. More specifically, the effect of deprivation or pre-incubation with serum (FBS) proteins was tested in the 24 h pre-exposure scenario. For protein-deprivation, i.e. avoiding contact between HEMA and serum proteins, the HEMA stock solution was prepared in serum free medium. In addition, the first 2 h of the

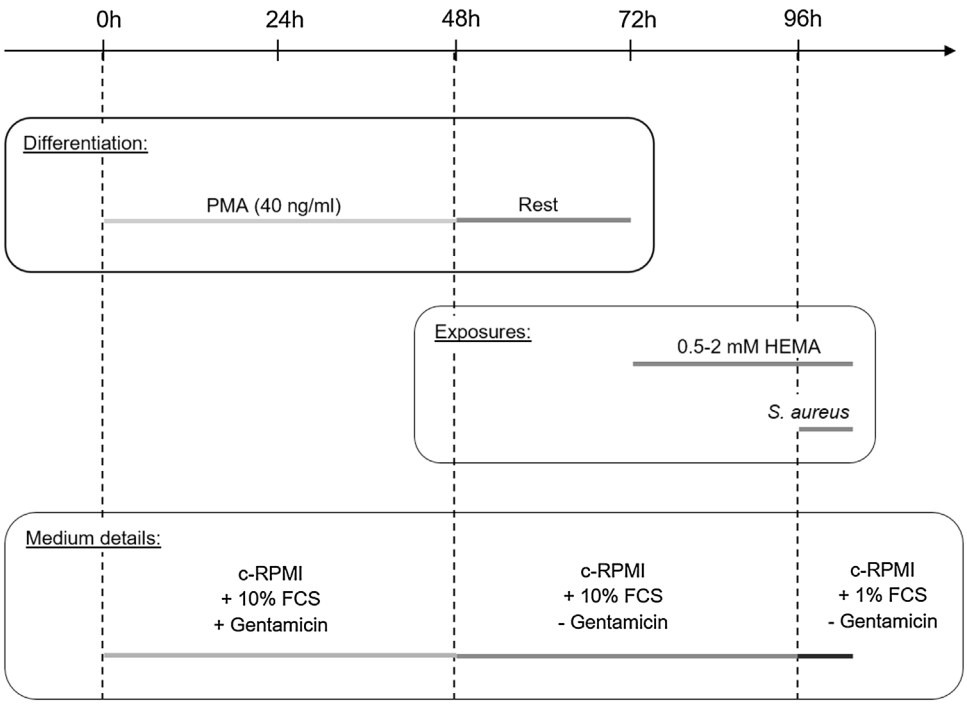

Fig. 1 – Schematic illustration of differentiation and exposure.
The figure illustrates the timeframes for PMA differentiation of THP-1 monocytes, including the resting period (upper panel), and exposure periods for HEMA and S. aureus (middle panel; for details regarding HEMA and *S. aureus* exposures, please see Figs. 2 and 3). The media specifications for the different phases of the culturing are also indicated (lower panel). To minimize the number of medium changes, the medium was not changed prior to HEMA exposure.

HEMA exposure was performed in serum-free medium, allowing for interaction with cells in the absence of proteins. For pre-incubation with serum proteins, c-RPMI containing 2 mM HEMA and 10% FBS (with antibiotics) was incubated over night at 37 °C to facilitate binding between HEMA and serum proteins. Upon exposure, the medium in the cell-culture well was replaced by this medium from the pre-incubation. Correspondingly, the medium in the control wells was replaced with freshly prepared pre-heated medium at the same time.

2.5. *Cytokine release*

For analysis of cytokine release, the supernatant was harvested at the end of the exposure period and centrifuged for 10 min at 100 × *g* to remove cells. Then the supernatant was transferred to a new tube and centrifuged again for 10 min at 5000 × *g* to remove bacteria, before storage at −80 °C until analysis. The levels of the pro-inflammatory cytokines IL-1β, TNF-α, MCP-1 and IL-8, and the anti-inflammatory cytokine IL-10 in the supernatants were analyzed using ELISA kits according to the manufacturer's manuals using a synergy H1 hybrid reader (BioTek).

2.6. *Cytotoxicity*

The level of lactate dehydrogenase (LDH) released from the cells was measured in supernatants as a measure of cytotoxicity. This was done using a colorimetric kit according to the manufacturers instructions, measuring the absorbance with a synergy H1 hybrid reader. To determine the maximal releasable LDH from the cell culture (max control), the cells in a control well were lysed with c-RPMI with 2% Triton x-100. To control for the LDH produced by S. aureus, the LDH levels were determined in Triton x-100 treated *S. aureus*. However, the measured LDH level was negligible compared to that from lysed cell cultures. The cytotoxicity was calculated as a percentage of the max control, i.e. the LDH level measured in the supernatant divided by the max control LDH level, and then multiplied by 100%.

2.7. *Phagocytosis and intracellular killing*

To determine the appropriate MOI for the phagocytosis experiments, THP-1 macrophages were exposed to a MOI of 1, 5 and 10 for 1 h in a titration experiment. This resulted in cytotoxicity of 2, 11 and 27% respectively. Based on these data a MOI of 5 was chosen for the experiments evaluating phagocytosis and intracellular killing. These assays were only performed for the 24 h pre-exposure to HEMA. For both assays, the medium was removed at the end of the HEMA exposure and replaced with 1 ml pre-heated medium containing live *S. aureus* corresponding to a MOI of 5 (c-RPMI, with 1% FBS, without antibiotics). After 1 h incubation at 37 °C, the cells were washed with 1 ml pre-heated c-RPMI with 1% FBS without antibiotics to remove extracellular bacteria. Then, the medium was replaced by 1 ml pre-heated c-RPMI with 1% FBS with antibiotics (0.3 mg/ml Gentamicin (6 μl/1 ml of 50 mg/ml stock solution), and incubated for 1 h at 37 °C to

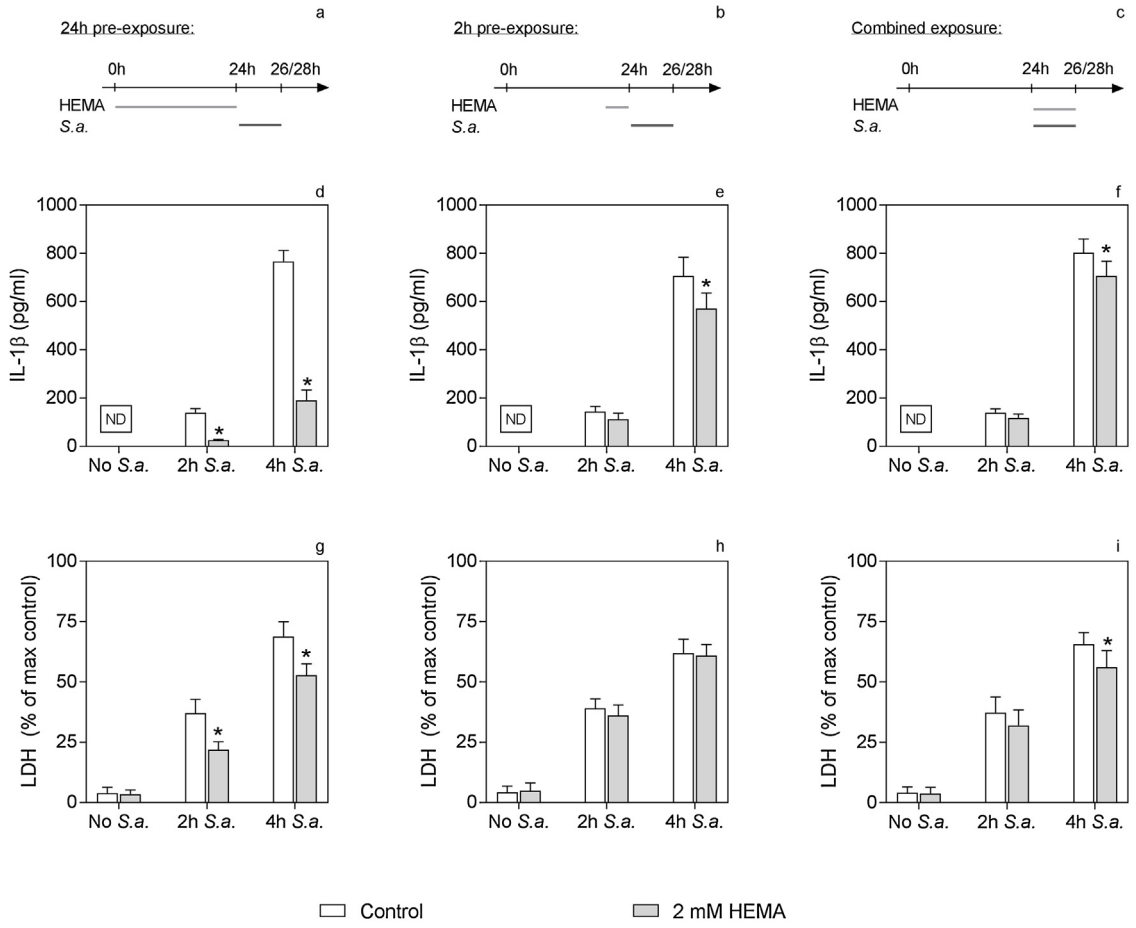

Fig. 2 – Comparison of three exposure scenarios for MOI 10.
PMA-differentiated THP-1 macrophages were exposed to 2 mM HEMA in three different exposure scenarios 24 h pre-exposure; (a, d, g), 2 h pre-exposure (b, e, h) and combined exposure (c, f, i). The exposure scenarios are illustrated in a–c. The graphs show release of IL-1β determined by ELISA (d–f) or cytotoxicity determined by LDH release (g–i) for exposure with or without 2–4 h *S. aureus* exposure at a MOI of 10. The * indicates significant decrease compared to control wells (2-way repeated measures ANOVA, Sidak post-tests, N = 4), while ND = not detected.

kill any remaining extracellular bacteria. Finally, the medium was replaced with pre-heated c-RPMI with 1% FBS, without antibiotics.

For the phagocytosis assay, immediately after the 2 h incubation described above, 1 ml dH_2O (MilliQ quality) was added to each well for lysis of the THP-1 macrophages. The contents of each well was mixed using a pipette, incubated at 37 °C for 15 min, mixed again and incubated for another 15 min at 37 °C. Then the cell lysates, containing the phagocytosed bacteria, were subjected to a 10x serial dilution resulting in a total of three dilutions in addition to the undiluted suspension; i.e. 1, 10, 100 and 1000× dilution. These were then plated on agar as 25 μl drops in duplicate. The agar plates were incubated over night at 37 °C before the number of colony forming units (CFU) were counted.

For the intracellular killing assay, after the 2 h incubation described above, the macrophages were incubated for another 1.5 or 3 h to allow for intracellular killing of bacteria. Finally, the cells were lysed using 1 ml dH_2O (30 min with mixing two times as described above). Then the resulting cell lysates were diluted, plated on agar and counted as described above.

2.8. *Statistics and data processing*

The number of independent experiments (N) are indicated in the figure legends. All columns in figures represent mean values while the error bars correspond to standard error of mean (SEM). All statistical analysis was performed in GraphPad Prism (GraphPad Software, CA, USA). As specified in the figure legends, either a two-way ANOVA with Sidaks post-test or a two-sided t-test was applied. All p-values <0.05 were considered as significant.

Since the IL-1β release from primary human macrophages showed large variation between individuals, these data are presented relative to the control level of *S. aureus*-induced cytokine release. To allow for ANOVA analysis after this normalization, a two-step procedure was used to retain variation in the controls but scale them to the same level. First, the data from each experiment (i.e. each individual) was divided by the mean value of all the data in that experiment. Then, all the data in this dataset (i.e. from all 6 individuals) were divided by the mean of the controls in these experiments, and multiplied by 100%.

The CFU data from the phagocytosis and intracellular killing assays in THP-1 cells also showed large variation between experiments, and were presented relative to their respective controls. Four independent phagocytosis experiments were performed, each with the control and 2 mM HEMA exposure in triplicates, resulting in 12 data-points for each treatment (Control and 2 mM HEMA) and time-point (0, 1.5 and 3 h). For each experiment, all the data were divided by the mean of the three controls in that experiment, retaining variation in the normalized control levels. After this normalization, the standard deviation of the data from a single experiment was in the same range as the standard deviation of all the data-points within each condition. Thus, the triplicate wells in each experiment were included as independent data-points in the statistical analysis.

For the intracellular killing, a similar normalization procedure was used. However, to assess cellular killing relative to the amount of bacteria phagocytosed initially (i.e. the phagocytosis data corresponding to 0 h), the Control and HEMA data were normalized separately. This was achieved by dividing all the data from one condition in each experiment to the mean value of their respective 0 h data. This retained the variation in the 0 h data for both Control and HEMA, and allowed for ANOVA analysis.

3. Results

3.1. Comparison of exposure scenarios

To establish the model system where live *S. aureus* bacteria induce cytokine release from THP-1 macrophages, different exposure scenarios and exposure times for HEMA were tested using MOI 10 (Fig. 2). The 2 or 4 h exposure to *S. aureus* alone in a MOI of 10 resulted in an IL-1β release of 200–800 pg/ml and a cytotoxicity of 30–70 % in the three different exposure scenarios.

Exposure to 2 mM HEMA reduced the *S. aureus*-induced IL-1β release with 75–85 % in the 24 h pre-exposure regime, while the effect was less pronounced in the 2 h pre- and combined exposure scenarios with reductions of 10–25 % (Fig. 2d–f). The IL-1β release was below the detection limit in the absence of *S. aureus*, thus HEMA exposure without *S. aureus* was excluded from the remaining experiments.

The *S. aureus*-induced cytotoxicity was reduced with 25–40 % due to 24 h pre-exposure to HEMA, while HEMA had a limited effect on the *S. aureus*-induced cytotoxicity in the two other exposure scenarios (Fig. 2g–i).

Since HEMA induced similar effects in the 2 h pre- and combined exposure scenarios both with respect to IL-1β release and cytotoxicity, only the combined and 24 h pre-exposure scenarios were included in the further experiments.

3.2. Titration of MOI

The 2–4 h incubation of the THP-1 macrophages with *S. aureus* at a MOI of 10 induced a 40–70 % cytotoxicity (Fig. 2g–i), with unspecific release of IL-1β due to rupture of the cell membrane as a possible consequence. In order to investigate controlled/regulated rather than cytotoxicity-induced IL-1β release, titration of the MOI to reach a non-cytotoxic level of *S. aureus* was performed (Fig. 3). A 4 h incubation with MOI of 0.02 and 0.1 resulted in negligible cytotoxicity, while MOI 0.5 increased the cytotoxicity to approximately 30% (Fig. 3f–h). The 24 h pre-exposure to HEMA generally resulted in a stronger effect on the IL-1β release than the combined exposure (Fig. 3c–e). HEMA induced a concentration dependent decrease in IL-1β release after 24 h pre-exposure, with a maximal reduction of 90–95 % for all MOIs investigated. In contrast, the effect of HEMA in the combined exposure scenario varied with the MOI (20–60 % reduction) and showed strongest effect for the two lowest MOI (Fig. 3d–e).

HEMA had similar effects on the *S. aureus*-induced cytotoxicity in the two exposure regimes for MOI 0.5, with 50 and 40% reductions in the 24 h pre- and combined exposure regimes, respectively (Fig. 3h). For MOI 0.1, HEMA had a stronger impact on *S. aureus*-induced cytotoxicity in the combined than pre-exposure regime (40 vs. 15% reduction) (Fig. 3g).

The effect of HEMA on IL-1β release from THP-1 macrophages induced by LPS from *E. coli* was similar to the *S. aureus*-induced release, with a 98% reduction in the pre-exposure regime (Fig. 4). Similarly, the LPS-induced cytotoxicity was reduced with 47% after 24 h pre-exposure to HEMA. In contrast to the *S. aureus*-induced responses, neither IL-1β release nor cytotoxicity were affected by HEMA in the combined exposure regime.

3.3. Primary human macrophages

To assess the translational value of the THP-1 macrophage model, the effect of 24 h pre-exposure to HEMA on *S. aureus*-induced IL-1β release was also tested in primary human macrophages (Fig. 5). Since initial experiments revealed that these primary macrophages were more robust in terms of withstanding *S. aureus*-induced toxicity, higher MOI were applied than in the THP-1 model. For the three MOI tested (0.1, 1 and 10), only the highest (MOI 10) induced a significant cytotoxicity of 60% compared to control. Moreover, the release of IL-1β was only significantly increased by the two highest MOIs (1 and 10). For MOI 1, resulting in significant cytokine release and no cytotoxicity, 2 mM HEMA reduced the IL-1β release with 60%, while an even stronger reduction in IL-1β release (75%) was observed for the cytotoxic MOI of 10. Interestingly, HEMA did not reduce the *S. aureus*-induced cytotoxicity in this model system using primary human macrophages, in contrast to the THP-1 macrophage model.

3.4. Release of other inflammatory mediators

To assess whether the effects of HEMA were limited to IL-1β, the levels of TNFα, IL-8, IL-10 and MCP-1 were determined in supernatants from experiments using MOI 0.1 (Fig. 6). The release of TNFα followed the same pattern as IL-1β after exposure to *S. aureus* and HEMA; the 24 h pre-exposure to HEMA resulted in a stronger effect than the combined exposure, with 95 and 40% reductions respectively (Fig. 6a). For IL-8, HEMA only induced a significant effect in the 24 h pre-exposure regime, with a 50% reduction in its release (Fig. 6b). Unfortunately IL-10 and MCP-1 levels were below the detection limit for the majority of the experiments. However, similar trends

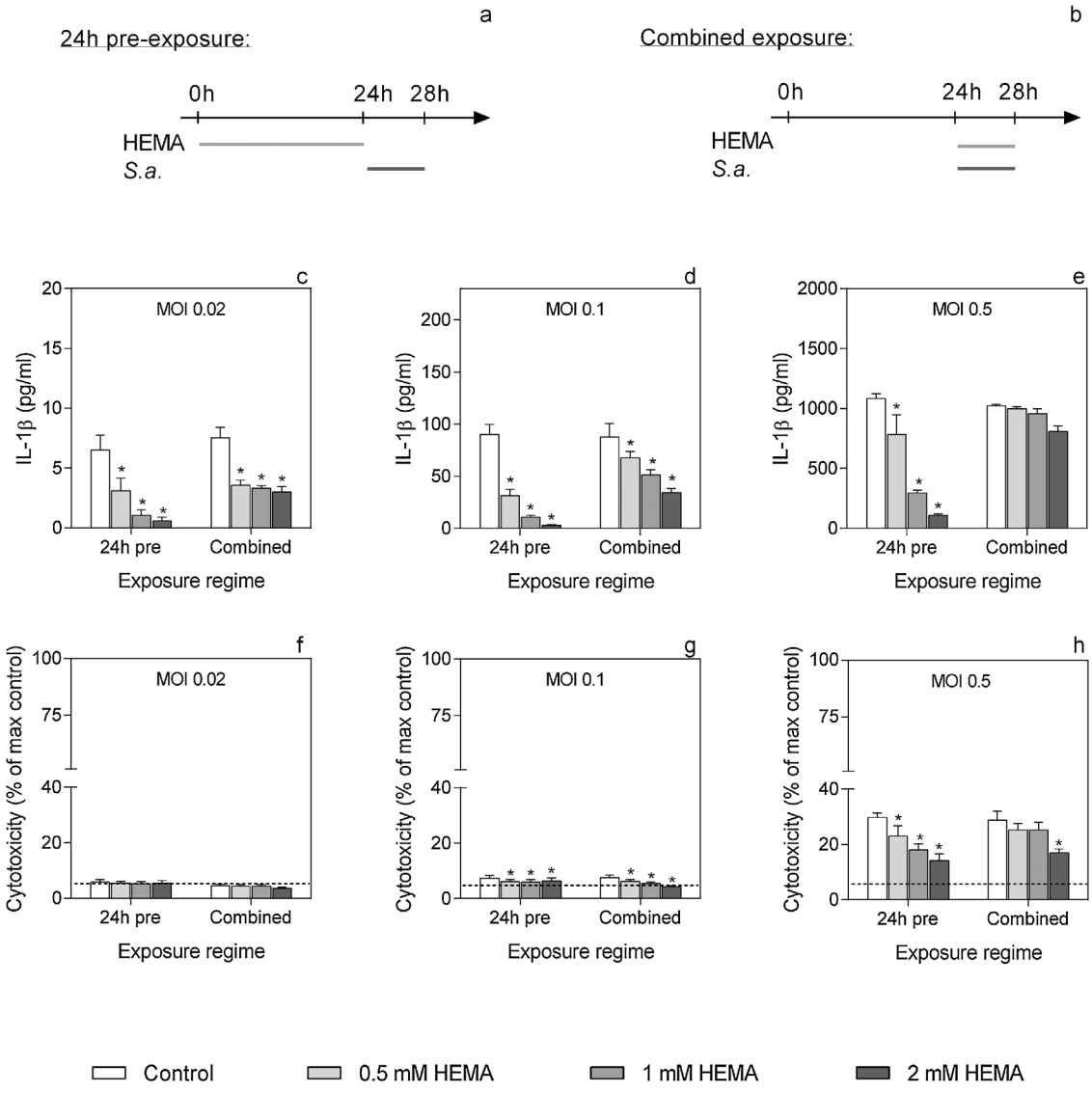

Fig. 3 – Titration of MOI to establish non-cytotoxic exposure conditions.
Schematic illustrations of the two applied exposure scenarios (a) 24 h pre-exposure to HEMA prior to 4 h *S. aureus* exposure and (b) 4 h combined exposure to HEMA and *S. aureus*. In c–h) PMA-differentiated THP-1 macrophages were exposed to 0.5–2 mM HEMA and *S. aureus* at a MOI of 0.02 (c, f), 0.1 (d, g) or 0.5 (e, h) according to the indicated exposure scenarios. After 4 h S. aureus exposure, the release of IL-1β was determined by ELISA in (c–e), while cytotoxicity was determined by the LDH assay (f–h). The * indicate significant decrease compared to control wells (2-way repeated measures ANOVA, Sidak post-tests, N = 4). The dotted line indicates the basal cytotoxicity level, e.g. in cells not exposed to *S. aureus*.

as for IL-1β and TNFα were observed with regard to HEMA-induced effects on the release of IL-10 and MCP-1 when these cytokines were detectable (data not shown).

3.5. *Intracellular cytokine levels*

To test whether the effects of HEMA on cytokine release were related to the transcriptional and translational regulation or to the actual release of the cytokines, the cells were lysed at the end of the *S. aureus* exposure to determine the remaining intracellular cytokine levels (Fig. 7). As for the IL-1β release, the *S. aureus*-exposure *per se* increased the intracellular cytokine levels compared to the basal levels (control bar vs. dotted line). The effect of HEMA on intracellular IL-1β was similar to its effects on IL-1β release, although not as strong. While the IL-1β release was reduced with 95 and 60% in the 24 h pre- and combined exposure scenarios (Fig. 3d), respectively, the corresponding intracellular levels were reduced with 70 and 20% (Fig. 7a). The effects of HEMA on intracellular TNFα and IL-8 on the other hand, was similar or more pronounced than the effects on their release. For TNFα, the intracellular levels were reduced with 95 and 50% reductions in 24 h pre- and combined exposure, respectively, while the corresponding reductions in release were 95 and 40% (Figs. 6a and 7 b). The IL-8 levels were only affected by HEMA after 24 h pre-exposure, and the 60% reduction in the intracellular level was slightly higher than

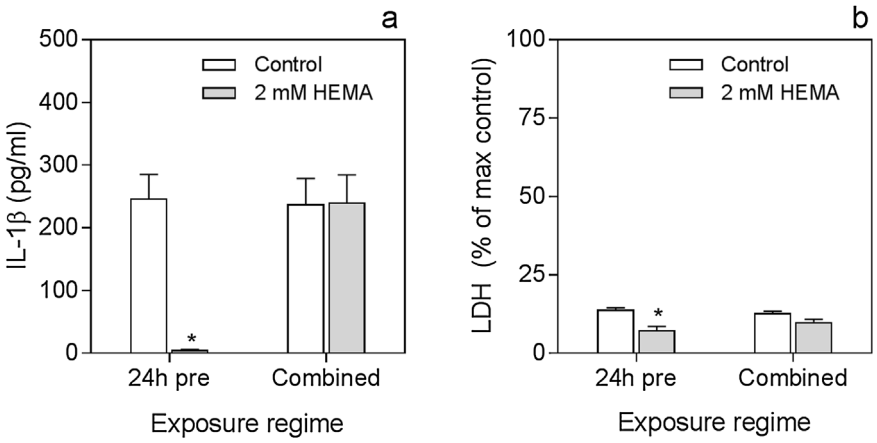

Fig. 4 – Effects of HEMA on LPS-induced IL-1β release.
PMA-differentiated THP-1 macrophages were either pre-exposed to 2 mM HEMA for 24 h followed by 4 h exposure to *E. coli* LPS (0.05 ng/ml) or subjected to a 4 h combined exposure to 2 mM HEMA and *E. coli* LPS (0.05 ng/ml). The release of (a) IL-1β was determined by ELISA while (b) cytotoxicity was assessed by the release of LDH presented as percent of the max control. * indicates significant decrease compared to respective control wells (2-way repeated measures ANOVA, Sidak post-tests, N = 3).

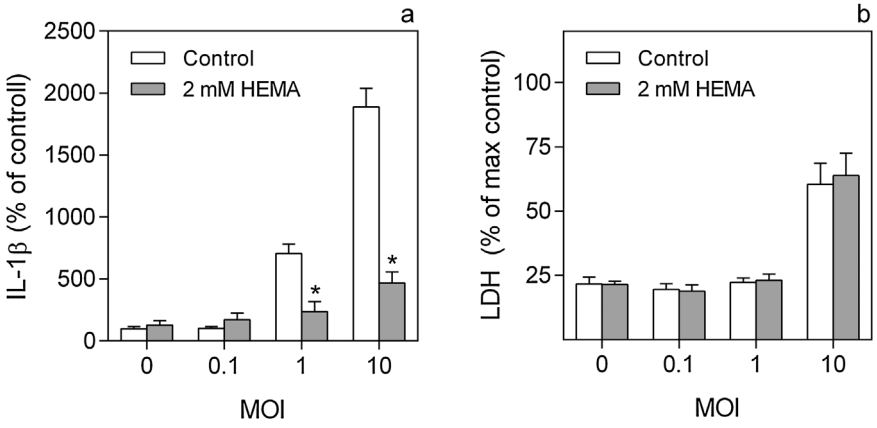

Fig. 5 – Effects of 24 h pre-exposure to HEMA in primary human macrophages.
Primary human macrophages from induced sputum were exposed to 2 mM HEMA and a MOI of 0.1, 1 or 10 for 4 h to determine (a) the release IL-1β by ELISA and (b) the cytotoxicity by the LDH assay. Since the basal and the *S. aureus*-induced IL-1β levels varied considerably between macrophages from different donors (basal: mean 20 pg/ml, range 2–55 pg/ml; induced: mean 300 pg/ml, range 50–480 pg/ml), these data were 2-way normalized to the level in the unexposed controls. The * indicates significant decrease compared to control wells (2-way repeated measures ANOVA, Sidaks post-tests, N = 6).

the 50% reduction in the release (Fig. 7c). On the contrary, the intracellular LDH level was not affected by either *S. aureus* or HEMA exposure (Figs. 6b and 7 d).

3.6. *Pre-incubation or deprivation of serum proteins*

Since conjugation with proteins has been proposed as a possible mechanism for the cellular effects of HEMA, the effects of deprivation or pre-incubation with proteins on *S. aureus*-induced IL-1β release were tested. Incubation in FBS-free medium the first 2 h of exposure or 24 h pre-incubation of HEMA with FBS at 2 mM in 37 °C resulted in HEMA-induced reductions in *S. aureus*-induced IL-1β release of 95 and 98%, respectively (Supplementary Appendix A, Fig. A.1). These reductions were similar to the 97% reduction in the pre-incubation exposure regime (left part of Fig. 3d).

3.7. *Phagocytosis and bacterial killing*

To address a different aspect of macrophage functionality, HEMA-induced effects on phagocytosis of *S. aureus* were investigated in PMA-differentiated THP-1 macrophages. After 24 h pre-exposure to HEMA, the phagocytosis was increased with 77% (±26%) compared to control (Fig. 8a). However, the intracellular killing of *S. aureus*, determined during the 3 h following the initial phagocytosis assessment in Fig. 8a, was not affected by HEMA exposure (Fig. 8b).

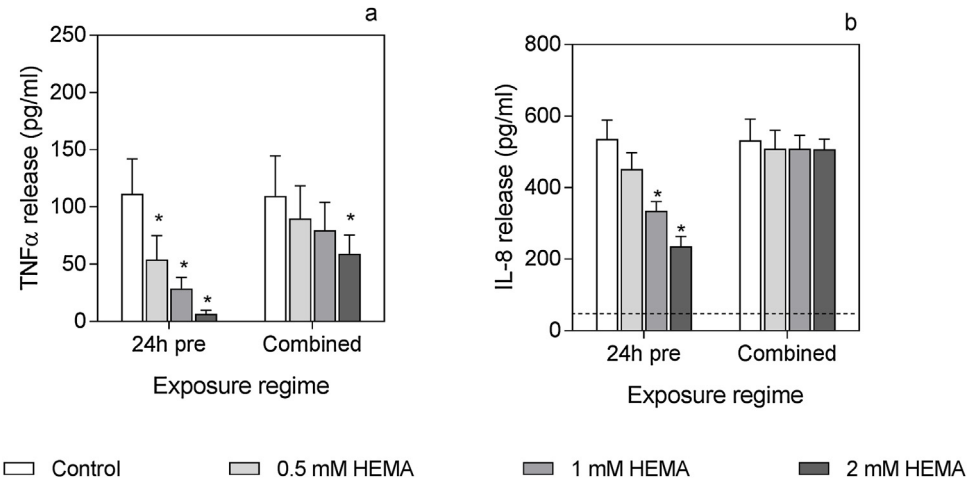

Fig. 6 – Effects of HEMA on S. aureus-induced release of TNF and IL-8.
PMA-differentiated THP-1 macrophages were exposed to 0.5–2 mM HEMA and *S. aureus* at a MOI of 0.1 for 4 h to determine release of (a) TNFα and (b) IL-8. The levels of MCP-1 and IL-10 were under the detection limit in the majority of the experiments, and are not displayed. The graphs are based on re-analysis of supernatants from experiments in Fig. 3d, and the * indicate significant difference from control wells (2-way repeated measures ANOVA, Sidaks post-test, N = 5). In the absence of *S. aureus*, the mean IL-8 levels ± standard deviation (range) was 52 ± 38 (14–99) pg/ml (indicated by a dotted line in b), while all the TNFα levels were below the detection limit in the absence of bacteria.

4. Discussion

Methacrylate monomer-containing dental materials are commonly applied in dentistry. Due to incomplete setting after placement, these materials cause oral exposures to methacrylate monomers. Although it is well documented that methacrylate monomers like HEMA attenuate cytokine release from murine RAW264.7 macrophages after exposure to LPS from *E. coli* [9,10,15,23,27], there is limited knowledge regarding effects of HEMA on macrophage responses to live bacteria. In the current study, a model using live *S. aureus* bacteria to induce cytokine release from THP-1 macrophages or primary human airway macrophages was successfully established. Moreover, HEMA attenuated *S. aureus*-induced cytokine release in both macrophage models, confirming the results from the murine macrophage cell-line. The effect of HEMA was stronger in the THP-1 macrophages, with 90–98 % reduction in *S. aureus*-induced IL-1β release, than in the airway macrophages, where the reduction was around 60%.

PMA differentiated THP-1 cells are commonly used as a human macrophage model [38,39]. However, since their phenotype has been reported to differ considerably from the tissue macrophage phenotype [40], primary airway macrophages were included in the current study as a complementary model system. The applied primary macrophages from induced sputum are available through a non-invasive method resulting in mature airway macrophages [37], hence no *in vitro* differentiation is required. The phenotype of tissue macrophages depends on the local environment and varies between different tissues [41]. Thus, macrophages in the oral cavity, both tissue macrophages and those recruited and differentiated from monocytes during acute inflammation, are likely to have a different phenotype than the applied primary airway macrophages. Nevertheless, the current data demonstrate that HEMA affects bacteria-induced IL-1β release from human macrophages.

S. aureus was chosen as a model-bacteria to study the effects of HEMA on bacteria-induced cytokine release since it is present in the oral cavity and known to induce a strong IL-1β signal [30–33,35,36]. Approximately 20% of the population are persistent nasal carriers of *S. aureus*, while around 60% are intermittent carriers and the remaining population are non-carriers [42,43]. In addition, the oral cavity and the perioral region is frequently colonized by *S. aureus*, with a carrier rate of 44.6% [44,45]. Also, oral *S. aureus* infections have recently been suggested to originate from the oral microbiota [34]. Although *S. aureus* is not strongly associated with chronic periodontitis or caries, it has been associated with therapy-resistant (refractory) cases of periodontitis and may also be associated with peri-implantitis or cause oral bacterial infections [46–48]. Such oral *S. aureus* infections will activate the innate immune response with macrophages as the first line of defense, supporting the choice of *S. aureus* as a model-bacteria.

The bacterial component LPS has been applied in the previous studies assessing the effects of HEMA on inflammatory macrophage responses. LPS interacts with toll-like receptor (TLR) 4 which causes activation of intracellular pathways resulting in activation of the transcription factor NFκB as well as other transcription factors [49]. In contrast, a number of cell wall components and proteins secreted from *S. aureus* contribute to the inflammatory response [50]. These bacterial factors interact with a range of receptors, including TLR2, Nod and TNFR1, that result in activation of NFκB and subsequent transcription of immune response genes including pro-inflammatory cytokines [50,51]. Although the interaction between *S. aureus* and immune cells is more complex than for LPS from *E. coli* and similar intracellular signaling pathways are activated [35], there are also distinct differences between

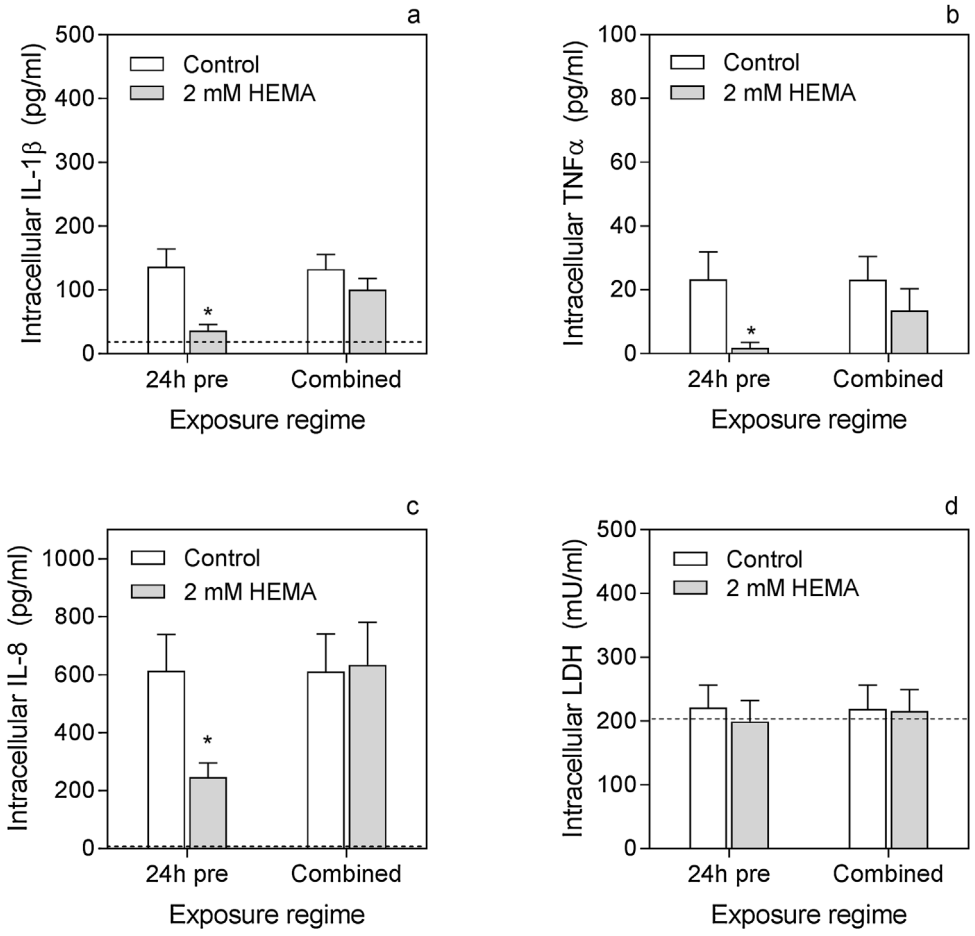

Fig. 7 – Remaining intracellular cytokine and LDH levels (lysed cells).
PMA-differentiated THP-1 macrophages were 24 h pre-exposed to 2 mM HEMA and *S. aureus* in MOI of 0.1 for 4 h. At the end of exposure the cells were lysed with Triton x-100 for analysis of a) intracellular IL-1β and b) TNFα, c) IL-8 and d) LDH levels. The * indicates significant decrease compared to control wells (2-way repeated measures ANOVA, Sidaks post-tests, N = 3). The dotted lines indicate the mean levels of intracellular IL-1β, IL-8 or LDH in unstimulated cells, while no TNFα could be detected in the lysates from unstimulated cells.

TLR2 and 4 signaling [49,52]. In the THP-1 macrophages, the *S. aureus* and LPS-induced IL-1β release was attenuated in a similar manner after 24 h pre-incubation with HEMA, with a 90–98 % reduction in release. In contrast, the effects of HEMA on *S. aureus* and LPS-induced IL-1β release differed dramatically for the 4 h combined exposure, where HEMA caused a 60% reduction in *S. aureus*-induced IL-1β release, while the LPS-induced release was not affected at all. However, in the RAW264.7 macrophages, HEMA affected LPS-induced IL-1β release to a similar extent in the 24 h pre-incubation and 24 h combined exposure regimes [15], suggesting that the lack of effect observed in the THP-1 cells may not be specific to the LPS stimuli. The applied exposure time to live *S. aureus* and LPS in the THP-1 cells was shorter than for LPS in RAW264.7 cells (4 vs 24 h) due to the cytotoxic effects of the live bacteria over time, and this difference in exposure time is a possible explanation for the different response patterns for LPS in the two macrophage models.

In the 24 h pre-exposure regime, the effect of HEMA on *S. aureus* induced IL-1β release from THP-1 macrophages was stable across the different MOI tested, with approximately 90–100 % reduction. In the combined exposure regime, however, the effect of HEMA varied across the different MOIs, and appeared to decrease with increasing levels of cytokine release. Indeed, when the percent reduction was plotted versus the cytokine level in the control well in the same experiment, there was a strong negative correlation ($R^2 = 0.81$) in the combined exposure regime, but not for the 24 h pre-exposure (Supplementary Appendix A, Fig. A.2). Thus, for high cytokine levels (i.e. high MOI) the effect of the bacteria exposure seems to 'override' the effect of the HEMA exposure in the combined exposure regime.

In accordance with the literature from LPS-stimulated RAW264.7 macrophages [15,27], HEMA reduced the *S. aureus*-induced cytotoxicity in THP-1 macrophages with up to 50%. When comparing the effects of pre-exposure and combined exposure to HEMA on cytotoxicity, there was no clear pattern with regard to the relative potency of HEMA in the two exposure regimes. However, in the combined exposure regime, the effect of HEMA seemed to depend on the MOI in a similar

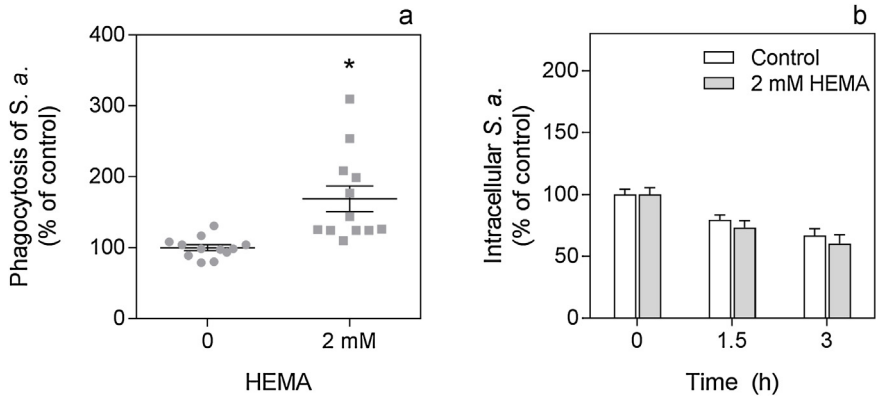

Fig. 8 – Effects of 24 h pre-exposure to HEMA on phagocytosis and intracellular killing of *S. aureus*.
PMA-differentiated THP-1 macrophages were pre-exposed to 2 mM HEMA for 24 h and then exposed to *S. aureus* at a MOI of 5 for 1 h. Extracellular bacteria where then removed by washing the wells, before 1 h incubation, lysis of cells and subsequent plating of suspension on agar for determination of phagocytosis (a). A total of 4 experiments were performed in triplicate, resulting in 12 measurements points for each condition, these were normalized to the mean of the control in each experiment (corresponding to displayed dots). To assess cellular killing of bacteria (b), the cells were incubated for an additional 1.5 and 3 h before lysis and agar plating. Then, Control and HEMA data were normalized to the mean of their respective 0 h control (corresponding to the data used to assess phagocytosis after 1 + 1 h incubation) to assess cellular killing of *S. aureus* relative to the amount of bacteria phagocytosed initially. In (a) * corresponds to significant increase compared to control (unpaired two-tailed *t*-test), while data in (b) were analyzed by 2-way ANOVA with Sidaks post-test (the columns correspond to the mean of the 12 individual measurement points).

manner as the IL-1β release, since the *S. aureus*-induced cytotoxicity decreased significantly with increasing cytotoxicity (i.e. high MOI) with a moderate negative correlation ($R^2 = 0.58$) (Fig. A.3). In the previous studies of HEMA reporting reduced LPS-induced cytotoxicity in RAW264.7 cells, activation of the cellular antioxidant defense system by HEMA has been proposed as the underlying mechanism [27]. Specifically, while the cytotoxic effect of LPS in RAW264.7 cells was mediated through NFkB and peroxynitrite, HEMA counteracted these effects by activation of the cytoprotective Nrf2 pathway [27]. In contrast to in the THP-1 and RAW264.7 macrophages, HEMA did not affect *S. aureus*-induced cytotoxicity in the primary human macrophages. Thus, this' protective' effect of HEMA seems to be cell type specific, and may have limited relevance for the *in vivo* setting since it was not observed in the primary macrophages.

The cytokine levels in THP-1 cell lysates were analyzed to assess whether HEMA-induced effects were due to pre- or post-translational mechanisms. When comparing the percent reduction in cytokine release (Figs. 3 and 6) and remaining intracellular levels (Fig. 7) in a 2-way ANOVA, there were no significant differences (data not shown). The similar effects of HEMA on *S. aureus*-induced cytokine release and intracellular levels, suggest that the effects of HEMA on *S.* aureus-induced cytokine release are mostly due to mechanisms pre-translation rather than mechanisms related to cytokine release, as suggested previously for LPS [15,27]. Schweikl et al. investigated the role of oxidative stress in LPS-induced cytokine release from RAW264.7 cells exposed to 1–8 mM HEMA, and suggested a role for hydrogen peroxide levels due to adduct formation of HEMA with GSH [27]. However, the hydrogen peroxide levels only increased for the higher concentrations of HEMA (4–8 mM). The molecular events preceding the HEMA-induced reduction in LPS stimulated cytokine release may differ between high and low HEMA concentrations [26]. In RAW264.7 cells, cellular GSH depletion by the GSH synthesis inhibitor butylsulfoximine (BSO) induced similar effects as exposure to 0.25–2 mM HEMA in terms of reduction of LPS-induced IL-1β release [15]. However, due to differences in the dynamics and magnitude of the effects between HEMA and BSO, GSH depletion alone could not explain the attenuation of LPS-induced IL-1β release by HEMA [15]. Based on the strong thiol reactivity of HEMA reported in the literature [12,13], formation of HEMA-protein conjugates was suggested as a possible candidate for the effects induced by low concentrations of HEMA [15].

Binding of HEMA to cysteine residues in proteins has also been proposed previously [13], based on the ability of HEMA to bind directly to cysteine in GSH and form GS-HEMA adducts [12,13]. Accordingly, dental monomers were reported to bind to serum proteins in protein binding assays [5,28], further supporting this hypothesis. Binding to serum proteins could, however, also reduce bioavailability of methacrylate monomers *in vitro* and thereby reduce their toxicity [5,28,53]. In the current study, the overnight pre-incubation of HEMA with serum protein, which is likely to result in reduced levels of unconjugated or 'free' HEMA, did not reduce the effect of HEMA on *S. aureus*-induced IL-1β release. Similarly, 2 h serum deprivation did not affect the effect of HEMA in the applied model. Thus, interaction between HEMA and serum proteins did not seem to play a role in the observed reduction in IL-1β release. This may point towards a specificity in the HEMA-protein conjugation, i.e. that it only occurs for certain proteins with specific topography around the SH group that

facilitates the HEMA-SH-group interaction (like GSH). Other possible hypotheses include that HEMA-protein conjugation occurs but is reversible or is not involved in the observed cellular effects. Overall, further studies are necessary to fully elucidate the role of HEMA-protein interactions in the cellular effects of HEMA.

Phagocytosis plays a critical role in the early innate immune response, and is essential for elimination of bacteria [54]. In contrast to the reduction in *S. aureus*-induced cytokine release, the phagocytosis of *S. aureus* was significantly increased with around 80% after 24 h pre-incubation with 2 mM HEMA. However, in human primary monocytes and neutrophils HEMA did not affect the phagocytosis of *E. coli* significantly, but decrease the average respiratory burst activity [55]. The observed increase in phagocytic activity due to HEMA exposure may suggest an enhanced macrophage immune response. In contrast, a possible consequence of the attenuated *S. aureus*-induced cytokine release, could be an impaired immune response due to insufficient recruitment of immune cells to the site of infection. Further experiments are required to elucidate the relative importance of the increased phagocytosis and the reduced cytokine release.

In contrast to the current findings, HEMA increased the LPS-induced mRNA expression of the pro-inflammatory mediators TNFα and Cox-2 in primary human gingival fibroblasts [24], while these mediators were not affected by TEGDMA during co-exposure with viable *S. mitis* [25]. Thus, the impact of methacrylate monomers may vary between different cell types and model systems and even be opposite. Similarly, contradictory findings have been reported with respect to the effect of GSH depletion on cytokine release, as both reduced and increased LPS-induced release was reported in different model systems [56,57]. Although macrophages are of major importance during oral infections, other cell types like epithelial cells, neutrophils and T-cells appear to play a more important role both during tissue homeostasis and development of oral disease [58–60]. Finally, while epithelial cells are continuously exposed to any monomers released from dental materials to saliva, macrophages are generally tissue resident and subjected to limited exposure to methacrylate monomers from saliva. These data point towards the importance of conducting further studies of methacrylate monomer-induced effects on the responses to bacteria and bacterial components in cell types with higher relevance for disease development and oral exposure.

In conclusion, a macrophage model with relevance for combined oral exposure to bacteria and dental monomers was successfully established. The current study confirmed that HEMA attenuates bacteria-induced cytokine release in human macrophages, indicative of an impaired immune response. However, since the significantly increased phagocytosis may point towards enhanced immune responses, further experiments are required for a better understanding of the clinical relevance of altered macrophage functionality due to HEMA exposure. Due to the complex interplay between immune cells and connective tissue in the oral immune responses, application of models including other cell types (e.g. epithelial cells and neutrophils) and bacterial species is highly recommended in future studies.

Funding sources

This research did not receive any specific grant from funding agencies in the public. Commercial or not-for-profit sectors.

Acknowledgements

Dr. Liv Ingunn B Sikkeland at the Department of Respiratory Medicine, Oslo University Hospital Oslo, Norway is acknowledged for providing access to facilities for sputum induction, sample processing and isolation of airway macrophages. Tonje Berg Steensen coordinated the sputum induction, as well as the processing of sputum samples and culturing of primary human macrophages.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.dental.2019.07.005.

REFERENCES

[1] Michelsen VB, Kopperud HB, Lygre GB, Bjorkman L, Jensen E, Kleven IS, Svahn J, Lygre H. Detection and quantification of monomers in unstimulated whole saliva after treatment with resin-based composite fillings in vivo. Eur J Oral Sci 2012;120(1):89–95.

[2] Gul P, Akgul N, Alp HH, Kiziltunc A. Effects of composite restorations on oxidative stress in saliva: an in vivo study. J Dent Sci 2015;10(4):394–400.

[3] Van Landuyt KL, Nawrot T, Geebelen B, J De Munck J, Snauwaert K, Yoshihara H, Scheers L, Godderis P Hoet, Van Meerbeek B. How much do resin-based dental materials release? A meta-analytical approach. Dent Mater 2011;27(8):723–47.

[4] Putzeys E, Cokic SM, Chong H, Smet M, Vanoirbeek J, Godderis L, Van Meerbeek B, Van Landuyt KL, Duca RC. Simultaneous analysis of bisphenol A based compounds and other monomers leaching from resin-based dental materials by UHPLC-MS/MS. J Sep Sci 2017;40(5):1063–75.

[5] Rothmund L, Shehata M, Van Landuyt KL, Schweikl H, Carell T, Geurtsen W, Hellwig E, Hickel R, Reichl FX, Hogg C. Release and protein binding of components from resin based composites in native saliva and other extraction media. Dent Mater 2015;31(5):496–504.

[6] Becher R, Kopperud HM, Al RH, Samuelsen JT, Morisbak E, Dahlman HJ, Lilleaas EM, Dahl JE. Pattern of cell death after in vitro exposure to GDMA, TEGDMA, HEMA and two compomer extracts. Dent Mater 2006;22(7):630–40.

[7] Krifka S, Hiller KA, Spagnuolo G, Jewett A, Schmalz G, Schweikl H. The influence of glutathione on redox regulation by antioxidant proteins and apoptosis in macrophages exposed to 2-hydroxyethyl methacrylate (HEMA). Biomaterials 2012;33(21):5177–86.

[8] Schweikl H, Hartmann A, Hiller KA, Spagnuolo G, Bolay C, Brockhoff G, et al. Inhibition of TEGDMA and HEMA-induced genotoxicity and cell cycle arrest by N-acetylcysteine. Dent Mater 2007;23(6):688–95.

[9] Krifka S, Petzel C, Hiller KA, Frank EM, Bosl C, Spagnuolo G, Reichl FX, Schmalz G, Schweikl H. Resin monomer-induced

differential activation of MAP kinases and apoptosis in mouse macrophages and human pulp cells. Biomaterials 2010;31(11):2964–75.
[10] Bolling AK, Samuelsen JT, Morisbak E, Ansteinsson V, Becher R, Dahl JE, et al. Dental monomers inhibit LPS-induced cytokine release from the macrophage cell line RAW264.7. Toxicol Lett 2013;216(2–3):130–8.
[11] Noda M, Wataha JC, Lockwood PE, Volkmann KR, Kaga M, Sano H. Sublethal, 2-week exposures of dental material components alter TNF-alpha secretion of THP-1 monocytes. Dent Mater 2003;19(2):101–5.
[12] Nocca G, Ragno R, Carbone V, Martorana GE, Rossetti DV, Gambarini G, Giardina B, Lupi A. Identification of glutathione-methacrylates adducts in gingival fibroblasts and erythrocytes by HPLC-MS and capillary electrophoresis. Dent Mater 2011;27(5):e87–98.
[13] Samuelsen JT, Kopperud HM, Holme JA, Dragland IS, Christensen T, Dahl JE. Role of thiol-complex formation in 2-hydroxyethyl- methacrylate-induced toxicity in vitro. J Biomed Mater Res A 2011;96(2):395–401.
[14] Krifka S, Spagnuolo G, Schmalz G, Schweikl H. A review of adaptive mechanisms in cell responses towards oxidative stress caused by dental resin monomers. Biomaterials 2013;34(19):4555–63.
[15] Bolling AK, Solhaug A, Morisbak E, Holme JA, Samuelsen JT. The dental monomer hydroxyethyl methacrylate (HEMA) counteracts lipopolysaccharide-induced IL-1beta release-possible role of glutathione. Toxicol Lett 2017;270:25–33.
[16] Schweikl H, Godula M, Petzel C, Bolay C, Hiller KA, Buchalla W. Critical role of superoxide anions and hydroxyl radicals in HEMA-induced apoptosis. Dent Mater 2017;33(1): 110–8.
[17] Kilian M, Chapple IL, Hannig M, Marsh PD, Meuric V, Pedersen AM, Tonetti MS, Wade WG, Zaura E. The oral microbiome — an update for oral healthcare professionals. Br Dent J 2016;221(10):657–66.
[18] Aruni AW, Dou Y, Mishra A, Fletcher HM. The biofilm community-rebels with a cause. Curr Oral Health Rep 2015;2(1):48–56.
[19] Mariano FS, Sardi JCO, Duque C, Höfling JF, Gonçalves RB. The role of immune system in the development of periodontal disease: a brief review. Rev Odonto Ciênc 2010;25(3):300–5.
[20] Merry R, Belfield L, McArdle P, McLennan A, Crean S, Foey A. Oral health and pathology: a macrophage account. Br J Oral Maxillofac Surg 2012;50(1):2–7.
[21] Hasturk H, Kantarci A, Van Dyke TE. Oral inflammatory diseases and systemic inflammation: role of the macrophage. Front Immunol 2012;3:118.
[22] Walker DM. Oral mucosal immunology: an overview. Ann Acad Med Singapore 2004;33(4 Suppl):27–30.
[23] Eckhardt A, Harorli T, Limtanyakul J, Hiller KA, Bosl C, Bolay C, Reichl FX, Schmalz G, Schweikl H. Inhibition of cytokine and surface antigen expression in LPS-stimulated murine macrophages by triethylene glycol dimethacrylate. Biomaterials 2009;30(9):1665–74.
[24] Di Nisio C, Zara S, Cataldi A, di Giacomo V. 2-Hydroxyethyl methacrylate inflammatory effects in human gingival fibroblasts. Int Endod J 2013;46(5):466–76.
[25] Di Nisio C, De Colli M, di Giacomo V, Rapino M, Di Valerio V, Marconi GD, Gallorini M, Di Giulio M, Cataldi A, Zara S. A dual role for beta1 integrin in an in vitro Streptococcus mitis/human gingival fibroblasts co-culture model in response to TEGDMA. Int Endod J 2015;48(9): 839–49.
[26] Becher R, Valen H, Olderbo BP, Bolling AK, Samuelsen JT. The dental monomer 2-hydroxyethyl methacrylate (HEMA) causes transcriptionally regulated adaptation partially initiated by electrophilic stress. Dent Mater 2018;35(1):125–34.
[27] Schweikl H, Gallorini M, Poschl G, Urmann V, Petzel C, Bolay C, Hiller KA, Cataldi A, Buchalla W. Functions of transcription factors NF-kappaB and Nrf2 in the inhibition of LPS-stimulated cytokine release by the resin monomer HEMA. Dent Mater 2018;34(11):1661–78.
[28] Moharamzadeh K, Van Noort R, Brook IM, Scutt AM. HPLC analysis of components released from dental composites with different resin compositions using different extraction media. J Mater Sci Mater Med 2007;18(1):133–7.
[29] Sandberg E, Bergenholtz G, Kahu H, Dahlgren UI. Low HEMA conjugation induces high autoantibody titer in mice. J Dent Res 2005;84(6):537–41.
[30] Vieira Colombo AP, Magalhaes CB, Hartenbach FA, Martins do Souto R, Maciel da Silva-Boghossian C. Periodontal-disease-associated biofilm: a reservoir for pathogens of medical importance. Microb Pathog 2016;94:27–34.
[31] Cuesta AI, Jewtuchowicz V, Brusca MI, Nastri ML, Rosa AC. Prevalence of Staphylococcus spp and Candida spp in the oral cavity and periodontal pockets of periodontal disease patients. Acta Odontol Latinoam 2010;23(1):20–6.
[32] Loberto JCS, de Paiva Martins CA, dos Santos SSF, Cortelli JR, Jorge AOC. Staphylococcus spp. In the oral cavity and periodontal pockets of chronic periodontitis patients. Braz J Microbiol 2004;35:64–8.
[33] O'Connor AM, McManus BA, Kinnevey PM, Brennan GI, Fleming TE, Cashin PJ, O'Sullivan M, Polyzois I, Coleman DC. Significant enrichment and diversity of the staphylococcal arginine catabolic mobile element ACME in Staphylococcus epidermidis isolates from subgingival peri-implantitis sites and periodontal pockets. Front Microbiol 2018;9, 1558-1558.
[34] Blomqvist S, Leonhardt Å, Arirachakaran P, Carlen A, Dahlén G. Phenotype, genotype, and antibiotic susceptibility of Swedish and Thai oral isolates of Staphylococcus aureus. J Oral Microbiol 2015;7:26250.
[35] Feezor RJ, Oberholzer C, Baker HV, Novick D, Rubinstein M, Moldawer LL, Pribble J, Souza S, Dinarello CA, Ertel W, Oberholzer A. Molecular characterization of the acute inflammatory response to infections with gram-negative versus gram-positive bacteria. Infect Immun 2003;71(10):5803–13.
[36] Shimada T, Park BG, Wolf AJ, Brikos C, Goodridge HS, Becker CA, Reyes CN, Miao EA, Aderem A, Gotz F, Liu GY, Underhill DM. *Staphylococcus aureus* evades lysozyme-based peptidoglycan digestion that links phagocytosis, inflammasome activation, and IL-1beta secretion. Cell Host Microbe 2010;7(1):38–49.
[37] Bolling AK, Steensen TB, Alexis NE, Sikkeland LIB. Isolating and culturing of sputum macrophages: a potential ex vivo/in vitro model. Exp Lung Res 2018:1–11.
[38] Chanput W, Mes JJ, Wichers HJ. THP-1 cell line: an in vitro cell model for immune modulation approach. Int Immunopharmacol 2014;23(1):37–45.
[39] Daigneault M, Preston JA, Marriott HM, Whyte MK, Dockrell DH. The identification of markers of macrophage differentiation in PMA-stimulated THP-1 cells and monocyte-derived macrophages. PLoS One 2010;5(1):e8668.
[40] Tomlinson GS, Booth H, Petit SJ, Potton E, Towers GJ, Miller RF, Chain BM, Noursadeghi M. Adherent human alveolar macrophages exhibit a transient pro-inflammatory profile that confounds responses to innate immune stimulation. PLoS One 2012;7(6):e40348.
[41] Ginhoux F, Guilliams M. Tissue-resident macrophage ontogeny and homeostasis. Immunity 2016;44(3):439–49.
[42] Kluytmans J, van Belkum A, Verbrugh H. Nasal carriage of *Staphylococcus aureus*: epidemiology, underlying

mechanisms, and associated risks. Clin Microbiol Rev 1997;10(3):505–20.
[43] Vandenbergh MF, Verbrugh HA. Carriage of *Staphylococcus aureus*: epidemiology and clinical relevance. J Lab Clin Med 1999;133(6):525–34.
[44] McCormack MG, Smith AJ, Akram AN, Jackson M, Robertson D, Edwards G. Staphylococcus aureus and the oral cavity: an overlooked source of carriage and infection? Am J Infect Control 2015;43(1):35–7.
[45] Smith AJ, Jackson MS, Bagg J. The ecology of *Staphylococcus* species in the oral cavity. J Med Microbiol 2001;50(11):940–6.
[46] Terpenning MS, Taylor GW, Lopatin DE, Kerr CK, Dominguez BL, Loesche WJ. Aspiration pneumonia: dental and oral risk factors in an older veteran population. J Am Geriatr Soc 2001;49(5):557–63.
[47] Archer NK, Mazaitis MJ, Costerton JW, Leid JG, Powers ME, Shirtliff ME. Staphylococcus aureus biofilms: properties, regulation, and roles in human disease. Virulence 2011;2(5):445–59.
[48] Heitz-Mayfield LJ, Lang NP. Comparative biology of chronic and aggressive periodontitis vs. peri-implantitis. Periodontol 2000;2010(53):167–81.
[49] Lu YC, Yeh WC, Ohashi PS. LPS/TLR4 signal transduction pathway. Cytokine 2008;42(2):145–51.
[50] Fournier B, Philpott DJ. Recognition of *Staphylococcus aureus* by the innate immune system. Clin Microbiol Rev 2005;18(3):521–40.
[51] Lewandowska-Sabat AM, Boman GM, Downing A, Talbot R, Storset AK, Olsaker I. The early phase transcriptome of bovine monocyte-derived macrophages infected with *Staphylococcus aureus* in vitro. BMC Genomics 2013;14:891.
[52] Oliveira-Nascimento L, Massari P, Wetzler LM. The role of TLR2 in infection and immunity. Front Immunol 2012;3:79.
[53] Nilsen BW, Ortengren U, Simon-Santamaria J, Sorensen KK, Michelsen VB. Methods and terminology used in cell-culture studies of low-dose effects of matrix constituents of polymer resin-based dental materials. Eur J Oral Sci 2016;124(6):511–25.
[54] Lim JJ, Grinstein S, Roth Z. Diversity and versatility of phagocytosis: roles in innate immunity, tissue remodeling, and homeostasis. Front Cell Infect Microbiol 2017;7:191.
[55] Andersson J, Dahlgren UI. Effect of 2-hydroxyethyl-methacrylate (HEMA) on the phagocytic and respiratory burst activity of human neutrophils and monocytes. Eur J Oral Sci 2008;116(4):369–74.
[56] Cho HY, Cho CW, Song YS. Antioxidative and anti-inflammatory effects of *Saururus chinensis* methanol extract in RAW 264.7 macrophages. J Med Food 2005;8(2):190–7.
[57] Haddad JJ. L-Buthionine-(S,R)-sulfoximine, an irreversible inhibitor of gamma-glutamylcysteine synthetase, augments LPS-mediated pro-inflammatory cytokine biosynthesis: evidence for the implication of an IkappaB-alpha/NF-kappaB insensitive pathway. Eur Cytokine Netw 2001;12(4):614–24.
[58] Dutzan N, Konkel JE, Greenwell-Wild T, Moutsopoulos NM. Characterization of the human immune cell network at the gingival barrier. Mucosal Immunol 2016;9(5):1163–72.
[59] Khan SA, Kong EF, Meiller TF, Jabra-Rizk MA. Periodontal diseases: bug induced, host promoted. PLoS Pathog 2015;11(7):e1004952.
[60] Meyle J, Dommisch H, Groeger S, Giacaman RA, Costalonga M, Herzberg M. The innate host response in caries and periodontitis. J Clin Periodontol 2017;44(12):1215–25.