Dental acrylates and methacrylates

Interactions with the immune system

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"*It always seems impossible until it's done*"

Nelson Mandela

Abstract

Professionals working in dentistry have reported adverse effects, such as allergic contact dermatitis due to exposure to different methacrylates/acrylates. Leakage of such monomers from dental fillings due to incomplete curing is also common. Monomers are released into the oral cavity and some may also diffuse through the dentin and reach the dental pulp. In the tissue, monomers will come in contact with different cells of the immune system. At present, there is a lack of knowledge of the effects these monomers might have on cells. The main objective of this thesis was to investigate possible effects of four different methacrylate/acrylate monomers on the immune system *in vivo* and *in vitro*. The studied methacrylates and acrylate were ethyl methacrylate (EMA), diethylene glycol diacrylate (DEGDA), 2-hydroxyethyl methacrylate (HEMA) and triethylene glycol dimethacrylate (TEGDMA). In summary, our *in vivo* and *in vitro* results showed that:

- HEMA affects the immune system by inducing formation of the NLRP3 inflammasome (**Study I**).
- TEGDMA has adjuvant properties and the ability to modulate cytokine production from peripheral blood mononuclear cells, which may lead to interference with the normal immune response to various agents, self or non-self, present in the pulp and the oral mucosa (**Study II**).
- HEMA acts as a mucosal adjuvant when applied sublingually together with ovalbumin (OVA) or *Streptococcus mutans* in mice (**Study III**).
- The different monomers affect the production, increase as well as decrease, of various cytokines *in vitro* by peripheral blood mononuclear cells. Antibody production and T cell activity to OVA was increased in mice immunized with OVA in combination with methacrylates (**Study IV**).

Bacteria and bacterial products such as hydrogen sulfide (H_2S) challenge the oral mucosa. We wanted to investigate whether H_2S also affects the immune system in similar way as HEMA, i.e., induce formation of the NLRP3 inflammasome.

- Human mononuclear leukocytes exposed to H_2S had an enhanced production of NLRP3 inflammasome-dependent secretion of IL-1β and IL-18. (**Study V**). Thus, this may be a mode for H_2S to contribute to the inflammatory host response and pathogenesis of periodontal disease

In summary, the different methacrylate/acrylate monomers frequently used in dental restoration materials may interfere with the immune system in many different ways. The increase as well as the decrease in cytokine production from human mononuclear cells is affected by all these methacryleate/acrylate monomers. The T and B cell activity is also affected by all tested methacrylates . Thus, this may be a model that provide some insight into the nature of the immune responses to methacrylates and acrylates, and may advance the development of more biocompatible restorative materials in the future for clinical use.

Keywords

Methacrylates, acrylates, cytokines, immunoglobulin, B cell, T cell, macrophages, mouse, adjuvant

Sammanfattning

på svenska

I Sverige har man i stort sett slutat använda amalgam som tandsubstansersättning. Istället används kompositmaterial som består av plast. Plaster är uppbyggda av stora molekyler (polymerer) som har bildats av att små molekyler (så kallade akrylat och metakrylatmonomerer) hakats ihop till välordnade nät. Parallellt med den ökade användningen av kompositfyllningar har man sett en ökad frekvens av allergiska hudbesvär hos tandvårdspersonal som handskas med dessa material dagligen. Dessutom anser ett antal patienter att de känner olika symtom p.g.a. sina plastfyllningar. Detta förknippas framförallt med metakrylatmonomererna 2-hydroxyethyl methacrylate (HEMA) och triethylene glycol dimethacrylate (TEGDMA) men även ethyl methacrylate (EMA) och diethylene glycol diacrylate (DEGDA).

Under de första 24 timmarna efter härdning (processen då monomererna går ihop och bildar polymerer) av kompositmaterialet läcker en stor del akrylat- och metakrylatmonomerer ut i munnen. En del monomerer kan även penetrera dentinet och komma i kontakt med olika celler som tillhör immunsystemet. Vårt immunsystem har till uppgift att försvara kroppen mot cancerutveckling och mot angrepp av patogena organismer. Immunsystemet består av vita blodkroppar som är specialiserade celler med olika roller i immunförsvaret. För att försvara sig reagerar kroppen oftast med en inflammation, där de vita blodkropparna har en viktig roll. Ett område som är inflammerat blir ömt, rött, svullet och varmt.

Det huvudsakliga syftet med avhandlingen har varit att undersöka olika effekter som HEMA, TEGDMA, EMA och DEGDA kan ha på immunsystemet. Vi har kunnat se att celler som exponeras för dessa akrylat/metakrylat monomerer producerar olika signaleringssubstanser som kan bidra till inflammation. Detta kan innebära att läckage av monomerer från tandfyllningar som kommer i kontakt med celler i orala vävnader leder till inflammation. Vi har även sett att metakrylaterna kan förstärka immunsystemets reaktion mot olika ämnen på munslemhinnan vilket eventuellt skulle kunna förklara de obehag som vissa patienter upplever efter behandlingar med akrylat och metakrylatbaserade material. Vi har även visat att vätesulfid, en metabolit från bakterier, har effekt på immunsystemet som liknar de som vissa metakrylater har.

Resultat från denna studie är av stor betydelse för att med en vetenskapligt förankrad kunskap kunna bemöta patienter som oroar sig för de dentala material de exponeras för. Dessutom behövs denna kunskap för att hitta material med god biokompatibilitet och med minimal risk för att påverka immunsystemet ofördelaktig.

List of papers

This thesis is based on the following studies, which are referred to in the text by their Roman numerals:

I. **Alizadehgharib S**, Östberg AK, Larsson L and Dahlgren U. *The immunomodulatory properties of 2-hydroxyethyl methacrylate are mediated by the NLRP3 inflammasome.* Submitted for publication.

II. **Alizadehgharib S**, Östberg AK and Dahlgren U. *Triethylene glycol dimethacrylate – adjuvant properties and effect on cytokine production*. Submitted for publication.

III. Östberg AK, **Alizadehgharib S** and Dahlgren U. *Sublingual administration of 2-hydroxyethyl methacrylate enhances antibody responses to co-administered ovalbumin and Streptococcus mutans.* Submitted for publication.

IV. **Alizadehgharib S**, Östberg AK and Dahlgren U. *Effects of the methacrylate/acrylate monomers HEMA, TEGDMA, DEGDA and EMA on the immune system*. Submitted for publication.

V. Basic A, **Alizadehgharib S**, Dahlén G and Dahlgren U. *Hydrogen Sulfide exposure induces NLRP3 inflammasome-dependent IL-1β and IL-18 secretion in human mononuclear leukocytes in vitro*. Clinical and Experimental Dental Research 2017. 3:115-120.

Content

Abbreviations

ALP	Alkaline phosphatase
Alum	Aluminum hydroxide
APC	Antigen-presenting cell
ASC	Apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain
DAMP	Damage-associated molecular patterns
DC	Dendritic cell
DEGDA	Diethylene glycol diacrylate
ELISA	Enzyme-linked immunosorbent assay
EMA	Ethyl methacrylate
GC	Germinal center
HCE	Hierarchical Clustering Explorer
HEMA	2-Hydroxyethyl methacrylate
HRP	Horseradish peroxidase
H_2S	Hydrogen sulfide
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
LPS	Lipopolysaccharide
MCP-1	Monocyte chemoattractant protein-1
NLRP3	NACHT, LRR and PYD domains-containing protein 3
NOD	Nucleotide-binding oligomerization domain
OVA	Ovalbumin
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PRR	Pattern recognition receptor
TEGDMA	Triethylene glycol dimethacrylate
TLR	Toll-like receptor
TNF	Tumor necrosis factor
VEGF	Vascular endothelial growth factor

Introduction

In recent years, amalgam tooth fillings have been replaced with resin-based composite materials that contain acrylate and methacrylate esters (Aalto-Korte *et al.*, 2007; Ansteinsson *et al.*, 2011; Goon *et al.*, 2006; Heil *et al.*, 2002; Nocca *et al.*, 2014; Rakich *et al.*, 1999; Schmalz *et al.*, 2011; Van Landuyt *et al.*, 2011). These resin esters are products formed when alcohol reacts with acrylic or methacrylic acid. Despite the growing usage of these materials, there are concerns regarding toxicity due to possible leakage of monomers (Bationo *et al.*, 2016; Gerzina *et al.*, 1996; Spahl *et al.*, 1998).

Dental materials are categorized as preventive, therapeutic, and restorative materials. Resin-based composite materials that contain acrylates and methacrylates belong to the group of restorative materials. Restorative materials are used to repair or replace tooth structure that have been lost due to oral disease such as caries, for repair of fractures, or they are used to change the appearance of the teeth. There are different classifications of resin-based filling composite materials, one of which is matrix resin. The matrix resin comprises of different elements, such as methacrylate monomers, and other additives, such as initiators (camphoroquinone and benzoylperoxide) (Schmalz, 2009). The process of forming polymers is referred to as polymerization, which requires free radicals that are generated during decomposition of the initiator. Once a free radical has reacted with a methacrylate/acrylate monomer, that monomer will contain a reactive carbon atom, which in turn can react with another free monomer. When all the free radicals have reacted, the polymerization process is terminated. However, the polymerization is never fully complete, and free residual monomers are present in the dental fillings and they leak out into the oral cavity over several days or weeks (Carol Dixon Hatrick, 2016; Schmalz *et al.*, 2011). Long-term exposure of mice to minute amounts of 2-hydroxyethyl methacrylate (HEMA) affects their general condition and impairs their growth (Andersson *et al.*, 2011b).

Three main routes for systemic intake of chemical components released by resin-based restorations are currently known: (i) the uptake of volatile components in the lungs (Hagberg *et al.*, 2005; Piirila *et al.*, 1998); (ii) the ingestion of released compounds in the gastrointestinal tract (Kwon *et al.*, 2015); and (iii) diffusion of released compounds through to the pulp (Schmalz *et al.*, 2011). The first route is of special importance for dental personnel (Aalto-Korte *et al.*, 2010; Hagberg *et al.*, 2005; Jacobsen *et al.*, 1996; Kanerva *et al.*, 2001; Piirila *et al.*, 1998), while the second and third routes are of greater importance for the patient. Adverse effects related to the uptake of compounds released from resin-based restorations, such as asthma and rhinoconjunctivitis, have been reported (Lindstrom *et al.*, 2002). It has also been established that methacrylate/acrylate monomers damage the soft tissues of the oral cavity *in vivo* (Geurtsen, 2000).

Dental methacrylates and acrylates

Two major components of dentin-bonding agents (DBAs) and dental resin-based composites in today's dentistry are: triethylene glycol dimethacrylate (TEGDMA), $CH_2=C(CH_3)COO(CH_2CH_2O)_3COC(CH_3)=CH_2$ (molecular weight = 286.32 g mol^{-1}); and HEMA, $CH_2=C(CH_3)COOCH_2CH_2OH$ (molecular weight = 130 g mol^{-1}) (Moharamzadeh *et al.*, 2007; Stanislawski *et al.*, 2003). Two other frequently used acrylates are: ethyl methacrylate (EMA), CH2=C(CH_3)$COOC_2H_5$ (molecular weight = 114 g mol^{-1}); and diethylene glycol diacrylate (DEGDA) $(H_2C=CHCO_2CH_2CH_2)_2O$ (molecular weight = 214 g mol^{-1}) (Fig. 1). These are all methacrylates / acrylates that are associated with contact dermatitis in dental personnel (Aalto-Korte *et al.*, 2007; Aalto-Korte *et al.*, 2010).

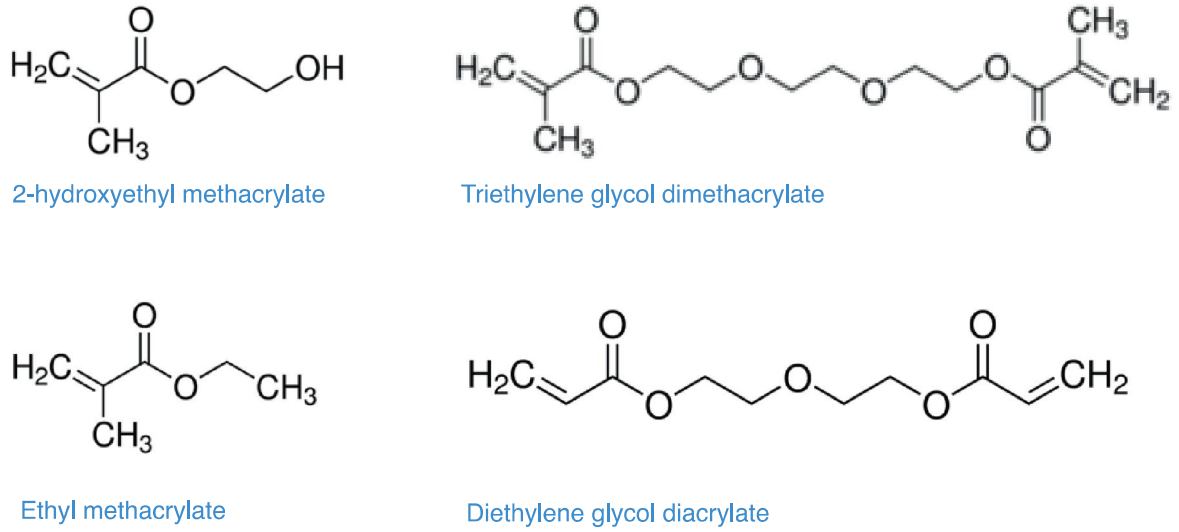

Figure 1. The chemical structures of the methacrylates and acrylate examined in the present thesis.

These methacrylates and acrylate are 'light' co-monomers that are used in resin matrices with "heavy" monomers, such as bisphenol A glycidylmethacrylate (Bis-GMA) (molecular weight = 513 g mol^{-1}), to enhance the bonding strength to dentin and to reduce the viscosity of the monomers (Altintas *et al.*, 2012).

Previous studies have shown that both DBAs and resin-based composites release unreacted HEMA and TEGDMA monomers into the oral cavity (Bationo *et al.*, 2016; Gerzina *et al.*, 1996; Spahl *et al.*, 1998). In deep cavities, the pulp can be exposed to concentrations of about 4 mmol/L TEGDMA or 1.5–8.0 mmol/L HEMA (Schmalz *et al.*, 2011). The monomers come in contact with leukocytes that are present in the pulp and oral cavity. The population of white blood cells found in the dental pulp consists of $CD4^+$ and $CD8^+$ T lymphocytes (T cells), as well as dendritic cells (DCs) (Langerhans cells), neutrophils and macrophages (Jontell *et al.*, 1998). Gingival crevicular fluid (GCF), flows into the gingival crevice through the junctional epithelium thereby transporting cells into the oral cavity. The population of cells in the GCF comprises of 95–97 % neu-

trophils, 1–2 % lymphocytes, with fewer T cells than B lymphocytes (B cells) and 2–3 % monocytes (Attstrom, 1970). Since many different cells are present in the oral cavity, pulp, and epithelium, a variety of cells may encounter free monomers. The dental methacrylates HEMA and TEGDMA have a multitude of effects on leukocytes of the innate branch of the immune system (neutrophils, monocytes, and macrophages) as well as the adaptive branch (B cells and T cells) (Table 1). However, not much is known about potential immunological effects of methacrylate EMA and the acrylate DEGDA.

Table 1. Previously reported inductive and suppressive effects of 2-hydroxyethyl methacrylate (HEMA), triethylene glycol dimethacrylate (TEGDMA), ethyl methacrylate (EMA), and diethylene glycol diacrylate (DEGDA) on the immune system.

	Promoted responses	Suppressed responses
HEMA	- T cell proliferation (Andersson *et al.*, 2011a) - Apoptotic cell death of murine macrophages (Paranjpe *et al.*, 2005) - Formation of reactive oxygen species in BEAS-2B cells (Morisbak *et al.*, 2015) and in rat submandibular salivary gland acinar cells (Samuelsen *et al.*, 2007) - Anti-OVA IgG but not IgM activity in BALB/c mice (Andersson *et al.*, 2011a) - IL-6 and IL-8 production (Trubiani *et al.*, 2012) - Phosphorylation of p38, JNK and ERK1/2 in rat submandibular salivary gland acinar cell (Samuelsen *et al.*, 2007)	- Migration of dental pulp cells (Williams *et al.*, 2013) - Type I collagen protein production (Grande *et al.*, 2015) - TNF-α production in splenocytes (Andersson *et al.*, 2011a) - Growth of human dental pulp mesenchymal stem cells (DP-MSCs) (Trubiani *et al.*, 2012) - Osteoclast differentiation of RAW-D cells and bone marrow-derived macrophages (Inamitsu *et al.*, 2017)
TEGDMA	- Phosphorylation of ERK1/2 in rat submandibular salivary gland acinar cell (Samuelsen *et al.*, 2007) - Apoptotic and toxic effect on THP1 cells (Harorli *et al.*, 2009) - IL-8 in human dental pulp cells (Golz *et al.*, 2016) - IL-6 in a three-dimensional model of human epithelium (Schmalz *et al.*, 2000) - IL-1β in an oral mucosa model (Moharamzadeh *et al.*, 2009)	- Osteoclast differentiation of RAW-D cells and bone marrow-derived macrophages (Inamitsu *et al.*, 2017) - LPS-induced release of IL-1β and TNF-α (Bolling *et al.*, 2013)
EMA	None reported.	None reported.
DEGDA	None reported.	None reported.

The oral microbiota

The oral cavity possesses various features that make a perfect environment for a number of different microorganisms. One of the features is the saliva that is continuously bathing the surfaces of the oral cavity at a limited temperature range (34 to 36° C) and a pH close to neutrality (Marcotte *et al.*, 1998).

The saliva contains $10^{7\text{-}9}$/ml bacteria (Martin, 2009) originating from 600 different oral bacterial strains (Aas *et al.*, 2005; Dewhirst *et al.*, 2010). These bacteria cover various surfaces of the oral cavity such as the tongue, the mucosal surfaces, the periodontal pockets, the gingiva and the teeth. The bacteria can be classified in many different ways, for example Gram-positive or Gram-negative based on cell wall structure, strict anaerobic or facultative anaerobic depending on their oxygen requirements and saccharolytic and proteolytic depending on the major source of nutrients. Normally, the oral microbiota constitutes of a stable community that exists in a balance with the host (microbial homeostasis). However, dysbiosis of the microflora due to changes in the environmental factors, such as access to nutrients (e.g., sugars in relation to caries and proteins/amino acids in relation to periodontal diseases), the pH level and the presence or absence of oxygen may result in different oral diseases such as caries and periodontal diseases (Kilian *et al.*, 2016).

Streptococcus mutans (*S. mutans*) is a bacterial species naturally present in the human oral microbiota but is also commonly associated with caries activity (Azizi *et al.*, 2016), mainly due to its acidogenic and aciduric properties. Streptococci including *S. mutans* are strongly saccharolytic and degrade most sugars including sucrose into lactic acid. The cariogenic property of *S. mutans* is due to glucosyltransferases, which is an essential virulence factor that facilitates the ability of this bacterium to produce polyglucans and to adhere and colonize the tooth surface when sucrose is present in the host diet (Yamashita *et al.*, 1993), forming a sticky biofilm (Grande *et al.*, 2015).

The acidic environment in the dental caries, formed by *S. mutans* and other streptococci is beneficial for other aciduric bacteria such as *Lactobacillus* species. *Lactobacillus* are extremely acid tolerant and is favored and increased in numbers in acidic environments such as caries lesions and in patients suffering from dry mouth.

Porphyromonas gingivalis (*P. gingivalis*) is a proteolytic bacterium specifically associated with chronic periodontitis (inflammation in tissue surrounding the teeth) (How *et al.*, 2016). *P. gingivalis* produces a number of metabolites such as various acids, ammonia and hydrogen sulfide (H_2S) (Greabu *et al.*, 2016), that may induce gingival inflammation, which may progress to loss of dental attachment and periodontitis (Hajishengallis *et al.*, 2012). H_2S is known as a toxic gas with an unpleasant smell, that may be found as bacterial waste product in the periodontal pocket (Persson, 1992). Due to the inflammatogenic potential of H_2S, it has been suggested to be part of the initiation and progression of periodontal disease. *P. gingivalis* also produce other virulence factors such as endotoxins that may cause damage to the host cells. Lipopolysaccharide (LPS) is an endotoxin found on the outer membrane of Gram-negative bacteria that may elicit tissue inflammation

(Schmalz *et al.*, 2011). As both bacteria and monomers can cause environmental stress to oral tissues, it is important to study the combined effect these substances may have on target cells.

Innate immunity

The innate immune system is an important first-line defense against pathogens and infections, and it activates the adaptive part of the immune system. Innate immune responses are generic i.e., not specifically directed against a particular pathogen and there is no memory involved in the response. The innate immune system is based on host proteins and phagocytic cells that quickly become activated and responds to pathogens (Alberts B, 2002).

The most important elements of the innate immune system are the physical epithelial barriers, DCs, phagocytic leukocytes (e.g., monocytes and neutrophils) and circulating plasma proteins.

The monocytes represent 2 - 10% of the circulating leukocytes and have a half-life of 3 days, whereas the neutrophils represent about 60% of the circulating leukocytes and have a circulating half-life of 6 - 8 h (Summers *et al.*, 2010).

The innate immune system recognizes danger-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs) that are associated with trauma, ischemia, and tissue damage (DAMP) and different groups of pathogens (PAMPs). These danger signals are recognized by pattern recognition receptors (PRRs) such as the membrane-bound Toll-like receptors (TLRs) (Mogensen, 2009). A prototypical PAMP is LPS, which is recognized by TLR4, a recognition receptor of the innate immune system.

Nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs)

Another group of the PRRs includes the NLRs, which are found in the cytoplasm. These intracellular receptors have a leucine-rich repeat (LRR) domain close to the carboxyl terminus, which recognizes conserved molecular patterns present on various pathogens in addition to danger signals such as ATP. Through activation of the transcription factor, nuclear factor kappa B (NF-κB), different proinflammatory genes are transcribed causing cytokines, chemokines, and adhesion molecules to be expressed.

Subfamilies of NLRs can be distinguished by the protein peptide domain located close to the amino terminus of the protein. The NOD subfamily has an amino-terminal, cysteine-aspartic acid proteases (caspase) recruitment domain (CARD), which interacts with caspase, an important factor in apoptosis. One of the subfamilies is the NLRP family, which has a pyrin domain (PYD) at the amino terminus and contains a NACHT-associated domain (NAD). These proteins are similar in structure to CARD, and they interact with other pyrin domains (Murphy, 2012). In humans, there are 14 different NLR proteins that contain a pyrin domain. The best-described one is NLRs and NACHT, LRR and PYD domains-containing protein 3 (NLRP3) inflammasome (also called NALP3 or cryopyrin), which has the ability to recognize cell damage/stress. In stressed cells (e.g., cells that have been exposed to infection), NLRP3 usually interact with caspase-1 and an

adaptor protein forming an inflammasome in the cytosol. The most common adaptor is apoptosis-associate speck-like protein containing a caspase recruitment domain (ASC) (Guo *et al.*, 2015).

There is a broad diversity of stimuli (e.g., ATP, cholesterol, monosodium crystals and alum) (Bauernfeind *et al.*, 2009; Duewell *et al.*, 2010; Eisenbarth *et al.*, 2008; Matias *et al.*, 2015; Sutterwala *et al.*, 2014) that cause formation of the NLRP3 inflammasome. Common to them all is that they cause efflux of cytoplasmic K^+ ions from the exposed cells (Eisenbarth *et al.*, 2008; Moretto *et al.*, 2013; Zhu *et al.*, 2011). By increasing the extracellular concentration of K^+ ions, the efflux of K^+ is inhibited and the formation of the NLRP3 inflammasome is prevented (Petrilli *et al.*, 2007). For activation of the NLRP3 inflammasome, two different signals are needed. The first signal is the priming step, and may be a TLR ligand (e.g., LPS), which induces the transcription of the genes for Pro-IL-1β, Pro-IL-18 and NLRP3 *via* NF-κB. The second signal (e.g., MSU or alum) triggers NLRP3 inflammasome and caspase-1 activation followed by IL-1β and IL-18 secretion (G. Chen *et al.*, 2010) (Fig. 2).

IL-1α and β, IL-18, IL-33, IL-36α, β and γ are cytokines that belong to the IL-1 family, a central mediator of the innate immunity and inflammation. For a balance between amplification of the innate immunity and uncontrolled inflammation, it is important with a tight regulation *via* anti-inflammatory cytokines such as IL-1Ra, IL-36Ra, and IL-38 that are additional members of the IL-1 family. The IL-1 family members affect all cells of the innate immune system and play a key role in the differentiation and function of polarized innate and adaptive lymphoid cells (Garlanda *et al.*, 2013).

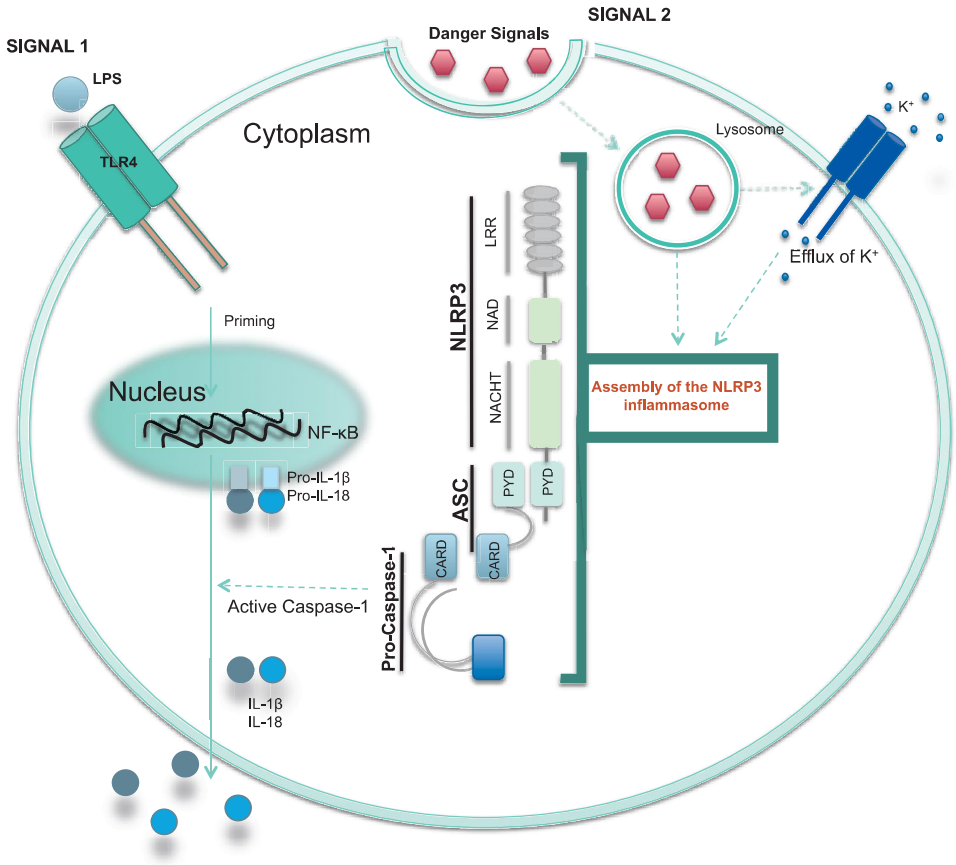

Figure 2. Assembly of the NLRP3 inflammasome.

Proinflammatory cytokines such as members of the IL-1 family, IL-6, IL-8, GROα, and TNF-α can contribute to inflammation. Inflammation is induced to remove harmful stimuli, including damaged cells, irritants, or pathogens and to start the healing process. Signs of inflammation are heat, pain, redness, swelling, and loss of function.

When foreign compounds such as dangers signals come in contact with different cells of the immune system, they may result in inflammation. Acrylate/methacrylate monomers that penetrate the mucosal epithelium of the mouth or the dentin, may work as danger signals when they interact with different cells of the immune system. This may lead to production of various cytokines with different effects such as inflammation in the surrounding tissues (Table 2. and Table 3.) (Reichl *et al.*, 2002). Some cytokines have the ability to trigger inflammation (proinflammatory cytokines), while others have the opposite effect and act to dampen the inflammatory response (anti-inflammatory cytokines, such as IL-1Rα).

Table 2. The cytokines produced during innate immune responses.

	Source(s)	Target(s)
IL-1	Macrophages, endothelia, epithelia	**Endothelia** (↑ coagulation, ↑ inflammation) **Hepatocytes** (↑ acute phrase proteins) **Hypothalamus** (↑ fever)
IL-18	Macrophages	NK lymphocytes (↑ IFN-II γ) T lymphocytes (↑ IFN-II γ)
TNF	Macrophages, T lymphocytes	**Endothelia** (↑ coagulation, ↑ inflammation) **Hepatocytes** (↑ acute phrase proteins) **Hypothalamus** (↑ fever) **Neutrophils** (↑ activation)
IL-6	Macrophages, endothelia, T lymphocytes	**Hepatocytes** (↑ acute phrase proteins), **B lymphocytes** (↑ proliferation)
IL-10	Macrophages, T lymphocytes	**Macrophages**, **dentritic cells** (↓ IL-12)
IL-12	Macrophages, dendritic cells	**T_H1 lymphocytes** (↑ differentiation), **CTL** (↑ IFN-II γ), **NK lymphocytes** (↑ IFN-II γ)
IL-15	Macrophages	**NK lymphocytes** (↑ proliferation) **T lymphocytes** (↑ proliferation)
IL-23	Macrophages, dendritic cells	**T lymphocytes** (↑ IL-17)
IL-27	Macrophages, dendritic cells	**T_H1 lymphocytes** (inhibition and/or differentiation) **NK lymphocytes** (↑ IFN-II γ)
IFN-1 (α)	Macrophages	All cells (↑ viral immunity, ↑ MHC class I), **NK lymphocytes** (↑ activation)
IFN-1 (β)	Fibroblasts	All cells (↑ viral immunity, ↑ MHC class I), **NK lymphocytes** (↑ activation)
Chemokines	Macrophages, endothelia, fibroblasts, epithelia	**Phagocyte** (↑ migration), **B lymphocytes** (↑ migration), **T lymphocytes** (↑ migration), ↑ wound repair

Adaptive immunity

The adaptive immune system (also known as the acquired immune system or specific immune system) is a subsystem of the overall immune system and is composed of highly specialized, lymphoid cells that combat infections and transformed cancer cells. Two main types of lymphocytes are involved in the immune response: **T cells**, which differentiate in the thymus, and **B cells**, which differentiate in the bone marrow. The two main groups of T cells are the T helper cells (T_H cells), which are crucial for orchestrating an appropriate immune response, and the cytotoxic T cells (T_C cells), which attack virus-infected cells and cancer cells. Meanwhile, activated B cells (**plasma cells**) are responsible for **antibody** production. Antibodies have the ability to bind to a specific epitopes on an antigen, which for instance facilitates elimination of the antigen.

The innate immune response and the adaptive immune responses are not separate events, but interplay to eliminate pathogens and any harmful molecules. Unlike the innate immune responses, the adaptive responses are highly specific to the particular pathogen that induced them, and usually provide long-lasting protection against the pathogen (Alberts B, 2002).

An important but difficult task for the adaptive immune system is the ability to distinguish between harmless and harmful external and internal substances. The high prevalence of autoimmune diseases and allergies is a reflection of this difficulty.

Antigens (antibody generators) are substances capable of eliciting a response from the adaptive immune system. In some instances, the adaptive immune system can be induced to react to harmless foreign substance, such as proteins. This can be done in a laboratory animal (e.g., a mouse), by injecting the molecule together with an **adjuvant** (usually a danger signal of microbial origin) that activates the innate immune system. This process is called immunization. This type of administration makes it possible for almost any macromolecule, as long as it is foreign to the recipient, to induce an adaptive immune response that is specific to the administered macromolecule (Alberts B, 2002). A commonly used adjuvant for immunization in animals is Freund‘s adjuvant, and in humans aluminum sulfate (alum). Alum has the ability to activate the NLRP3 inflammasome (Eisenbarth *et al.*, 2009).

B cells

As mentioned above, the adaptive immune responses are mediated by B cells and T cells. B cells represent 10 - 15% of the lymphocytes found in the blood and 40 - 45 % of the lymphocyte population in the spleen (Abbas, 2007).

An individual B cell, upon activation is committed to producing one type of antibody that is specific for a given epitope on an antigen. When the B cell is activated, it starts to proliferate and differentiate into plasma cells, which secrete antibodies. Some of the B cells do not become plasma cells, but instead become long-lived **B-memory cells**.

Immunoglobulins (Ig), i.e., the collective of antibodies, are glycoproteins that consist of two heavy and two light peptide chains interlinked via disulphide bridges. They are divided into different classes and subclasses depending on the amino acid sequence of the heavy chain constant region. The basic structure of a generic immunoglobulin molecule

is Y-shaped, where the "arms" of the Y, also called the 'Fab' fragments (where 'Fab' stands for 'fragment, antigen-binding'), are the sites that selectively bind to antigen. The "tail" of the Y, also called the Fc fragment ('Fc' for 'fragment, crystallizable'), binds to receptors on the surfaces of cells, such as macrophages (Fig. 3). The roles of the antibodies in the bloodstream are to agglutinate pathogens, activate the complement system, and act as opsonins to facilitate phagocytosis.

The different antibody classes in human are **IgA**, **IgE**, **IgD, IgM** and **IgG** each with a specific role in an immune response. IgA is an immunoglobulin that dominates in human secretions. IgE is the immunoglobulin located on surfaces of mast cells and basophils and is responsible for type I allergic reactions. IgD is located on the surface of naïve B cells and is present in small amounts in serum. IgG is the immunoglobulin with the highest concentration in blood and it has a long half-life. It dominates during the secondary immune response whereas IgM is the short-lived antibody that is produced during the primary immune response.

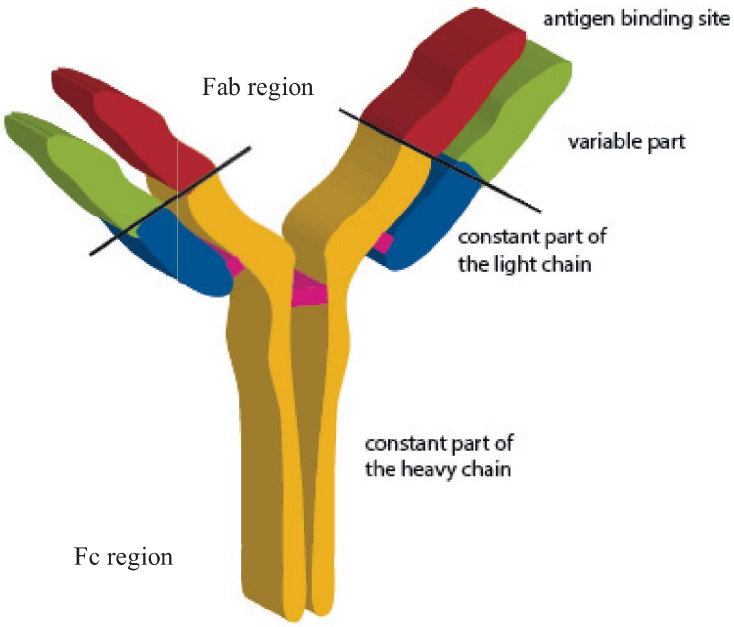

Figure 3. Immunoglobulin molecule. Source: Ulf Dahlgren.

T cells

T cells represent 85 - 90% of the lymphocytes found in blood and 50 - 60% of the lymphocyte population in the spleen (Abbas, 2007). T cells bind specifically with their T cell receptor to peptides presented on various cells. Thus the original intact antigen must be processed and fragmented to peptide pieces, called epitopes, which are presented on the surfaces of the antigen-presenting cells (APCs). The epitopes are presented by special membrane glycoproteins, the expression of which is determined by genes of the major histocompatibility complex (MHC). MHC is a group of genes that encode the peptide presenting membrane glycoproteins (HLA in humans). There are two different classes of HLA molecules: HLA class I that is found on all nucleated cells and platelets; and HLA class II, which is found on professional APCs, i.e., B cells and DCs.

The different HLA classes activate different T cells subsets. The two most common subsets are T_H cells that are identified by the presence of the CD4 surface marker, and T_C cells, which express CD8 on the cell surface. The T_H cells initiate the activation of B cells and other T cells. T_H cells are activated by peptides presented by class II molecules on the surface of B cells and DCs. The two most common subtypes of T_H cells are T_H1 and T_H2. T_H1 cells promote T_C cell activation and thus destruction of virus infected cells, while T_H2 cells produce cytokines, which are needed for B cell activation and antibody production.

T_C cells are specialized to target and kill virus infected and cancer-transformed cells. Direct contact between the T_C cell and its target cell is required. These cells secrete perforins and fragmentins, which cause the target cell to lyse or undergo apoptosis. Just like the B memory cells, some of the T cells, become T memory cells. The antigen-activated B and T cells that differentiate into memory cells are responsible for the rapid and enhanced responses to subsequent exposure to the antigens.

Table 3. Cytokines produced by cells of the adaptive immune system.

	Source(s)	Target(s)
IL-2	**T lymphocytes**	**T lymphocytes** (↑ survival, ↑ proliferation, ↑ cytokines) **B lymphocytes** (↑ proliferation, ↑ antibody production) **NK lymphocytes** (↑ proliferation, ↑ activation)
IL-4	**T_H2 lymphocytes**	**B lymphocytes** (↑ isotype switch IgE) **T_H2 lymphocytes** (↑ proliferation, ↑ differentiation) **Macrophages** (↓ IFN-II Y response), **Mast cells** (↑ proliferation)
IL-5	**T_H2 lymphocytes**	**B lymphocytes** (↑ proliferation, ↑ isotype switch IgA) **Eosinophils** (↑ proliferation, ↑ activation)
IL-13	**T_H2 lymphocytes, NK lymphocytes, mast cells**	**B lymphocytes** (↑ isotype switch IgE) **Macrophages** (↑ collagen) **Fibroblasts** (↑ collagen) **Epithelia** (↑ mucus)
IL-17	**T lymphocytes**	**Endothelia** (↑ chemokines) **Macrophages** (↑ cytokines/chemokines) **Epithelia** (↑ G-CSF and GM-CSF)
IFN-γ	**T_H1 lymphocytes** **CTL** **NK lymphocytes**	**B lymphocytes** (↑ isotype switch) **T_H1 lymphocytes** (↑ differentiation) **Macrophages** (↑ activation) **Various cells** (↑ antigen processing and ↑ MHC class I)

Organs of the immune system

The organs of the immune system include: the generative organs, also termed the **primary lymphoid organs** (bone marrow and thymus) in which the leucocytes are produced; and the peripheral group, also termed the **secondary lymphoid organs** (lymph nodes and spleen), where naive lymphocytes are activated by antigens. In the bone marrow, stem cells for all types of blood cells are found and all leucocytes except T cells mature in the bone marrow. Precursors for T cells leave the bone marrow and differentiate to T cells in the thymus.

Antigens mainly enter the body through the mucosal membranes. These areas, including the oral cavity are lined by epithelia that contain DCs. The DCs belong to the group of professional APCs and are also found in the subepithelial tissue. The functions of DCs are to display peptide antigens for recognition by T cells and thereby promote their activation. When pathogens enter the body, DCs capture the pathogen derived antigens and up-regulates lymph node-homing receptors and start to migrate *via* afferent lymphatic vessels to the draining **lymph nodes**. Lymph nodes consist of a capsule divided into an outer cortex, which contains lymphatic nodules, and an inner medulla. Within the lymph nodes, B cells respond to the antigens present in the lymph, and T cells respond to peptides presented by DCs and B cells.

The T cells that are activated in the lymph nodes are naïve T cells. The activated T cells exit the lymph nodes via the efferent lymphatic vessels and enter into the bloodstream and migrate to the site of inflammation or other peripheral tissue where they can mount a local immune response. When naïve B cells and T cells become activated and gain the ability to produce molecules capable of eliminating foreign antigens, they are called 'effector cells'. Effector B cells are antibody producing plasma cells and effector T cells comprise cytokine-secreting T_H cells and cytotoxic T_C cells.

The remaining activated T cells located in the lymph nodes become follicular helper T cells, which assist the B cells in forming germinal centers (GCs). The GC is the site in the lymph nodes where B cells proliferate and produce antibodies that exits the lymph nodes, thus contributing to the immunoglobulins found in blood.

Another lymphoid organ is the **spleen**, which in contrast to the lymph nodes (which traps antigens arriving in the lymph) captures antigens present in the blood. Arterial blood enters the spleen *via* the splenic artery and venous blood exits *via* the splenic vein. Similar to the lymph nodes, the spleen is also organized into B cell zone and the T cell zone. A capsule surrounds the spleen and in the middle, a splenic pulp is found. The pulp is divided into white pulp and red pulp. The white pulp consists mainly of lymphocytes while the red pulp is composed of erythrocytes. The spleen is supplied by small arteries branching from the trabeculae that are surrounded by a layer of lymphocytes, the lymphatic sheath, and therefore known as the sheathed (central) arteries. These sheathed arteries also divide into branches called follicular arteries that supply the lymphatic nodules. The lymphatic sheath is mainly composed of T cells, whereas the lymphatic nodules contain mostly B cells. Following exposure to an antigen, GCs are assembled in the nodules. The activated B cells migrate towards the marginal zone, which is the zone between the white and the red pulp, where they mature into plasma cells and secrete mainly IgG antibodies into the venous blood of the sinusoids. In the white pulp, the blood is filtered, and cellular fragments and old blood cells are engulfed by the macrophages in the red pulp. Therefore, the lymph nodes are filters for the lymph, while the spleen is the filter for the blood.

Lymphatic nodules, which are one of the most diffuse lymphoid tissues, are located in the loose connective-tissue spaces beneath most epithelial membranes (e.g., in the gastrointestinal tract and the respiratory system). Many cells of the lymphatic system are found in these spaces, and an antigen may come in contact with these cells, and thereby activate the B cells and induce them to proliferate. These localized aggregations of cells that are produced in response to antigen exposure are referred to as nodules. The nodules lack a capsule and are considered as temporary structures that are developed due to antigen stimulation. However, permanent groupings of lymphatic nodules can be found in the tonsils, the small intestine (Peyer's patches), and the appendix. Leucocytes that are found in the gut are referred to as the Gut-Associated Lymphoid Tissue (GALT). The white blood cells found in the airways are referred to as the Bronchus-Associated Lymphoid Tissue (BALT). A collective term for these parts of the immune system is the Mucosa-Associated Lymphoid Tissue (MALT). The cells that are found in the epithelium that covers the Peyer's patches in the gut are called the M-cells, and they possess the ability

to transport intraluminal antigens to the lymphocytes and other white blood cells present in the patches.

Routes of immunization

The different routes through which an antigen can be administered affect the type and magnitude of the immune response that is induced. The most common routes used for introduction of an antigen experimentally into the body are *via* **subcutaneous injection** into the fatty layer just below the dermis and oral administration, which includes topical **sublingual immunization**. Subcutaneous injection of HEMA and an antigen has been shown to increase the production of IgG and IgE antibodies directed against the co-administered antigen (Andersson *et al.*, 2011a; Sandberg *et al.*, 2005c).

Subcutaneous immunization

Antigens injected subcutaneously are taken up by Langerhans cells (DCs) in the skin, which transports the antigens to local lymph nodes where an immune response is initiated.

The skin, which is the largest organ in the human body, constitutes an important physical barrier between the host and the external environment. It is an active participant in host defense, as it has a cutaneous immune system with lymphocytes and APCs, which have the abilities to generate and support local immunological and inflammatory reactions.

Entry of foreign antigens into the body is often through ruptured or even intact skin. The majority of cells found in the epidermis are keratinocytes, melanocytes, epidermal Langerhans cells, and T cells. The keratinocytes produce several cytokines that can contribute to innate immunological reactions and cutaneous inflammation, while the Langerhans cells are the immature DCs of the cutaneous immune system. Langerhans cells capture the antigens that enter through the skin and, thereby become activated through the engagement of TLRs. The cells detach from the epidermis, enter the lymphatic vessels, start to express the CCR7 chemokine receptor, and migrate to the T cell zones of the draining lymph nodes in response to chemokines that are produced at that location. Thus, the peripheral tissue inflammation causes a significant increase in T cell influx into lymph nodes draining the site of inflammation.

Sublingual immunization

During sublingual immunization an antigen is applied to the mucosa under the tongue. The antigen normally penetrates the mucosal surface rapidly and therefore, sublingual immunization is regarded as being equivalent to intravenous injection of the immunogen. Previous studies have shown that the sublingual mucosa is highly vascularized and can rapidly absorb drugs *via* APCs, such that they enter the bloodstream without first passing through the liver or intestine (Song *et al.*, 2008; Zhang *et al.*, 2002).

Like the skin, the mucosal epithelium is a barrier between the internal and external environments and is in fact the most important site of entry for microbes and other antigens.

Once the antigen penetrates the mucosal surface, DCs transport it *via* afferent lymph to the draining lymph nodes (submandibular lymph nodes and cervical lymph nodes) for presentation of the antigen to T cells (Song *et al.*, 2009).

In recent years, many studies have been conducted on sublingual vaccine therapy (Shim *et al.*, 2013; White *et al.*, 2014). The mucosa under the tongue harbors high density of DCs and T cells and low numbers of mast cells, basophils and eosinophils. Unlike in humans, the sublingual mucosa in mice is keratinized, which makes it more difficult for the antigens to pass through the mucosal barrier. A previous study has shown that sublingual application in BALB/c mice of OVA in combination with adenovirus 2 fiber protein (OVA-Ad2F) or adenovirus 2 fiber protein (OVA-Ad2F) generates significantly higher levels of IgG and IgA anti-OVA antibodies than in control mice (Jun *et al.*, 2012).

Aims

The overall aim of the present thesis was to study the interaction between HEMA, TEGDMA, EMA, DEGDA and hydrogen sulfide on the immune system with the following specific aims:

Study I

To investigate whether HEMA interacts with the immune system to induce the formation of the NLRP3 inflammasome, thereby promoting the production of IL-1ß and IL-18.

Study II

To examine the cytokine production profiles of cells exposed to TEGDMA and the adjuvant properties of TEGDMA *in vivo*.

Study III

To test the hypothesis that HEMA may act as a mucosal adjuvant.

Study IV

To compare the effects of DEGDA, EMA, HEMA and TEGDMA on the production of cytokines and to elucidate the effects of these compounds *in vivo* in mice.

Study V

To investigate if hydrogen sulfide induces formation of the NLRP3 inflammasome, and thereby the production of IL-1β and IL-18.

Materials and Methods

Isolation of mononuclear cells from human blood (Studies I, II, IV, and V)

Peripheral blood mononuclear cells (PBMCs) have round nuclei, and include monocytes and lymphocytes. Other cell types, such as erythrocytes and platelets lack nuclei, while granulocytes (neutrophils, basophils, and eosinophils) have multi-lobed nuclei. PBMCs are extracted from whole blood using a hydrophilic polysaccharide (Ficoll-Paque) that separates blood into layers such that the cell types can be purified by gradient centrifugation. Thus, the top layer contains the plasma, followed by a layer of PBMCs, and the bottom fraction consists of neutrophils, eosinophils, and erythrocytes.

To study the production of cytokines from mononuclear leucocytes exposed to dental methacrylate/acrylate monomers and H_2S, blood cells from eight healthy blood donors were obtained from Sahlgrenska University Hospital in Gothenburg, Sweden. The cells were resuspended in phosphate-buffered saline (PBS) purchased from Sigma Chemical Co., St. Louis, MO_2 USA, centrifuged, and then resuspended in Dulbecco's Modified Eagle's Medium (D-MEM: Invitrogen, Lidingö, Sweden) that was supplemented with 5% heat-inactivated human AB serum (Sigma-Aldrich, Steinheim, Germany), 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin (Invitrogen). Cell viability was determined by staining with 0.4% trypan blue (Sigma-Aldrich) and counting the cells using a Bürker chamber.

Animals (Studies I, II, III and IV)

For all four studies, 6-week-old female BALB/c mice (Charles River Laboratories, Sulzfeld, Germany) were used and housed in the animal facility according to governmental regulations. All the studies were approved by the Ethical Committee for Animal Experimentation in Gothenburg, Sweden (nr. N186/15)

BALB/c mice

BALB/c is an inbred strain, which can be defined as colonies that are produced by a minimum of 20 generations of brother-sister breeding that can be traced to a single founding pair. The inbred mice are animals genetically identical within each strain, which means that they are almost free of genetic variations that could lead to increased variability in the experimental results.

BALB/c mice are albinos, with a white coat and pink eyes, and they belong to the set of most commonly used inbred strains for animal experiments. The strain was originally called "Bagg albino" and was developed by H.J. Bagg in 1913 with a stock from a pet dealer in Ohio. In 1923, the strain became inbred through the efforts of E.C McDowell,

and the name “Bagg albino” was eventually changed to “BALB” to which the genotype of the color locus “c/c” was added, creating the new name of “BALB/c” (Potter, 1985).

BALB/c mice have broad research applications and are highly suitable for studying different immunologic responses. The oral microflora of the BALB/c mice is well-described, with "*Lactobacillus murinus*" (*L. murinus*) (38%) and *Staphylococcus aureus* (37%) being described as the predominant species (Trudel *et al.*, 1986). BALB/c mice are therefore a suitable inbred strain to use for studying the effect of HEMA on the immune response to the oral microbiota.

Studies I and V

PBMCs at 2×10^6 per well were pre-stimulated with LPS from *Escherichia coli* (Sigma-Aldrich) at 10 μg/ml for 3 h and cultured with or without 1000 μM HEMA (**Study I**) or 1000 μM H_2S (**Study V**) in a 24-well plate (**Study I**), in a 96-well plate (**Study V**) at 37°C (humidified atmosphere, 5% CO_2). To block NLRP3 inflammasome activation, the cells were cultured with HEMA/H_2S and 130 mM KCl (Merck, Whitehouse Station, NJ, USA). After 24 h, the cell cultures were frozen for subsequent cytokine analyses. Cell viability was assessed prior to freezing.

Human monocyte cell lines

THP1-Null cells are derived from THP1 human monocytic cells, and designed to study the signals involved in inflammasome activation. These cells express high levels of pro-caspase-1, NLRP3, and ASC. Upon stimulation with inflammasome inducers, such as alum, caspase-1 is activated and pro-IL-1β and pro-IL-18 are cleaved to the active forms of IL-1β and IL-18, respectively.

The human monocyte cell line THP1 was purchased from InvivoGen (San Diego, CA, USA). Three different cell lines were used in our studies: THP1-Null control cells (which are capable of forming the NLRP3 inflammasome); THP1-defASC cells, which express negligible levels of ASC; and THP1-defNLRP3 cells, which have reduced NLRP3 expression. The cells were seed at 2×10^5 per well in 96-well plates and pre-stimulated with LPS at 10 μg/ml for 3 h. Cells were cultured with or without HEMA (**Study I**) or H_2S (**Study V**) for 24 h at 37°C (humidified atmosphere, 5% CO_2). Alum (Merck) was added at 200 μg/ml as a positive control for NLRP3 inflammasome-dependent IL-1ß production. The plates were stored at -80°C until analyses of the cytokine concentrations in the culture supernatants.

HEMA exposure *in vivo*

To investigate the involvement of the NLRP3 inflammasome in the inflammatory properties of HEMA *in vivo*, we used two different experimental setups.

In the first experimental protocol, two groups of mice (n=5 per group) were injected subcutaneously in the tail with 50 μl of 20 μmol HEMA with or without 100 mM KCl. After 3 weeks, the animals were given an identical booster injection. Two weeks after the last injection, the mice were sacrificed and splenectomized.

In the second experimental setup, HEMA, with or without 100 mM KCl, was injected subcutaneously into the tails of two groups of mice (n=7 per group). The mice were administrated two similar injections at 3-weeks intervals to allow evaluations of the local inflammation induced by HEMA. After the last inoculation, the injection site was examined daily for 4 days, after which the mice were sacrificed.

An ordinal scale was used in a blinded manner to score the severity of the inflammation. According to this scoring system, mice with no signs of inflammation on the injected tail were given a score of 0, those with light-red reactions were given a score of 1, and those with dark-red reactions were assigned a score of 2 (Fig. 4).

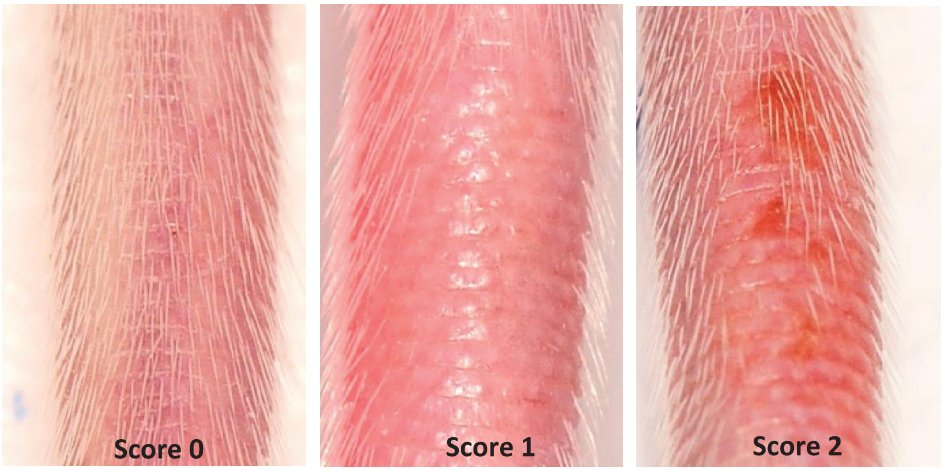

Figure 4. The scoring system used for scoring the local inflammation caused by 2-Hydroxyethyl methacrylate injections into the tail of the mouse.

Cell proliferation assay

Cell proliferation measurements are used to determine the number of cells that are growing in the absence or presence of certain proliferation-affecting agents, such as an antigen or a mitogen. A mitogen is a molecule that has the ability to activate T cells and/or B cells regardless of the antigen-specificity of the cells.

The method is based on that a pre-defined number of cells are seeded in the wells of a 96-well plate. At the same time, a proliferation-affecting agent is added (stimulated cells) or not (unstimulated cells; baseline splenocyte proliferation). As a positive control, concanavalin A (Con A; mitogenic for murine cells) is added to the cells. The cells are incubated for a specified time (e.g., 72–96 h) at 37°C, and thereafter ^{3}H-thymidine (thymine is one of the nucleotides in DNA and thymidine is a nucleoside that closely resembles thymine, which means that it is readily incorporated into the DNA strands) is added to the wells and the plates are incubated for additional period of time (e.g., 24 h). The ^{3}H-thymidine is incorporated into the cellular DNA at each cell division, so the more cell divisions the more radioactivity is incorporated into the DNA.

The cells are harvested after the final incubation, and washed out of the wells of the 96-well plates with distilled water. During the wash, the cells are damaged and the radioac-

tive DNA is set free. The cell fragments and the radioactive DNA are then passed through a membrane (glassfiber) where only particles smaller than 1,5 µm could pass the filter. Intact DNA will not be able to pass through the filter and is instead collected on the membrane. The filter membrane is dried and the amount of radioactivity (incorporated ^{3}H-Thymidine) will be counted in a scintillation counter.

Proliferation in Study I

Cell viability was determined by staining with 0.4% trypan blue and the stained cells were counted in a Bürker chamber. Cells were seeded at 2×10^{5} cells per well in 96-well plates and cultured for 3 days at 37°C (humidified atmosphere, 5% CO_2). Proliferation was assayed by adding [methyl-^{3}H]-thymidine (PerkinElmer, Väsby, Sweden) to the cultures for the final 20 h of incubation, after which the cells were frozen until harvesting. Cells were harvested on glass-fiber filters (Filtermat A 1450-421; Wallac, Turku, Finland), and the incorporation of ^{3}H-thymidine was assayed using the Microbeta Trilux (PerkinElmer).

Study II

PBMCs at 2×10^{6} per well were cultured with or without 500 µM or 1000 µM TEGDMA in 24-well plates at 37°C (humidified atmosphere, 5% CO_2). After 24 h, the cell cultures were frozen and supernatants were collected for subsequent cytokine analyses.

TEGDMA exposure *in vivo*

To study the adjuvant properties of TEGDMA, a primary injection of 50 µl of test solution was administered to two groups of mice (n=8), subcutaneously at the base of the tail. The first group received a solution that contained OVA at 50 µg/animal. The second group received OVA at 50 µg/animal plus TEGDMA (Sigma-Aldrich) (at 20 µmol/animal. To induce a substantial immune response, the animals were given an identical booster injection (re-exposure to the immunizing antigen) 3 weeks later. The mice were sacrificed 2 weeks after the booster injection, and blood samples were drawn from the axillary plexus.

Study III

Preparation of bacteria for sublingual immunization

L. murinus (strain 45959, Culture Collection, University of Gothenburg) was cultured on MRS agar plates for 48 h in a 5% CO_2 atmosphere at 37°C. On the day of immunization, bacterial colonies were transferred into sterile PBS buffer and centrifuged at 4000 × g for 15 min at 4°C. The supernatant was discarded and the bacteria were washed twice in PBS before the optical density was measured and compared against a standard curve, to determine the bacterial concentration.

S. mutans (in-house patient isolate strain IB16) was cultured on blood agar plates (Acumedia Manufacturers, Lansing, MI, USA) containing 50 ml/L defibrinated horse blood

for 24 h in a 10% CO_2 and 90% N_2 atmosphere. Just prior to immunization, the bacterial colonies were transferred to PBS buffer and prepared according to the protocol described above.

Sublingual immunization of HEMA

In order to study the leakage of un-polymerised HEMA onto the sublingual mucosa and antigen encounter events, we used two different experimental setups, with each group containing 7-8 mice. The animals were anaesthetized using Isoflurane (IsoFlo®vet, Abbott Laboratories Ltd, Queenborough Kent, UK) and administered with 10 µl of HEMA (20 µmol) together with OVA (50 µg) or live bacteria (2.5×10^6) under the tongue using a micropipette on four occasions at weekly intervals. Cholera toxin (CT) (10 µg) was used as a positive control, since it is known to act as an adjuvant by stimulating T and B cells (Raghavan *et al.*, 2010) (Mohan *et al.*, 2013). One week after the last immunization, blood was drawn from the axillary plexus, and the experiments were ended. For estimations of the anti-OVA and anti-bacterial antibody responses, sera were prepared from the blood samples and analyzed in ELISAs.

Study IV

PBMCs at 2×10^6 per well were cultured with or without 500 or 1000 µM HEMA, TEGDMA, EMA or DEGDA (all purchased from Sigma-Aldrich) in 96-well plates at 37°C (humidified atmosphere, 5% CO_2). After 24 h the cultures were frozen and supernatants were collected for later cytokine analyses.

Exposure to dental methacrylate and acrylate monomers *in vivo*

A primary injection (immunization) was administrated to five groups of mice (n= 8 per group) subcutaneously at the base of tail with 50 µl of test solution. The groups received a solution that contained OVA 50 µg/animal together with either 20 µmol/animal of HEMA, TEGDMA, EMA, DEGDA or OVA alone. To ensure the induction of a substantial immune response, the animals were given an identical booster injection (re-exposure to the immunizing antigen) 3 weeks later. The mice were sacrificed 2 weeks after the booster injection, and blood was drawn from the axillary plexus.

Enzyme-linked immunosorbent assay (ELISA)

ELISA is a plate-based assay technique used for detecting and quantifying substances such as proteins and antibodies.

In a capture assay (**Studies I and V**) also called a sandwich ELISA, a capture antibody is attached on the bottom of a 96-well plate. The sample (with the antigen of interest) is added to bind to the specific capture antibody, after which a biotin-labeled detecting antibody is added, which will bind to the antigen. Avidin-horseradish peroxidase (HRP) is added, and the avidin binds to the biotin of the detection antibody. Thereafter, 3,3',5,5'-tetramethylbenzidine (TMB) is added, which acts as a hydrogen donor for the reduction of HRP. Upon oxidation, TMB forms a water-soluble blue reaction, and upon acidifica-

tion (with sulfuric acid), the reaction is stopped and the yellow product has an absorbance peak at 450 nm, which is measured in an ELISA plate reader (Fig. 5).

In a direct ELISA assay (**Studies II-IV**), an antigen (e.g., OVA, *L. murinus* or *S. mutans*) is fixed to the bottom of the wells of a 96-well plate and the sample is added. If the sample contains specific antibodies against the antigen, they will bind to the immobilized antigen. An alkaline phosphatase (ALP)-labeled antibody against human-IgG or IgE is added in the next step. The ALP-labeled antibody can only bind if an antibody against the antigen is present in the sample. Then, a substrate is added to the wells, which changes color upon chemical conversion by the ALP. The color is then quantified using a spectrophotometer, and the obtained values can be translated using a standard curve to the level of antibodies in the sample of interest (Fig. 5).

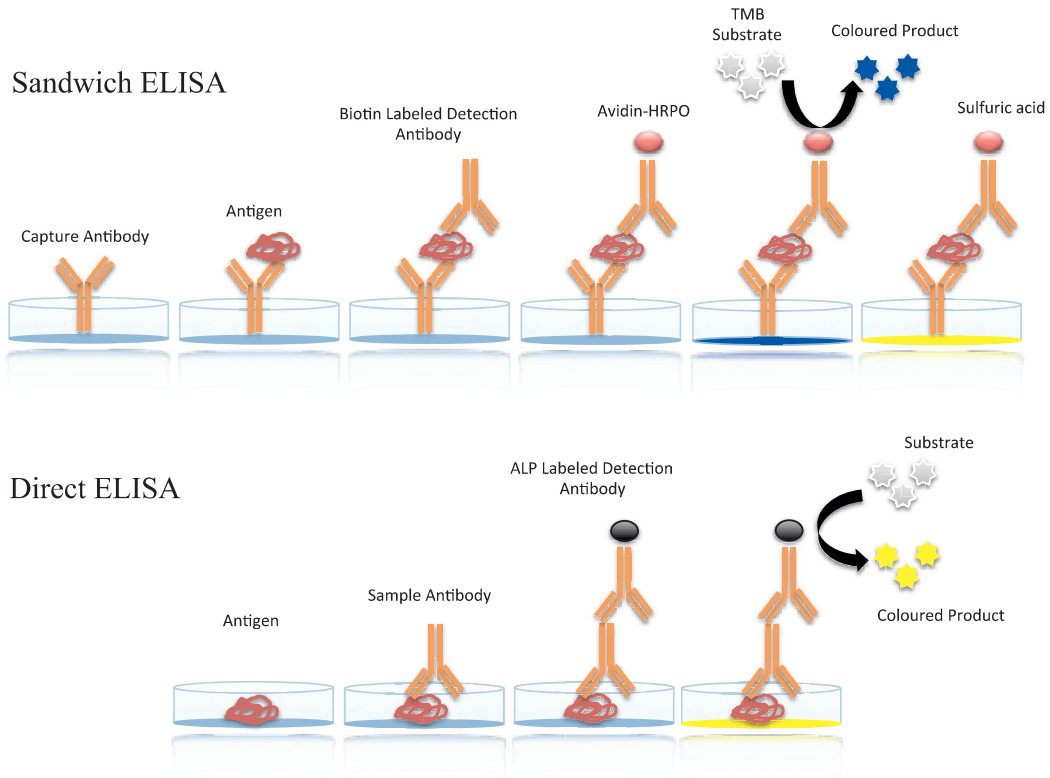

Figure 5. Schematic of the procedures used for the sandwich ELISA and direct ELISA.

Bio-Plex Pro™ Human Cytokine Assay (Studies I, II, and IV)

Color-coded beads are coupled with antibodies directed to the desired biomarker, e.g., IL-1β. Antibodies directed against different biomarkers have different colored beads to which they are attached. The sample is added, and the beads react with the biomarker of interest present in the sample. After a series of treatments with detergents to remove unbound proteins, a biotinylated detection antibody (used to create a sandwich complex) is added. The final detection complex is formed when streptavidin-phycoerythrin (SA-PE)

conjugate is added to bind to the biotinylated antibody. Phycoerythrin acts as a fluorescent indicator, or reporter.

The samples are analyzed using a BioPlex 200 instrument equipped with BioManager analysis software (BioRad, Hercules, CA, USA), using red and green lasers to detect the different colors on the beads while measuring the fluorescence intensity using a standard curve. The red (635 nm) laser and the green (532 nm) laser measure concentration (pg/ml) and median fluorescence intensity (MFI). The concentration of the analyte bound to each bead is proportional to the MFI of the reporter signal (Fig. 6).

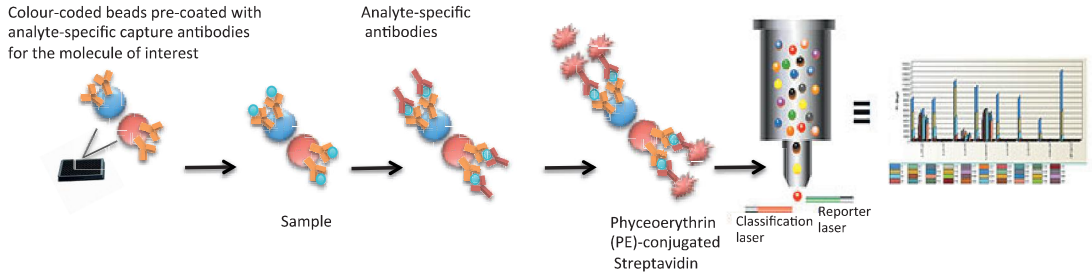

Figure 6. Schematic of the protocol used for the Multiplex Immunoassay.

Hierarchical Clustering Explorer (HCE)

The HCE software (University of Maryland, College Park, MD, USA) was used to create a heat map of the expression levels of selected cytokines produced from exposed and unexposed PBMCs. This was done to simplify understanding of the structures and patterns in the dataset.

The levels of each cytokine in the culture supernatants were measured with a multiplexed bead-based cytokine immunoassay. The median value was calculated for each cytokine and the values were normalized and transformed using HCE into color codes representing higher (red), intermediate (black), and lower (green) expression levels of each cytokine.

Statistics

The GraphPad Prism Software (GraphPad Software Inc., San Diego, CA, USA) was used to create all the artwork and analyses.

Statistical comparisons of paired samples were made using the Wilcoxon matched-pairs signed-rank test. For unpaired samples, the Mann-Whitney U-test was used. For all tests, a p-value of <0.05 was considered statistically significant.

Results

Study I. To investigate whether HEMA interacts with the immune system by inducing the formation of the NLRP3 inflammasome, leading to the production of IL-1ß and IL-18.

Does HEMA affect the immune system by inducing the formation of the NLRP3 inflammasome?

To address this question, PBMCs were cultured with or without HEMA for 24 h, and the concentrations of IL-1ß and IL-18 in the culture supernatants were determined by ELISA. There were significantly higher levels of IL-1ß (p=0.0391) and IL-18 (p=0.0156) in the supernatants of the cultures exposed to HEMA than in the supernatants of cultures of unexposed cells. The PBMC cultures exposed to HEMA in combination with increased KCl, did not lead to IL-1ß production whereas cells that were exposed to HEMA alone did produce IL-1ß (p= 0.0078). When the THP1-Null cells were exposed to HEMA, the production of IL-1ß was significantly increased (p=0.0312), as compared to untreated THP1-null cells. The ASC-deficient and NLRP3-deficient cells did not produce IL-1ß when treated with HEMA.

The splenocytes isolated from mice that were immunized with HEMA alone or HEMA plus KCl were cultured for 4 days. The level of proliferation *in vitro* was significantly higher for the splenocytes of mice that were exposed to HEMA *in vivo* than for the splenocytes of mice that received HEMA in combination with KCl *in vivo* (p=0.0079).

Mice that were injected subcutaneously in the tail with HEMA alone developed a significantly more prominent skin inflammation at the site of injection than mice that were injected with both HEMA and KCl (p=0.0148).

Study II. To study the immunomodulatory properties of TEGDMA *in vivo* and *in vitro.*

Does TEGDMA exhibit adjuvant properties in vivo?

To study the adjuvant properties of TEGDMA, BALB/c mice were immunized subcutaneously in the base of the tail with TEGDMA in combination with OVA. Mice that were immunized with OVA plus TEGDMA had significantly higher levels of IgG anti-OVA antibodies in their blood samples than mice that were immunized with OVA alone (p=0.0104). Mice that were immunized with OVA in combination with TEGDMA also had significantly higher levels of IgE anti-OVA antibodies in their blood samples than mice immunized with OVA alone (p=0.0468).

Does TEGDMA interact with human white blood cells and affect their cytokine production in vitro?

Those cultured PBMCs that were exposed to various concentrations of TEGDMA that had median cytokine production levels >10 pg ml-1 (total of 20 different cytokines) were included in a heat map, which was based on the median values of the measured concentrations from eight donors. Furthermore, two cytokines that are involved in activation of the NLRP3 inflammasome (IL-1β and IL-18) were included in the heat map.

Exposure of PBMCs to TEGDMA led to increased production of certain cytokines (IL-9, IL-12, IL-1RA, VEGF, MIP-1α, IL-1β, IL-6, GROα, IL-1α, IL-8, IL-18, MCP-1 and TNF-α (at concentration 500 μM)), while at the same time, the production of other cytokines was inhibited (M-CSF, IP-10, TRAIL, HGF, MIF, IFN-α2, MIP-1β, MCP-3 and IL-16). There was a trend towards higher levels of cytokines in the PBMC cultures exposed to the lower concentration of TEGDMA (500 μM).

Study III. To test the hypothesis that HEMA may act as a mucosal adjuvant.

Does HEMA enhance the IgG and IgE anti-OVA antibody levels after sublingual application of HEMA plus OVA?

To study the adjuvant properties of HEMA and its potential to enhance the antibody response to OVA when administered via the oral mucosal route, we sublingually immunized mice with OVA with or without HEMA. The OVA-specific IgG and IgE antibody levels in the sera of the immunized mice were measured. Significantly higher levels of IgG (p=0.0019) and IgE (p=0.0019) anti-OVA antibodies were detected in the sera of mice immunized with OVA in combination with HEMA than in mice that were immunized with OVA alone

Does HEMA increase the IgG anti-S. mutans antibody response and the anti-L. murinus antibody response after sublingual application?

Next, we studied the effects of HEMA on the induction on IgG anti-*L. murinus* and anti-*S. mutans* antibodies. For this purpose, mice were sublingually immunized with *L. murinus* or *S. mutans* together with HEMA or alone.

No significant differences in the IgG antibody responses were detected in the sera obtained from mice that were immunized with *L. murinus* together with HEMA, as compared to the other groups (p=0.0650). However, a significantly increased IgG anti-*S. mutans* antibody response was detected in the sera of mice that were immunized with *S. mutans* together with HEMA, as compared to mice immunized with *S. mutans* alone (p=0.0006).

Study IV. To compare the effects of DEGDA, EMA, HEMA and TEGDMA on the production of cytokines by PBMCs and on the immune responses *in vivo* in mice.

Do the different methcrylate/acrylate monomers affect the cytokine production profiles of PBMCs in different ways?

PBMCs were exposed for 24 h to the methacrylate/acrylate monomers HEMA, EMA, TEGDMA, and DEGDA at 500 μM or 1000 μM, and the cytokine expression patterns was presented in a heat map (Fig. 7). From the heat map, it is clear that the levels of most of the cytokines were increased by exposure to HEMA, EMA or TEGDMA. However, DEGDA caused a significant increase only in the production of IL-18.

Do the different methacrylates and acrylate exhibit the same adjuvant properties?

Mice were immunized subcutaneously at the base of the tail with OVA alone or in combination with HEMA, TEGDMA, EMA or DEGDA. The levels of the IgG anti-OVA antibodies in the sera from the mice were measured.

Significantly higher serum IgG anti-OVA antibody levels were detected in the mice that were injected with OVA plus HEMA (p=0.0070), OVA plus TEGDMA (p=0.0207) or OVA plus EMA (p=0.0207), as compared to the mice immunized with OVA alone. There were no significant differences in serum IgG anti-OVA antibody levels between the groups that were immunized with OVA in combination with the different methacrylates. Remarkably, All of the animals that received a booster injection with OVA in combination with DEGDA died.

Study V. To study the effects of H_2S on IL-1β and IL-18 production and to investigate if such production is dependent upon formation of the NLRP3 inflammasome.

Does H_2S affect the inflammatory response by inducing formation of the NLRP3 inflammasome, thereby promoting the secretion of IL-1ß and IL-18 from PBMCs?

When PBMCs were exposed to NaHS in culture, significantly increased production levels of IL-1ß (p=0.023) and IL-18 (p=0.008) were observed, as compared to untreated PBMCs. When the concentration of K^+ in the PBMC cultures was increased the production levels of both IL-1ß and IL-18 (both p=0.008) were decreased, as compared to cultures without increased extracellular K^+.

When the control human THP1-Null monocytes were exposed to NaHS, the levels of secreted IL-1ß (p=0.0006) and IL-18 (p=0.002) were increased compared to the unexposed cells. However, the THP1-defASC and THP1-defNLRP3 cells that were exposed to NaHS did not exhibit any increased levels of IL-1ß and IL-18, as compared to the respective unexposed cells.

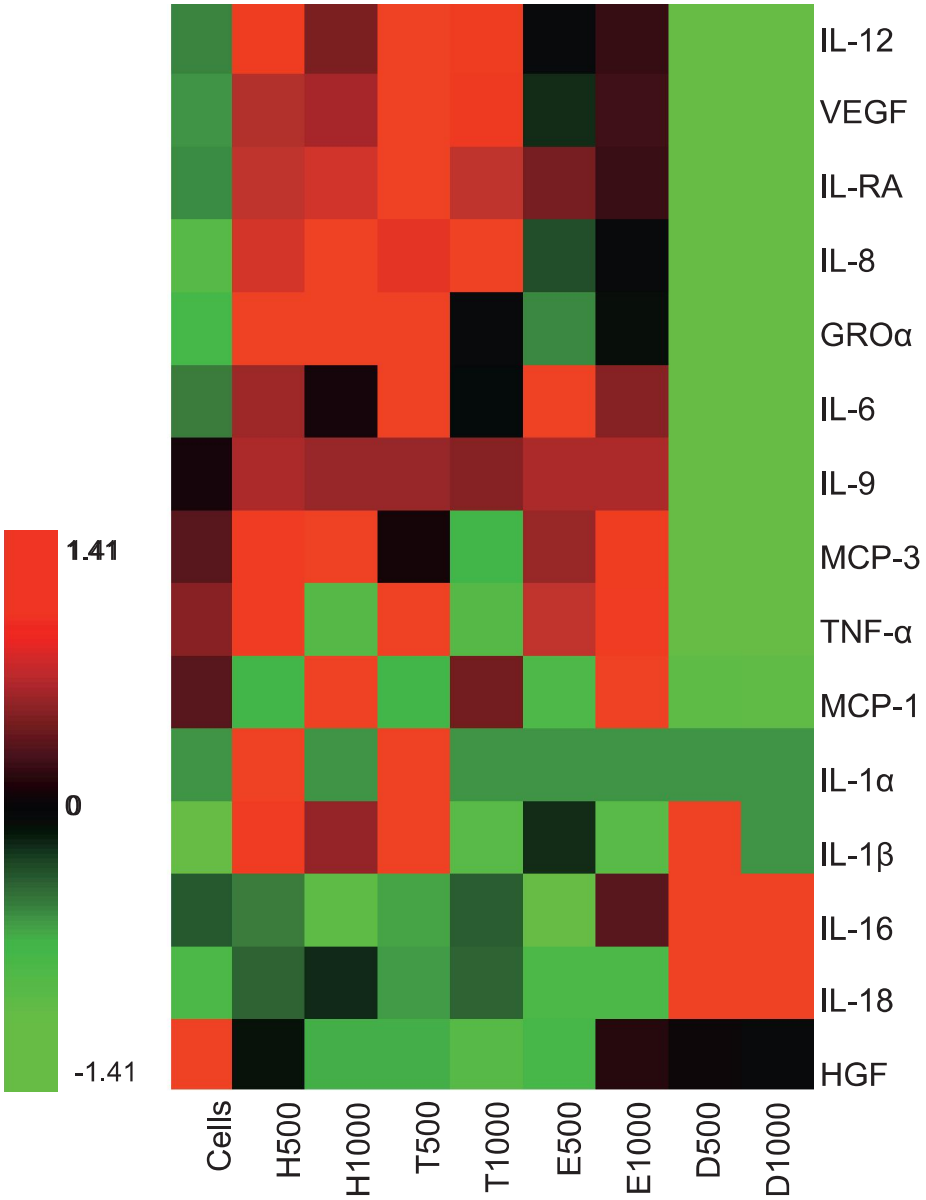

Figure 7. Human peripheral blood mononuclear cells (PBMCs) (n=8) were exposed *in vitro* to two different concentrations (500 μM, and 1000 μM) of HEMA (H), TEGDMA (T), EMA (E), or DEGDA (D). After 24 hours of culturing, the levels of cytokines IL-12, VEGF, IL-1RA, IL-8, GROα, IL-6, IL-9, MCP-3, TNF-α, MCP-1, IL-1α, IL-1β, IL-16, IL-18 and HGF in the culture supernatants were measured with a multiplexed bead-based cytokine immunoassay. The median level for each cytokine was calculated and the values were normalized and transformed into a heat map using Hierarchical Clustering Explorer and color codes that depict higher (red), intermediate (black), and lower (green) expression of each cytokine.

Main findings

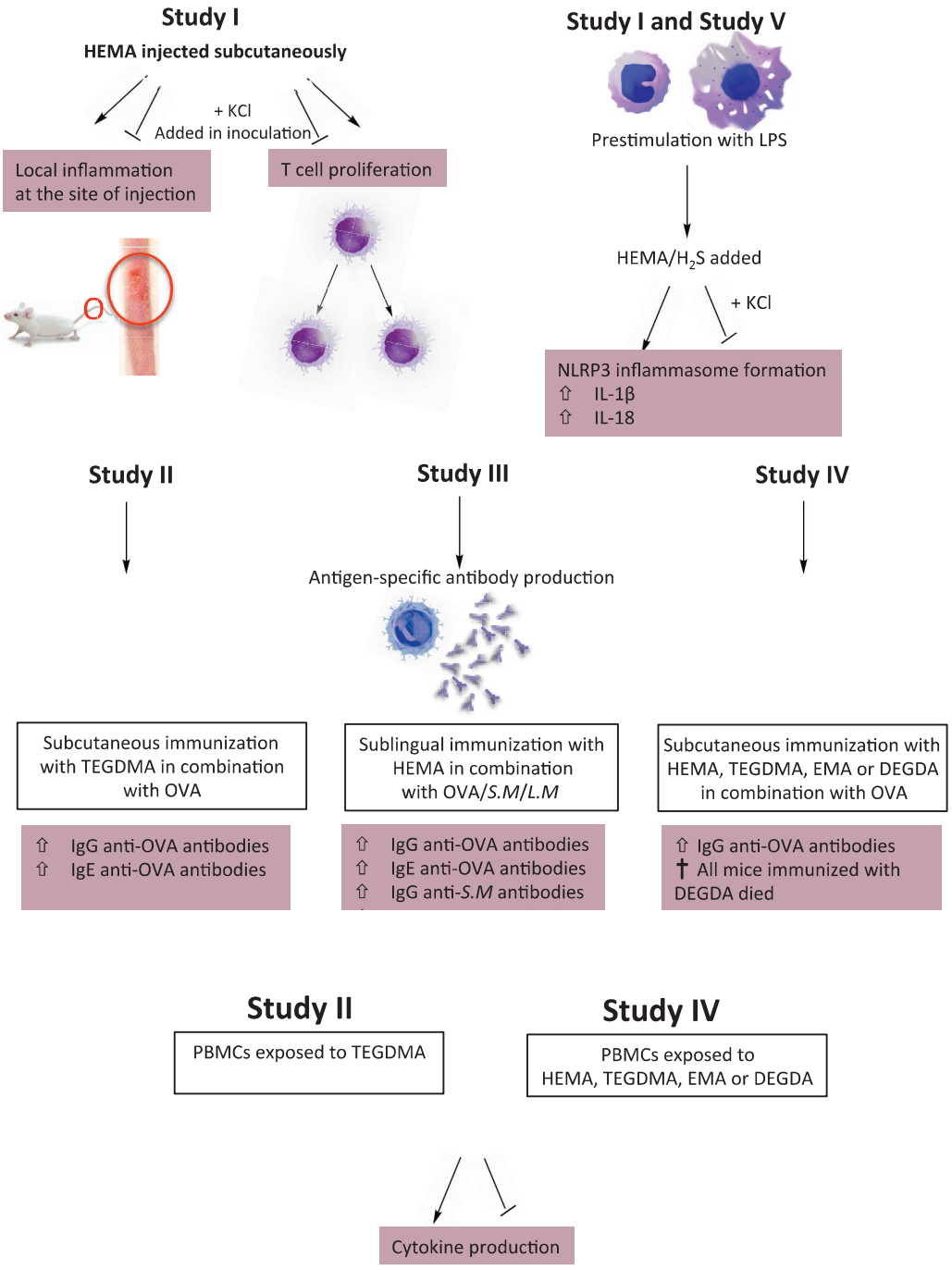

Figure 8. Summary of the experiments and results from all the studies included in the present thesis. DEGDA, diethylene glycol diacrylate; EMA, ethyl methacrylate; H_2S, hydrogen sulfide; HEMA, 2-hydroxyethyl methacrylate; *L.M*, *Lactobacillus murinus*; *S.M*, *Streptococcus mutans*; TEGDMA, triethylene glycol dimethacrylate.

Discussion

In recent years, there have been many advances in the design and application of dental biomaterials. However, despite these improvements, the perfect material from all perspectives does not yet exist. The ideal material would bond permanently to the tissue of tooth structures, be esthetic and useful in repairing missing substance and most importantly, be biocompatible.

It is important to study the biological properties of each dental material and to recognize that all materials contain potentially noxious ingredients. Reactions induced by the different dental materials can include toxicity, postoperative sensitivity, and hypersensitivity. Some dental materials may be acceptable for use of construction on hard tissues, but not acceptable for use on soft tissues. Other materials may be irritating or toxic during prolonged contact with tissues or when present in larger quantities, but constructive in small amounts or if in contact with tissues for only a short time. Previous studies have shown that the residual monomers can diffuse through the dentin into the pulp or come in contact with the oral mucosa and cause inflammation (Bationo *et al.*, 2016; Carol Dixon Hatrick, 2016; Gerzina *et al.*, 1996; Hensten-Pettersen, 1998; Spahl *et al.*, 1998). It is, therefore, important to study the relationships between dental materials and inflammatory reactions, as chronic inflammatory responses, such as pulp inflammation.

Inflammation that is initiated by the innate immune system is a nonspecific response, as compared to the pathogen-specific responses of immune system, the adaptive immunity. The innate immune cells that are involved and present in the inflamed tissues are macrophages, fibroblasts, mast cells, DCs as well as neutrophils.

Macrophages and DCs recognize pathogen invasion or cell damage with surface-expressed or intracellular PRRs. One group of the cytosolic PRRs is the NLR family, which is known for the ability to induce secretion of the proinflammatory cytokines IL-1β and IL-18. Many chemically different molecules may contribute to the formation of the NLRP3 inflammasome (Bauernfeind *et al.*, 2009; Duewell *et al.*, 2010; Eisenbarth *et al.*, 2008; Matias *et al.*, 2015; Sutterwala *et al.*, 2014). A common feature shared by these compounds is the ability to promote the efflux of cytoplasmic K^+ ions from the exposed cells (Eisenbarth *et al.*, 2008; Moretto *et al.*, 2013; Zhu *et al.*, 2011). Increasing the extracellular concentration of K^+ ions prevents this efflux, which means that the NLRP3 inflammasome cannot be formed (Petrilli *et al.*, 2007).

The formation of the NLRP3 inflammasome

In Study I, we hypothesized that the previously reported immunomodulatory effects of HEMA (Andersson *et al.*, 2010, 2011a, 2011b; Sandberg *et al.*, 2005a; Sandberg *et al.*, 2006; Sandberg *et al.*, 2005c) could be attributed to HEMA being a member of a diverse group of molecules that can drive NLRP3 inflammasome formation. We have previously

demonstrated that HEMA injected subcutaneously in mice results in dermatitis at the site of injection (Sandberg *et al.*, 2005b). An increase in the spontaneous proliferation of splenocytes from mice immunized with HEMA in combination with the model antigen OVA was also observed (Andersson *et al.*, 2011a). In the present thesis, we propose that the local inflammation and the increased baseline proliferation of splenocytes are due to the ability of HEMA to drive the formation of the NLRP3 inflammasome, as both these effects can be suppressed by adding KCl to the injected emulsion.

We have previously reported on the adjuvant properties of HEMA when injected subcutaneously in combination with the model antigen OVA (Sandberg *et al.*, 2005c). A common adjuvant used in vaccines in human is alum, which has the ability to activate the NLRP3 inflammasome (Eisenbarth *et al.*, 2008). In a previous study, using NLRP3, ASC and caspase-1 knockout mice, it was shown that the NLRP3 inflammasome is a crucial component in the adjuvant activity of alum (Eisenbarth *et al.*, 2008). This is in concordance with the conclusion drawn in the review article (Awate *et al.*, 2013). Combining these studies, we propose that, in similarity to alum, the adjuvant properties of HEMA are linked to its ability to induce the formation of the NLRP3 inflammasome.

To corroborate the results of our *in vivo* studies, we carried out experiments in which it was discovered that PBMCs that were cultured with HEMA produced more IL-1ß and IL-18 than PMBCs that were cultured in medium alone. Since reducing the intracellular concentration of K^+ ions is a prerequisite for NLRP3 inflammasome formation, we inhibited this process by increasing the concentration of extracellular K^+. PBMC cultures that were exposed to HEMA together with a high extracellular concentration of KCl did not produce any IL-1ß. Furthermore, when the NLRP3-deficient and ASC-deficient THP1 cell lines were exposed to HEMA there was almost no production of IL-1β. In contrast, the original THP1-null cells produced high levels of IL-1β in response to HEMA exposure.

Induction of the cytokines IL-1β and TNF by PRRs provokes the amplification of inflammatory response by promoting NF-κB and mitogen-activated protein kinases (MAP-Kinases) activation (Newton *et al.*, 2012). Previous studies have shown that HEMA treatment of rat submandibular salivary gland acinar cells results in ROS formation and the phosphorylation of ERK, JNK and p38 (Samuelsen *et al.*, 2007), leading to the activation of MAP-Kinases (Krifka *et al.*, 2010). This may reflect the ability of HEMA to cause the formation of the NLRP3 inflammasome, thereby increasing the production of IL-1β.

Many different chemical substances can promote formation of the NLRP3 inflammasome. As we have previously shown, some of the inflammatory responses induced by HEMA reflect its ability to initiate assembly of the NLRP3 inflammasome. We considered it of interest to study whether the mechanism underlying the proinflammatory properties of H_2S could also be linked to the formation of the NLRP3 inflammasome. H_2S, which is a toxic waste product of bacteria, is present in the subgingival pocket and in the gingival crevicular fluid at a concentration of 1.9 mM (Persson, 1992). H_2S is produced as a result of biofilm degradation of proteins (Kuester *et al.*, 1964). Given the proinflammatory properties of H_2S, it has been implicated in the pathogenesis of the bacterially induced inflammatory response in periodontal disease (Greabu *et al.*, 2016). Proinflammatory responses induced by H_2S, such as the increased production of the pro-

inflammatory cytokine IL-8 by gingival and oral epithelial cells *in vitro*, have been reported previously (W. Chen *et al.*, 2010).

We investigated the involvement of NLRP3 inflammasome formation with respect to the proinflammatory properties of H_2S. In PBMC cultures that were exposed to NaHS, increased secretion of IL-1β and IL-18 was observed, whereas in PBMC cultures to which KCl was added the NaHS-induced production of these cytokines was inhibited. In addition, higher levels of secretion of IL-1ß and IL-18 were noted for THP1 cells exposed to NaHS than for unexposed controls, NLRP3-deficient or ASC-deficient THP1 cells. Taken together, these results suggest that H_2S, in similarity to HEMA, belongs to the diverse group of chemical substances that have the ability to induce formation of the NLRP3 inflammasome. Moreover, NLRP3 inflammasome formation may be the mechanism through which H_2S contributes to the inflammatory host response and disease development.

H_2S belongs is one of the many bacterial waste products that, together with the bacteria themselves, cover the surfaces of the oral cavity. As more than 600 bacterial species have been identified in the oral cavity (Aas *et al.*, 2005; Dewhirst *et al.*, 2010), their presence must be taken into consideration when studying the different oral aspects of the immune system.

The adjuvant effects of HEMA on the immune response when applied on the oral mucosa

The leakage of acrylate/methacrylate monomers from newly placed filings is a well-known phenomenon. HEMA monomers released from resin-based composite materials have been demonstrated in saliva (0.015-0.19 μg ml^{-1}) (Michelsen *et al.*, 2012). We have previously demonstrated that HEMA possess adjuvant properties that lead to significantly higher IgG1 and IgE anti-OVA antibody responses when it is included with OVA in the injected emulsion. Considering that HEMA can penetrate intact skin and that subcutaneous co-administration of HEMA and OVA led to augmented antibody response to OVA, we consider it likely that exposure of the oral mucosa to HEMA modifies the immune responses to substances that are usually present in the oral cavity. We applied OVA in combination with HEMA to the sublingual mucosa of mice. The anti-OVA antibody levels were thereafter measured in the sera of the mice. Significantly increased levels of IgG and IgE anti-OVA antibodies were detected in the sera from mice that received OVA in combination with HEMA as compared to mice that received OVA alone.

Since the oral mucosa is constantly exposed to environmental antigens numerous species of bacteria (Paster *et al.*, 2001), we thought that it would be of interest to study if exposure of the oral mucosa to HEMA in combination with either an indigenous bacterium or a bacterium that is not part of the murine commensal flora would affect the immune response to that bacterium. For the indigenous bacterium, we chose the Gram-positive bacterium *L. murinus*, which belongs to the group of predominant resident bacteria in the oral mucosa of BALB/c mice (Rodrigue *et al.*, 1993; Rodrigue *et al.*, 1996; Trudel *et al.*, 1986). We report that the adjuvant activity of HEMA was not robust enough to disrupt the tolerance to the normal flora since HEMA caused no significant enhancement in the IgG antibody response to *L. murinus*. This can be interpreted that HEMA monomers that

leak from dental fillings will not promote an immunological reaction once they together with an indigenous bacterium present in the oral cavity come in contact with the oral mucosa.

Next, we studied the ability of HEMA to affect the immune response to an invading bacterium, which in this instance was the caries-associated bacterium *S. mutans* that is not normally present in BALB/c mice (Loesche, 1986). The results show that HEMA significantly enhances the IgG antibody response to *S. mutans* after sublingual application of the bacteria together with HEMA. In summary, acrylates/methacrylates may affect the normal immune response of the oral tissues to microorganisms present in the mouth. These findings are of interest, since allergic contact stomatitis due to residual monomers has been observed in some dental patients after restorative treatment (Fisher, 1954; Giunta *et al.*, 1979; Koutis *et al.*, 2001; Venables *et al.*, 2016). Contact stomatitis is an inflammatory reaction of the oral mucosa caused by contact with irritants or allergens. Beside the possibility that the residual monomers may initiate formation of the NLRP3 inflammasome thereby causing a local inflammation, it might be possible that due to the adjuvant properties of the residual monomers to a foreign bacterium, a hypersensitivity reaction is induced.

The immunomodulatory effects of HEMA, TEGDMA, EMA and DEGDA

As a multitude of immunomodulatory effects of HEMA has been shown ((Aalto-Korte *et al.*, 2007; Andersson *et al.*, 2010, 2011a; Samuelsen *et al.*, 2007; Sandberg *et al.*, 2005a; Sandberg *et al.*, 2006; Sandberg *et al.*, 2005c), we investigated whether other methacrylate monomers, e.g., TEGDMA, have similar properties *in vivo* and *in vitro*. Mice that were subcutaneously immunized with OVA in combination with TEGDMA had higher levels of IgG and IgE anti-OVA antibodies in sera than control mice that were immunized with OVA alone. From this we conclude that TEGDMA, just as in the case of HEMA, has the ability to act as an adjuvant *in vivo.*

In addition, the results showed that human white blood cells exposed to TEGDMA had an increased secretion of diverse cytokines with different functions. In the present project, six proinflammatory cytokines and one chemokine were further investigated. Previous studies have shown that TEGDMA-exposure of cells resulted in increased production of IL-1β (Moharamzadeh *et al.*, 2008; Moharamzadeh *et al.*, 2009), IL-6 (Schmalz *et al.*, 2000), IL-8 (Golz *et al.*, 2016) and MCP-1 (Gregson *et al.*, 2008). These outcomes are in concordance with our results, whereby PBMCs exposed to TEGDMA showed increased production of the proinflammatory cytokines GRO-α, IL-1β, IL-6, IL-8, IL-18, TNF-α and MCP-1. These cytokines play important roles in various inflammatory reactions, such as those in the inflamed dental pulp (Barkhordar *et al.*, 1999; Silva *et al.*, 2009). The increased levels of IL-1β and IL-18 might be attributable to the formation of the NLRP3 inflammasome. However, further studies are required to confirm the involvement of the NLRP3 inflammasome in the TEGDMA-induced increase in IL-1β and IL-18 production. Exposing the cells *in vitro* to 500 μM TEGDMA resulted in higher cytokine production than in cultures containing 1000 μM. Since cell viability was similar in cultures with 500 μM and 1000 μM TEGDMA, the decreased cytokine production at the

higher concentration of TEGDMA indicates a disturbed function of the cells. This is an interesting finding that needs further investigation.

We compared the effects of HEMA, TEGDMA, EMA and DEGDA on the cytokine production from human mononuclear cells *in vitro* as well as their *in vivo* adjuvant effects in combination with OVA by measuring the serum IgG anti-OVA antibody production after subcutaneous immunization. The cytokine production in supernatants from spleen cell cultures from mice immunized with the four different methacrylates/acrylate in combination with OVA or OVA alone was also measured.

PBMC viability and cytokine production were estimated after exposure to the different methacrylate/acrylate monomers. Results show that 90–95% of the cells that were exposed to HEMA, TEGDMA and EMA were viable after 24 h of *in vitro* culturing. Conversely, <50% of the cells exposed to DEGDA were viable after 24 h. We observed trends towards increased cytokine production after exposure to HEMA or TEGDMA. Most of the PBMCs died after exposure to DEGDA, although the IL-18 production was significantly increased in the cultures that were exposed to DEGDA. This indicates that the IL-18 was produced early after DEGDA exposure, i.e., before most of the cells died. The concentrations to which the PBMCs were exposed to *in vitro* lie within the range of concentrations that may be found clinically so the experiments appear to mimic the reactions that may occur in patients (Noda *et al.*, 2002).

To compare the *in vivo* effects of the methacrylates/acrylate, BALB/c mice were immunized with OVA alone or in combination with one of the methacryales/acrylate. Remarkably, all of the mice that were immunized with OVA in combination with DEGDA, died after the booster injection, a finding that is congruent with the *in vitro* toxicity of DEGDA. This indicates that DEGDA produces a potent memory-type immune response to OVA following the primary injection. Upon first exposure, it takes time for the antigen to be presented to lymphocytes, and for memory cells to be produced that are directed against that antigen. Following the secondary exposure to the same antigen, the memory cells will recognize the antigen and a much faster and a stronger response to the antigen is initiated. It can be speculated that memory cells are produced during the first exposure to OVA and DEGDA, that after the second immunization, a faster and much stronger response to OVA leads to the death of the animals. In addition, one can speculate that DEGDA induces the production of IL-18, which is a product of inflammasome activation. During NLRP3 inflammasome activation, pro-caspase-1 is cleaved to caspase-1, which has the ability to cause pyroptosis, which may explain the cell death *in vitro* (Miao *et al.*, 2011). It is possible that the NLRP3 inflammasome activation may also have a role in the *in vivo* effect of DEGDA causing the mice to die. Activation of the NLRP3 inflammasome may cause stimulation of the innate immune responses and the expression of costimulators on APCs, which works as a second signal usually required for adjuvants. However, no significant increased production of IL-1β was observed therefore further studies are required in order to be able to establish the involvement of the NLRP3 inflammasome. Another explanation may be that acrylates have been shown to normally be more toxic than methacrylates both *in vivo* and *in vitro*. The cytotoxicity of acrylates/methacrylates have been reported to be associated with the partition coefficient, the presence or absence of the methyl group, the presence or absence of a hydroxyl group

and the length of the polyoxyethylene chain (Dillingham *et al.*, 1983; Tanii *et al.*, 1982; Yoshii, 1997). Either way, this is an observation that deserves further investigation.

Spleen cells from mice that were immunized with the different monomers, were exposed to OVA *in vitro*. All the groups had a tendency toward an increased IL-6 levels as compared to the control group (mice immunized with OVA alone). However, a significantly increased production of IL-6 was only observed in the group that was immunized with OVA in combination with HEMA. Some of these results are in agreement with the outcomes of our previous study (Andersson *et al.*, 2011a). The production of IL-2 (also called T cell growth factor), which is a reflection of the T cell proliferation in the splenocyte cultures (Boyman *et al.*, 2012), was significantly increased in the cultures from the animals immunized with OVA in combination with TEGDMA. IL-2 is mainly produced by activated T cells, which indicates that TEGDMA interferes with the activity of T cells. We could not detect any significant difference for the levels of KC (the murine equivalent of IL-8), although the group that was immunized with HEMA plus OVA seemed to have higher levels of KC secretion than the other groups.

The serum samples from the mice that received OVA in combination with HEMA, TEGDMA or EMA showed higher IgG anti-OVA antibody levels than the mice that were immunized with OVA alone. HEMA and TEGDMA appeared to enhance serum IgG anti-OVA antibody production, as compared to mice that were immunized with OVA in combination with EMA. However no significant differences in the antibody productions were observed between the mice that received the various methacrylate monomers. These adjuvant properties of the methacrylates may be linked to the previously reported abilities of these compounds to cause allergic contact dermatitis (ACD) (Kiec-Swierczynska, 1996; Wrangsjo *et al.*, 2001a). ACD is a cutaneous delayed-type hypersensitivity (DTH) reaction, which is monocyte and T cell-mediated rather than antibody-mediated. This secondary cellular response appears 24-72 h after antigen exposure, as compared to immediate hypersensitivity response, which usually appears within minutes of an antigen challenge (Janeway CA Jr, 2001). Cutaneous hypersensitivity response is divided into two phases, a sensitization and elicitation. During the sensitization phase, cutaneous Langerhans' cells take up and process antigen, and migrate to regional lymph nodes where T cells get activated and memory T cells are produced. The memory T cells end up in the dermis, and during the elicitation phase (further exposure to the sensitizing chemical/antigen) the antigens are presented to them, which lead to release of T cell cytokines such as IFN-γ and IL-17. This leads to stimulation of keratinocytes of the epidermis to release proinflammatory cytokines such as IL-1 (IL-1β and IL-18), IL-6, TNF-α and the chemokine IL-8. These cytokines/chemokine enhance the inflammatory response by inducing the migration of monocytes into the lesion and by attracting more T cells (Janeway CA Jr, 2001). Recent studies have suggested that formation of the NLRP3 inflammasome, causing IL-1β and IL-18 release that activates T cells (Buters *et al.*, 2017; Watanabe *et al.*, 2007; Yazdi *et al.*, 2007). Therefore, it is possible that the formation of the NLRP3 inflammasome may also be involved in ACD development caused by methacrylates.

Since EMA and TEGDMA, in similarity to HEMA, have the capacity to act as adjuvants *in vivo*, they may contribute to the initiation of immune responses and/or allergy in patients to other substances, such as bacteria and food particles that are present in the oral

cavity. Furthermore, methacrylates/acrylates may contribute to the initiation of allergy to other substances, such as the latex proteins from gloves, which may affect dental personnel. A study conducted among Swedish dentists has shown that natural rubber latex glove-related hand eczema is associated with IgE-mediated allergy (Wrangsjo *et al.*, 2001b). A theory is that the methacrylates/acrylates may, in a similar way to the increased IgE anti-OVA antibody levels, also enhance the IgE anti-latex antibody levels.

EMA and TEGDMA, in similarity to HEMA, have the ability to act as an adjuvants to enhance the antibody response to OVA. It would be of interest to investigate wether these monomers can, just as in the case of HEMA, act as adjuvant after sublingual application.

There appears to be coherence between the cytokine responses of the PBMCs, and the antigen-specific antibody responses induced by the methacrylates. Important roles for the cytokines are to induce maturation of APCs, induction of cytotoxic NK cells and T_C cells, and differentiation of T_H1 and T_H2 cells. These are all important functions for inducing protection against pathogens and harmful substances. Therefore, cytokines have become alternative vaccine adjuvants for enhancing the immune responses to pathogens (Kayamuro *et al.*, 2010). Previous studies have shown the important roles of the cytokines CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP-1β), and CXCL8 (IL-8) (Seubert *et al.*, 2008) in association with the widely used adjuvants alum and the oil-in-water emulsion MF59. In addition, it has been suggested that the cytokines IL-25 and/or IL-6 may promote a T_H2 immune response to alum (Serre *et al.*, 2008). A mechanism recently associated with the adjuvant properties of alum is the NLRP3 inflammasome (Eisenbarth *et al.*, 2008). Taking together the previously reported mechanisms for the adjuvanticity of alum, it seems likely that different mechanisms underlie the adjuvant properties of HEMA, TEGDMA, and EMA.

One mechanism implicates the involvement of the cytokines secreted in response to exposure to the different methacrylates/acrylates, while an alternative mechanism may be due to the possibility that HEMA causes the formation of the NLRP3 inflammasome. In a previous study, Kool *et al*., have reported that the activation of adaptive cellular immunity to OVA-alum is initiated by monocytic DC precursors that induce the increase of antigen specific T cells in NLRP3-dependent manner (Kool *et al.*, 2008). A similar scenario is possible for the methacrylates/acrylates. Kayamuro *et al*. purposed that the IL-1 family members are potential mucosal vaccine adjuvants with the ability to induce antigen specific immune responses for protection against infectious pathogens (Kayamuro *et al.*, 2010). Taking this into consideration, the adjuvant properties of HEMA are most probably due to the NLRP3 inflammasome-mediated IL-1 production. Therefore, it would be interesting to establish whether the NLRP3 inflammasome is also involved in the immunomodulatory properties of TEGDMA and EMA. Moreover, further studies on DEGDA are warranted, and indeed are underway in our research group, to define the concentration at which DEGDA starts to become cytotoxic and the mechanisms behind its lethal effects *in vivo*.

The current studies described in this thesis have some limitations, so interpretation of the results should take these into account. The concentrations of the methacrylate/acrylate monomers used in the *in vivo* studies were higher than those usually found in a clinical setting. Higher concentrations had to be used because we wanted to establish the maximum effects induced by the methacrylates and the acrylate, in order to be able to study their mechanisms and examine how they affect the immune responses. Nevertheless, similar concentrations have been used previously in other studies (Rustemeyer *et al.*, 1998). However, it is important to have in consideration that amounts of monomers reaching the pulp vary. Some important factors that may affect the amount are; concentration of the methacrylate monomers used in the dentin adhesive and the thickness of the dentin.

Another aspect, worth considering is the possibility that responses in mice may not occur in precisely the same way in humans. However, mice are still the main *in vivo* model used for studying human immunology and are crucial for progress in our understanding of the immune system (Mestas *et al.*, 2004).

Concluding remarks

In conclusion, it is important to be aware that substances that leak out from dental materials (e.g., methacrylate monomers) can alter the responses of cells, such as monocytes thereby result in biological consequences. The cellular alterations that occur initially may be due to the toxic effects of the leaked substances. Monocytes control chronic inflammatory and immune responses, and they also secrete many substances that alter the actions of other cells. This may affect the normal response of the immune system to oral bacteria. Considering all the results described in this thesis, we concluded that; (i) methacrylates/acrylate seem to be able to interfere with different parts of the immune system; (ii) DEGDA is more toxic than the other methacrylate monomers that were analyzed; (iii) HEMA and TEGDMA initiate stronger responses from both the innate and the adaptive immune systems than does EMA; and (iv) H_2S induce formation of the NLRP3 inflammasome.

It is difficult to be able to compare fully the different methacrylates/acrylates, since they all have different properties, sizes, and chemical structures. However, taking all the present results into consideration, it seems likely that EMA is the least noxious of the methacrylates in our test group, while DEGDA is highly toxic. However, investigations of other methacrylates/acrylates are needed, to allow the identification of methacrylates/acrylates that exhibit good biocompatibility. The findings of the present thesis provide some insights into the nature of the immune responses to methacrylates and acrylates that are in clinical use, and may advance the development of more biocompatible restorative materials in the future.

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