# Immunomodulatory Properties of 2-Hydroxyethyl Methacrylate

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Cover Illustration: "Invading organisms are attacked by the Y-shaped antibody molecules. Antibodies attach to the invaders and make it easier for them to be destroyed by the immune system. At the top, invaders (gold) are eaten by a macrophage, which processes their antigens and presents them to a helper T-cell (purple). The helper cells then activate their corresponding B-cells (blue). These divide repeatedly and become plasma cells that produce vast quantities of antibodies (orange) that attack the invader (gold)." 

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# Abstract

## Immunomodulatory Properties of 2-Hydroxyethyl Methacrylate

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Professionals working in dentistry have reported adverse effects, such as allergic contact dermatitis, following exposure to 2-hydroxyethyl methacrylate (HEMA). Furthermore unpolymerized HEMA monomers leaking from cured fillings can reach the dental pulp, where HEMA could come into contact with leukocytes.

The aims of this thesis were to study specific effects of HEMA exposure on the phagocytic and respiratory burst activity of human phagocytes (study I), human immunoglobulin production (study II), antibody production (study III, study IV), leukocyte proliferation and leukocyte cytokine production (study III, study IV).

Using fluorescently labeled *Escherichia coli* it was demonstrated that HEMA does not impair the phagocytic activity of either monocytes or neutrophils *in vitro*. By using dihydrorhodamine, a substrate for hydrogen peroxide, it was further shown that HEMA exposure decreases neutrophil respiratory burst activity and thus impairs the bactericidal capacity.

By exposing pokeweed stimulated human B cells to HEMA for six days *in vitro* it was shown that HEMA specifically increases the production of the immunoglobulin IgG1 *in vitro* at lower concentrations, while at higher concentrations HEMA reduces IgG1 and IgM production *in vitro* as well as B cell proliferation. The IgA production *in vitro* appeared insensitive to HEMA exposure.

The effect of long-term exposure to HEMA *in vivo* was analyzed by implanting osmotic pumps, filled with different concentrations of HEMA, subcutaneously in mice. Pumps were left *in situ* for 40 days, during which time the animals were injected with ovalbumin (OVA), dissolved in bicarbonate buffer, on two occasions. Control animals received pumps filled with saline. Mice exposed to high concentrations of HEMA had an impaired weight gain throughout the exposure period and a lower splenocyte interleukin(IL)-2 production *in vitro*. Mice exposed to low concentrations of HEMA had an impaired weight gain in the beginning of the exposure period and lower concanavalin A stimulated splenocyte proliferation *in vitro*, splenocyte IL-2 production *in vitro* and serum IgA anti-OVA antibody activity, compared to control mice.

The *in vivo* effect of HEMA was further studied by injecting mice subcutaneously with HEMA dissolved in bicarbonate buffer, in the presence or absence of OVA. Mice exposed to HEMA, on two separate occasions, had a reduced splenocyte tumor necrosis factor alpha production *in vitro* compared to control animals injected with only buffer. Further both baseline and concanavalin A stimulated splenocyte proliferation *in vitro* was higher compared to controls. Mice exposed to HEMA and OVA in bicarbonate buffer had a higher IgG anti-OVA antibody activity relative to the corresponding IgM anti-OVA antibody activity, compared to animals that were injected with only OVA in buffer.

In conclusion our results suggest that HEMA can suppress as well as enhance immunological responses, specifically affecting neutrophil bactericidal function, immunoglobulin/antibody production, cytokine production and leukocyte proliferation.

**Key Words:** 2-Hydroxyethyl Methacrylate; Granulocyte; Respiratory burst; Immunoglobulin; B cell; Interleukin; Mouse

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# Preface

The present thesis is based on the following papers, which will be referred to in the text by their Roman numerals.

I. Andersson, J. & Dahlgren, U.I. Effect of 2-hydroxyethyl-methacrylate (HEMA) on the phagocytic and respiratory burst activity of human neutrophils and monocytes. *European Journal of Oral Sciences* **116**, 369-374 (2008).

II. Andersson, J. & Dahlgren, U. HEMA enhances IgG1 production by human B-cells *in vitro*. *Journal of Dental Research* **89**, 1461-1464 (2010).

III. Andersson, J. & Dahlgren, U. Effects on mouse immunity of long-term exposure *in vivo* to minute amounts of HEMA. *European Journal of Oral Sciences* **119**, 109-114 (2010).

IV. Andersson, J. & Dahlgren, U. HEMA promotes IgG but not IgM antibody production *in vivo* in mice. Accepted for publication in *European Journal of Oral Sciences*.

# Abbreviations

| | |
|---|---|
| ACD | Allergic contact dermatitis |
| AID | Activation induced cytidine deaminase |
| ALP | Alkaline phosphatase |
| ANOVA | One-way analysis of variance |
| BCR | B cell receptor |
| $B_F$ | Follicular B cells |
| $B_M$ | Marginal zone B cells |
| BPI | Bactericidal permeability increasing protein |
| BSA | Bovine serum albumin |
| CHS | Contact hypersensitivity |
| Con A | Concanavalin A |
| CSR | Class switch recombination |
| DHR 123 | Dihydrorhodamine 123 |
| D-MEM | Dulbecco's Modified Eagles Medium |
| DTH | Delayed-type hypersensitivity |
| *E. coli* | *Escherichia coli* |
| ELISA | Enzyme linked immunosorbent assay |
| FACS | Fluorescence activated cell sorter |
| Fc | Fragment crystallizable |
| FDC | Follicular dendritic cells |
| FITC | Fluorescein |
| GCF | Gingival crevicular fluid |
| HEMA | 2-hydroxyethyl methacrylate |
| HRP | Horseradish peroxidase |
| HSA | Human serum albumin |
| $H_2SO_4$ | Sulfuric acid |
| $H_2O_2$ | Hydrogen peroxide |
| [$^3$H] | Tritium |
| ICD | Irritant contact dermatitis |
| IFN | Interferon |
| Ig | Immunoglobulin |
| IL | Interleukin |
| J gene | Joining gene |
| LPS | Lipopolysaccharide |
| MFI | Mean fluorescence intensity |
| MHC | Major histocompatibility complex |
| MPO | Myeloperoxidase |
| MSA | Mouse serum albumin |
| NADPH | Nicotinamide adenine dinucleotide phosphate |
| NE | Neutrophil elastase |

| | |
|---|---|
| NET | Neutrophil extracellular trap |
| NOD | Nucleotide-binding oligomerization domain |
| OVA | Ovalbumin |
| $O_2$ | Oxygen |
| $O_2^-$ | Superoxide |
| PAMP | Pathogen associated molecular pattern |
| PBMC | Peripheral blood mononuclear cells |
| PBS | Phosphate buffered saline |
| PI | Propidium iodide |
| PRR | Pattern recognition receptor |
| R 123 | Rhodamine 123 |
| Rac2 | Ras-related C3 botulinum toxin substrate 2 |
| RIG | Retinoic acid inducible gene |
| ROS | Reactive oxygen species |
| STAT-6 | Signal transducer and activator of transcription-6 |
| TGF-β | Transforming growth factor-beta |
| TLR | Toll-like receptor |
| TNF-α | Tumor necrosis factor-alpha |
| V gene | Variable gene |

# Introduction

## *Acrylates and Methacrylates*

The majority of materials used for dental restorations today contain acrylate and methacrylate monomers [1]. Acrylates and methacrylates are esters, derived from the reaction of an alcohol with either acrylic or methacrylic acid. Previous studies have reported on the adverse effects of methacrylate monomers, primarily on professionals working in dentistry [2-7]. The reported prevalence of methacrylate contact allergy varies from 1.3% to 22% [8-10]. Methacrylates have also been implicated in cases of asthma and conjunctivitis [11].

## *Contact Dermatitis*

A common reaction to methacrylate monomers is contact dermatitis, which can be irritant (ICD) or allergic (ACD) in nature [12]. ICD is caused by the innate immune system. Patients with ICD exhibit an infiltration of primarily neutrophils at the site of exposure. ACD involves a T cell-mediated, delayed-type hypersensitivity (DTH) response to low molecular weight molecules (*i.e.* haptens), of which acrylates and methacrylates are two examples [13, 14]. For ACD to occur an individual has to be sensitized. The ability of a hapten to sensitize an individual depends on the haptens inflammatogenic capacity, *i.e.* to act as a "danger signal". The hapten also has to be capable of binding to amino acids of cutaneous proteins, thus providing new antigenic determinants. Insight into the pathological mechanisms behind ACD has been gained by using animal models. In these models the animal is first sensitized by painting the hapten on the abdomen or on the back. A few days later the animal is challenged by painting the hapten on the ear, which results in an inflammatory reaction referred to as contact hypersensitivity (CHS). The effector cells of CHS is believed to be interferon (IFN)-γ producing CD8+ T cells while it has been proposed that interleukin (IL)-10/IL-4 producing CD4+ T cells play a down-regulatory role [15, 16]. However CD4+ T cells can be cytotoxic against keratinocytes presenting haptenated peptides, which suggest that CD4+ T cells may cooperate with cytotoxic CD8+ T cells in damaging the tissues during CHS [17]. B cells also play a role in CHS. Following hapten sensitization naïve B cells, located in the peritoneal cavity, are induced to migrate to the spleen where they start to produce hapten specific IgM antibodies [18]. Upon challenge these hapten specific IgM antibodies form immune complexes when binding to the challenging hapten. The immune complexes can then activate the complement system, which leads to the generation of the complement fragment C5a. C5a in turn activates mast cells. Mast cells drive the influx of neutrophils by secreting tumor necrosis factor (TNF)-α and macrophage inflammatory protein – 2 [19]. The resolution phase of CHS is believed to be driven by CD4+ regulatory T cells and IL-10 producing mast cells [20, 21].

This thesis focuses on the immunomodulating properties of 2-hydroxyethyl methacrylate (HEMA), one of the methacrylate allergens most frequently reported to cause problems among dental personnel.

## *HEMA*

### *Characteristics and Function in Dental Resin Materials*

HEMA ($CH_2=C(CH_3)COOCH_2CH_2OH$) is an ester monomer found in dentin adhesives and resin glass ionomer cements [1, 22]. HEMA has several characteristics that make it a utile constituent of materials used for dental restorations. For instance the HEMA monomer is relatively small (molecular weight = 130 g $mol^{-1}$) and hence has a relatively low viscosity. This confers that HEMA can function as a solvent for other larger monomers, such as bisphenol A-glycidyl methacrylate (molecular weight = 511 g $mol^{-1}$), thereby improving their polymerization rate. Further, HEMA is a polar molecule with one hydrophobic end and one hydrophilic end. The polar nature of HEMA makes it capable of functioning as a coupling agent, promoting the adhesion of a composite resin to demineralized dentin, by binding to both the hydroxyl groups on collagen fibers in demineralized dentin and to the composite resin. To be able to function as a coupling agent HEMA has to saturate the collagen fibers, which requires the displacement of surrounding water. When performing dental restorations water is displaced by solvents such as ethanol and acetone. That HEMA is soluble in ethanol and acetone is therefore another useful characteristic. Using scanning electron microscopy it has been shown that pretreatment of dentin with HEMA prior to application of resin composites and adhesives results in an increase in the thickness and the length of the hybrid layer, *i.e.* the layer of a dental filling that consists of a mixture of polymerized resin monomers and demineralized dentin. In other words HEMA increases the bond strength of resin composites to demineralized dentin [23, 24].

HEMA is a functional monomer, which means that upon curing HEMA will form a linear polymer [1, 22]. Free radicals are required for polymerization to occur. In dental adhesives these radicals are generated via the decomposition of an initiator, *e.g.* camphoroquinone and benzoylperoxide. The initiator can be either photo or redox activated. Once a free radical has reacted with a HEMA monomer, that HEMA monomer will contain a reactive carbon atom, which in turn can react with another free HEMA monomer. In this way the polymerization of monomers will progress. In theory the polymerization terminates when all the free radicals have reacted. However polymerization is never complete, some free monomers always remain.

### *HEMA Leakage and Leukocytes in the Oral Cavity*

In the oral cavity there are different areas where cells of the immune system are present. Antigen-presenting, dendritic cells called Langerhan's cells reside in the oral epithelium [25, 26]. Previous studies have shown that neutrophils represent 95% - 97%, lymphocytes 1% - 2% and mononuclear cells 2% - 3% of the leukocyte population present in a normal gingival crevice [27]. Further, there are less T cells than B cells present in the gingival crevice [28]. Gingival crevicular fluid (GCF) flows into the gingival crevice through the junctional epithelium [29]. GCF brings neutrophils, monocytes, B cells and T cells into the oral cavity. The superficial layers of the oral epithelium and the base of the gingival crevice are primarily populated by neutrophils, while macrophages and lymphocytes predominately reside in the basal layers of the junctional epithelium [30]. In the dental pulp dendritic cells, T cells and macrophages can be found [31, 32].

Previous studies have shown that leakage of unpolymerized HEMA monomers occurs after curing of resin-based materials [33, 34]. Further, HEMA monomers can penetrate through dentin [35-38]. In addition HEMA can bind to proteins [39, 40]. Free HEMA monomers leaking from a cured filling could thus bind to proteins and the HEMA/protein conjugate could then come into contact with neutrophils, monocytes, macrophages, B cells and T cells. Contacts like these could affect the physiological functions of leukocytes belonging to both the innate branch (neutrophils, monocytes and macrophages) and the adaptive branch (B cells and T cells) of the immune system.

### *Innate Immunity: Neutrophils and Monocytes*

Innate immunity represents the first line of defense against foreign invaders that have managed to penetrate the skin barrier. Innate immunity relies on the recognition of pathogen-associated molecular patterns (PAMPS), *e.g.* lipopolysaccharide (LPS), viral ribonucleic acid, zymosan, peptidoglycan and oxidized lipoproteins [41]. The presence of PAMPS is sensed by pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), the mannose receptor, RIG-I helicase (senses double-stranded ribonucleic acid), NOD1 and NOD2 receptors (senses peptidoglycan) and scavenger receptors [42-48]. Microorganisms can also be recognized indirectly, by way of opsonins like IgG and complement fragments [49, 50].

Monocytes/macrophages and granulocytes, of which the neutrophil is the most numerous representative, contribute to the innate army against infection.

*Monocytes*

The monocyte is 12 μm - 20 μm in diameter and has a U-shaped nucleus [51]. In a human adult monocytes represent 5% - 6% of the circulating leukocytes. Circulating monocytes have a half-life of about 3 days [52]. Human monocytes are divided into subsets based on the expression of CD14, an LPS receptor, and CD16, the FcγRIII receptor [53, 54]. The majority (80% - 90%) of circulating monocytes are characterized by a high expression of CD14 and a lack of CD16 expression, so-called $CD14^{high}CD16^{neg}$ monocytes. Another class of monocytes is the $CD14^{low}CD16^{high}$ monocytes, which represent about 10% of the circulating monocyte population. $CD14^{low}CD16^{high}$ monocytes are referred to as proinflammatory monocytes, due to an increase in cell number during acute infections and to the production of TNF-α following LPS stimulation [55-57]. Furthermore, the two monocyte subsets differ in their expression of the Major Histocompatibility Complex (MHC) class II protein, $CD14^{low}CD16^{high}$ monocytes having the higher MHC class II expression [58]. Monocytes, not being fully differentiated upon leaving the bone marrow, can differentiate further into for instance alveolar dendritic cells, splenic dendritic cells, Langerhan's cells, lung macrophages, lymph node macrophages and splenic macrophages [59-63].

*Neutrophils and Their Granules*

The neutrophil is 12 μm - 15 μm in diameter [51]. In a human adult neutrophils represent about 60% of the circulating leukocytes. Neutrophils have a short half-life, ranging from 6 hrs to 8 hrs, with $5\text{-}10x10^{10}$ neutrophils being released from the bone marrow every day [64]. Neutrophils are referred to as polymorphonuclear granulocytes, due to their multi-lobed nucleus and the presence of several granules in their cytoplasm [65-67]. There are three types of cytoplasmic granules: azurophilic granules, specific granules and gelatinase granules. In addition there are secretory vesicles, which are a source of neutrophil cell membrane receptors, *e.g.* CD14, CD16 and receptors for formylated bacterial peptides [68, 69].

The cytoplasmic granules are packed with anti-microbial proteins and proteases [70]. Specific granules contain lactoferrin, cathelicidins and neutrophil lysozyme. Furthermore, specific granules release matrix metalloproteinases, which degrade the extracellular matrix, thereby clearing the path for incoming neutrophils. Gelatinase granules, like specific granules, contain matrix metalloproteinases and lysozyme. The azurophilic granules contribute to the arsenal of anti-microbials with myeloperoxidase (MPO), α-defensins, LPS-binding bactericidal permeability increasing protein (BPI), azurocidin and the serine proteases cathepsin G, neutrophil elastase (NE) and protease 3. The anti-microbial effects of the granule proteins involve sequestering nutrients essential to microbes, *e.g.* the iron binding capacity of lactoferrin, and permeabilizing microbial cell membranes, *e.g.* α-defensins, lysozyme, cathelicidins and BPI [71-74]. Granule proteins are also involved in the formation of neutrophil extracellular traps

(NETs) [75]. These consist of secreted decondensed chromatin, MPO and NE bundled together into extracellular fibers. NETs enable extracellular killing of bacteria by neutrophils.

Granule proteins have other functions besides acting as anti-microbials. For instance, the cathelicidins LL-37 and azurocidin promotes chemotaxis of monocytes and T cells [76-78].

*Phagocytosis and The Respiratory Burst: The Radical Armor of Monocytes and Neutrophils*

Neutrophils, monocytes and macrophages are capable of performing a process called phagocytosis, *i.e.* they are phagocytes (Fig. 1) [66]. Phagocytosis ensures the removal of harmful pathogens as well as apoptotic cells [79, 80]. Neutrophils, monocytes and macrophages have a variety of PRRs, which upon interaction with their respective PAMPs promote phagocytosis.

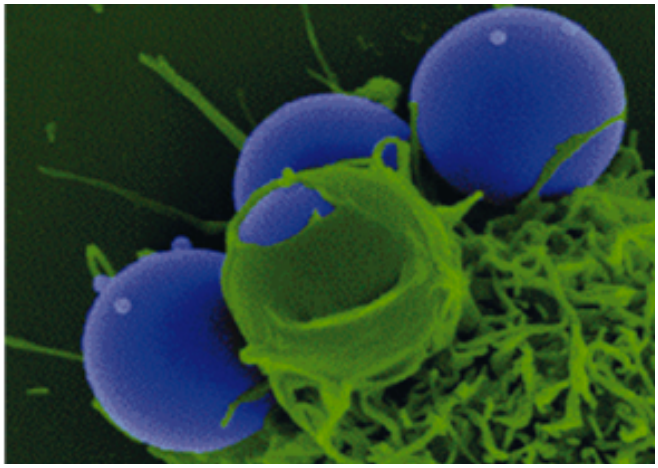

**Figure 1. In the beginning of phagocytosis the cell membrane protrudes, forming phagocytic cups. Shown is a scanning electron micrograph of a phagocyte in the process of engulfing beads. Huynh KK *et al*. Fusion, fission, and secretion during phagocytosis. *Physiology (Bethesda)* 2007; 22: 366-372. © The American Physiological Society.**
**http://physiologyonline.physiology.org/content/22/6/366.full**

Following recognition of a PAMP, the plasma membrane of the phagocyte invaginates and eventually pinches off, trapping the pathogen/apoptotic host cell together with extracellular fluid inside a phagosome [81]. The phagosome subsequently fuses with early endosomes, late endosomes and finally lysosomes, thus creating a phagolysosome [82]. During the phagosome maturation process the environment for the trapped pathogen becomes increasingly acidic due to the presence of V-ATPases, which pumps protons into the phagosome lumen [83, 84]. The acidic pH hampers microbial metabolism and increases the activity of host hydrolytic enzymes. Furthermore, azurophilic and

specific granules fuse with the phagolysosome, releasing their anti-microbial proteins [85].

Contributing to the armor of the phagocyte is a multisubunit NADPH oxidase [86]. When phagocytosis is initiated the NADPH oxidase subunits (gp91$^{phox}$, p22$^{phox}$, p40$^{phox}$, p47$^{phox}$, p67$^{phox}$, Rac2) assemble at the emerging phagosome membrane [87-90]. Once assembled, the NADPH oxidase oxidizes cytosolic NADPH, transferring two electrons from NADPH to two oxygen ($O_2$) molecules inside the phagolysosome, resulting in the generation of two superoxide ($O_2^-$) ions. The consumption of $O_2$ for the production of $O_2^-$ is referred to as the respiratory burst (Fig. 2). An efflux of chloride anions and a simultaneous rise in intracellular calcium levels, which occurs upon recognition of a pathogen, stimulate the respiratory burst [91, 92].

$O_2^-$ belongs to a group of molecules known as reactive oxygen species (ROS) [93]. Once formed by the action of NADPH oxidase, $O_2^-$ can subsequently be converted to hydrogen peroxide ($H_2O_2$) by the action of superoxide dismutase. Neutrophil MPO potentiates the anti-microbial effect of the respiratory burst by converting $H_2O_2$ into hypochlorous acid, hypobromous acid or hypoiodous acid, all of which belong to the ROS family. $H_2O_2$ can also react with metal ions to generate highly oxidizing hydroxyl radicals. ROS released into the phagolysosome damages microbial proteins, lipids and nucleic acids.

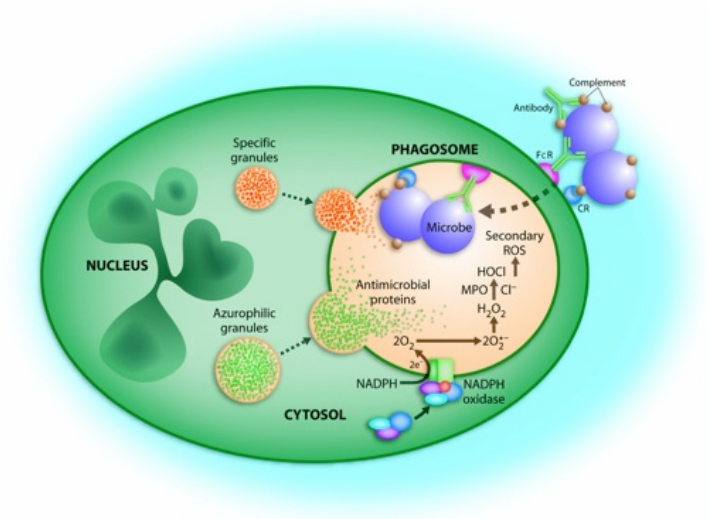


**Figure 2. The neutrophil respiratory burst is triggered by complement and Fc receptors. Figure originally published in *Clinical Science*. Quinn MT *et al*. The expanding role of NADPH oxidases in health and disease: no longer just agents of death and destruction. *Clin Sci*. 2006; 111: 1-20. © The Biochemical Society.**

### *Effects of HEMA on the Life of Monocytes, Macrophages and Neutrophils*

*In vitro* studies have revealed several effects of HEMA on monocytes/macrophages.

Monocyte/Macrophage activities suppressed by HEMA:

The respiratory burst [94]
IL-1β and TNF-α production [95-97]
Cell proliferation [98]
Heat shock protein 72 expression [99]
Levels of the radical scavenger glutathione protein [100, 101]

Monocyte/Macrophage activities promoted by HEMA:

Vascular endothelial growth factor expression [102]
Recognition of carbohydrate ligands [103]
Cyclooxygenase-2 expression [104]

Further, HEMA induces DNA fragmentation in monocytes and macrophages [105, 106].

*In vitro* studies on the effect of HEMA exposure on neutrophils remain to be reported.

## *Adaptive Immunity: B Lymphocytes and Antibodies*

Activated B lymphocytes (plasma cells) have a diameter of up to 20 μm [51]. B lymphocytes represent 10% - 15% of the lymphocytes found in blood and 40% - 45 % of the spleen lymphocyte population [107]. Activated B cells produce antibodies that confer antigen specificity to the immune system.

### *B Cell Subsets*

B cells can be divided into B1 B cells and B2 B cells. Murine B1 B cells develop from hematopoietic stem cells in the fetal liver [108]. B1 B cells spontaneously secrete IgM directed at polysaccharides, without an apparent immunization. They represent the first line of defense against blood-borne encapsulated bacteria.

The majority of human and murine B cells are B2 B cells. B2 B cells are derived from hematopoietic stem cells in the bone marrow. In the spleen B2 B cells mature into either marginal zone B cells ($B_M$) or follicular B cells ($B_F$) (Fig. 3) [109]. $B_M$ reside at the marginal sinus in the murine spleen and like B1 B cells, respond rapidly to multivalent, blood-borne antigens, such as polysaccharides [108]. $B_M$ can also respond to glycolipids due to expression of the glycolipid receptor CD1. Following primary immunization $B_M$ cells rapidly develop into plasmablasts, secreting large amounts of antigen specific IgM

antibodies [110]. $B_F$ are referred to as recirculating B cells due to their migratory behavior, traveling via blood and lymph to lymph nodes, Peyer's patches and the spleen (Fig 3.) [111]. In the lymph node, $B_F$ respond to protein antigens with a T helper cell dependent antibody production. However $B_F$ can also recirculate through the bone marrow where they can respond to antigen in a T cell independent manner [112].

*B Cell Activation and Maturation*

In the lymph node antigen peptides can be presented on the surface of macrophages, dendritic cells and B cells by MHC class I or MHC class II molecules [113-116]. Antigens can also be displayed in the form of immune complexes, bound to either complement receptor 2 or Fcγ receptors on the surface of follicular dendritic cells (FDC) [117, 118]. Furthermore, $B_F$ can acquire soluble antigens that diffuse into the B cell follicle from the subcapsular sinuses (Fig 3.) [119].

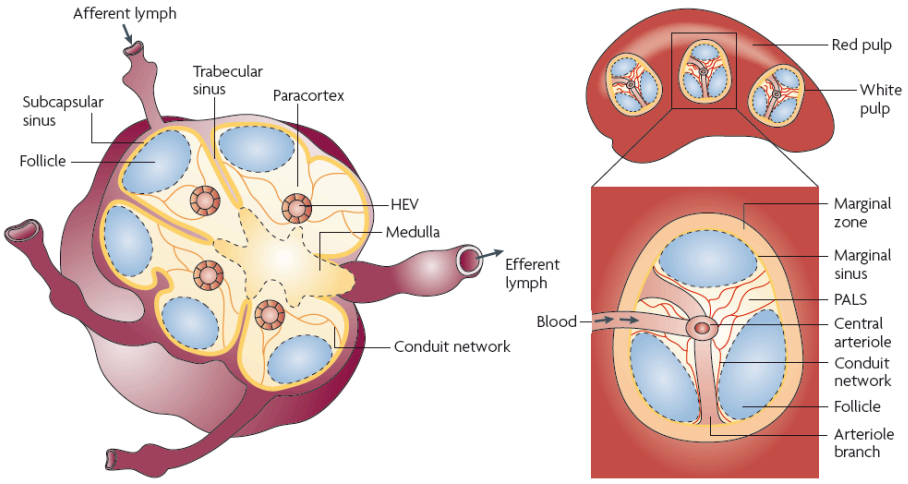


**Figure 3. A schematic representation of the lymph node and the spleen. HEV = high endothelial venule, PALS = periarteriolar lymphoid sheath. Adapted by permission from Macmillan Publishers Ltd: Batista FD *et al.* The who, how and where of antigen presentation to B cells. *Nat. Rev. Immunol.* 9: 15-27. © 2009. http://www.nature.com/nri/index.html**

B cells recognize antigens by way of the membrane-bound B cell receptor (BCR). The antigen specificity of the BCR is determined by gene recombinations that occur in the bone marrow during B cell development [120]. The extensive rearrangements of variable- (V), diversity-, joining (J) gene segments (heavy chain locus) and V- and J gene segments (light chain locus) that occur in the bone marrow are the basis for creating a large variety of BCR specificities and hence B cell clones.

Following antigen internalization, B cells migrate to the T cell zone, known as the paracortex in the lymph node and the periarteriolar lymphoid sheath (PALS) in the

spleen, where they are activated by interactions with T helper cells. Activated B cells migrate into primary follicles where intense proliferation ensues, forming secondary follicles and eventually germinal centers [121]. It has been estimated that it takes about 12 hrs for a germinal center B cell to duplicate itself [122]. In the germinal center somatic hypermutation (primarily single nucleotide exchanges) of immunoglobulin V region genes, affinity maturation and class switch recombination (CSR) take place. Finally the B cells exit the lymphoid organ as either memory B cells or antibody-producing blast cells [123]. Memory B cells, characterized by high-affinity antigen receptors, quickly initiate the production of large amounts of antibodies upon reexposure to an antigen [124]. Long-lived plasma cells residing in the bone marrow maintains a high titer of antigen-specific antibodies following an initial antigen exposure [125, 126]. Plasma cells represent 0.1% - 1% of the cells present in human bone marrow. The longevity of plasma cells in the bone marrow depends on several factors. For instance IL-6 produced by bone marrow stromal cells enhances plasma cell survival. Plasma cell survival also depends on engagement of the chemokine receptor CXCR4 by its ligand CXCL12.

### *Antibodies: Classes, Structure and Functions*

Antibodies (immunoglobulins) can be divided into different classes and subclasses based on the amino acid sequence of the heavy chain constant (C) region [127]. Humans have several classes of soluble immunoglobulins, some of which can be further divided into subclasses. These are IgA1, IgA2, IgE, IgM, IgG1, IgG2, IgG3 and IgG4 [128]. In mice, IgG are divided into the subclasses IgG1, IgG2a, IgG2b and IgG3 [129]. Like humans, mice also produce soluble IgA, IgE and IgM immunoglobulins. IgD is mainly a membrane bound immunoglobulin expressed by naïve B cells [130]. However IgD secreting plasma cells exist, for instance in the tonsils and the nasal mucosa.

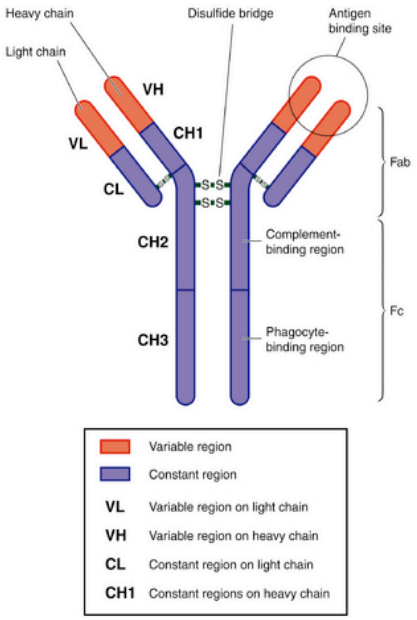


**Figure 4. Immunoglobulin architecture.**

Antibodies consist of two identical heavy chains and two identical light chains (kappa or lambda) giving them a Y-shaped morphology (Fig. 4) [127, 131, 132]. Heavy and light chains are linked by disulfide bonds, as are the two heavy chains. Each chain has one or more C regions and one V region [133-135]. The C- and V regions are composed of varying numbers of Ig domains. Heavy chain C regions are responsible for the biological functions of the antibody [136]. The antigen-binding site consists of six hypervariable loops, the V regions of heavy and light chains contributing with three loops each [137, 138]. The antigen-binding site is complementary to three-dimensional structures (*i.e.* epitopes) of a specific antigen, which is why the hypervariable loops are referred to as complementarity determining regions 1, 2 and 3 [107]. A hinge region allows for the recognition of widely spaced epitopes. The antibody repertoire of an individual may comprise more than $10^9$, and theoretically at least $10^{15}$, different antigen specificities.

Antibodies are involved in several immunological responses against antigens, *e.g.* neutralization of toxins (IgG and IgA antibodies), degranulation of Mast cells (IgE antibodies), complement-dependent cytotoxicity (IgG and IgM antibodies), opsonization and antibody-dependent-cellular cytotoxicity by way of Fc receptors (IgG antibodies) [127, 139-141]. Furthermore, one class of antibody can regulate the effector functions of another class. For instance, serum IgA antibodies can down-regulate IgG mediated phagocytosis and respiratory burst in neutrophils [142, 143].

### *Antigens: Immunogens and Haptens*

B cell antibody production can be initiated by various antigens, *e.g.* proteins, lipids, LPS and viruses [144-146]. These antigens are all immunogens, *i.e.* they can stimulate an immune response. Small chemicals like methacrylates, referred to as haptens, are only immunogenic when associated with a carrier protein [147]. It has been shown that B cell IgG and IgE anti-hapten antibody production requires the presence of T helper cells [148, 149]. Further, Palm *et al.* demonstrated that immunizing mice with the hapten dinitrophenyl coupled to human serum albumin (HSA), resulted in high titers of IgG1 anti-dinitrophenyl antibodies but low titers of IgG1 anti-HSA antibodies [150]. This was in contrast to the antibody response in animals immunized with HSA together with LPS, where the titer of IgG1 anti-HSA antibodies was high. The authors suggest that in contrast to PRR stimulating adjuvants (*e.g.* LPS) haptens primarily stimulate an antibody response that is hapten specific, as opposed to specific for the carrier protein (*e.g* HSA). In addition it has been shown that hapten density affects the diversity of anti-hapten antibody specificities [151].

*Effects of HEMA Exposure on Antibody Production*

Previous studies performed in mice have revealed various effects of HEMA exposure on antibody production:

- Mice injected with HEMA-conjugated mouse serum albumin (MSA) have an elevated IgG anti-MSA antibody activity in blood compared to mice injected with native MSA. That is HEMA stimulates the production of auto-antibodies [40].

- The number of HEMA monomers conjugated to MSA affects the level of IgG and IgE anti-MSA autoantibody activity and the total amount of serum IgE immunoglobulin [152].

- Injecting mice with HEMA-conjugated MSA leads to the production of anti-HEMA antibodies [152].

- A bicarbonate solution containing HEMA acts as an adjuvant in mice and promotes antibody production [153].

# Aims

The main objective of the present studies was to investigate effects of HEMA exposure on the innate and adaptive immune system.

*The specific aims were as follows:*

- To examine whether HEMA affects the phagocytic and respiratory burst activity of human peripheral blood phagocytes *in vitro*.
- To examine if HEMA affects the immunoglobulin production of human B cells *in vitro*.
- To study effects of long-term HEMA exposure *in vivo* on the general health and the immune system of mice.
- To investigate further the specific effects of HEMA exposure *in vivo* on antibody and cytokine production.

# Materials & Methods

## *2-hydroxyethyl methacrylate (HEMA)*

Before HEMA (Sigma-Aldrich, Stockholm, Sweden) was used in the studies it was passed through a column of polystyrene-co-divinylbenzene beads in order to remove the inhibitor monomethyl-ether-hydroquinone.

## *Effects of HEMA on the Phagocytic and Respiratory Burst Activity of Granulocytes and Monocytes (I)*

### *Study Subjects and Sampling*

Following informed consent, venous blood was collected from five, healthy female volunteers, aged 50-58 yrs.

### *HEMA Exposure in vitro*

Blood cells were exposed to 7.5 mmol $L^{-1}$ and 15 mmol $L^{-1}$ HEMA, using 0.85% NaCl as a diluent, for 2 hrs at room temperature. Blood samples left unexposed to HEMA served as controls. Cell viability following HEMA exposure was verified by trypan blue staining.

### *Flow cytometric Assay of Phagocyte Immune response in Activated whole blood (FAPIA)*

To determine the phagocytic activity, HEMA exposed blood cells were incubated with fluorescein (FITC)-conjugated, opsonized *Escherichia coli* (*E. coli*) ($1x10^7$ bacteria) (PHAGOTEST®, Orpegen Pharma, Heidelberg, Germany). Unconjugated, opsonized *E. coli* ($1x10^7$ bacteria) (PHAGOBURST®, Orpegen Pharma) and dihydrorhodamine (DHR) 123 were used to determine the respiratory burst activity of HEMA exposed blood cells. DHR 123 is oxidized to fluorescent rhodamine (R) 123 by the action of ROS [154, 155]. Blood cells incubated with PBS served as matched controls. Prior to flow cytometric acquisition erythrocytes were lysed by incubating the blood samples (room temperature, dark) in a hypotonic solution (FACS lysing solution, BD Biosciences, San José, CA, USA). Erythrocytes were then discarded and remaining blood cells resuspended in sheath fluid (FACSFlow, BD Biosciences) for subsequent flow cytometric analysis.

For each blood sample 50 000 events were collected using a flow cytometer (FACSCalibur®, BD Biosciences) equipped with a 488 nm laser. Each collected event corresponds to a photon of light emitted from one fluorescently labeled cell, as a result of being struck by a laser beam. Fluorescence emissions of FITC-*E. coli* and R 123 were detected at 530 nm. Granulocytes and monocytes were identified by their

characteristic forward and side scatter profiles (Fig. 5). Data, collected as the number of granulocytes and monocytes capable of performing phagocytosis and respiratory burst (% gated cells) and the phagocytic and respiratory burst activity (MFI), was analyzed with CellQuest Pro software (BD Biosciences).

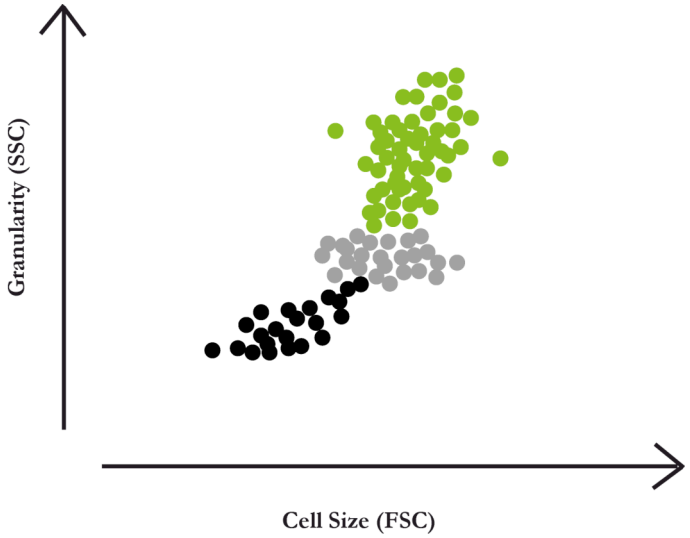


**Figure 5. A schematic dot plot of human whole blood. Each dot represents one cell detected by a flow cytometer. Illustrated are the granulocytes (green circles), the monocytes (grey circles) and the lymphocytes (black circles). FSC = Forward scatter and SSC = Side scatter.**

### *Cell Membrane Integrity - Propidium Iodide Staining*

Propidium iodide (PI), being positively charged, does not enter intact cells [156, 157]. Any disruption of cell membrane integrity leads to uptake of PI, which binds to nucleic acids in the cell. When excited by a laser beam, PI emits red fluorescence.

The effect of HEMA exposure on cell membrane integrity was analyzed by incubating HEMA exposed/unexposed blood cells with PI (Phagotest DNA staining solution, Orpegen Pharma). PI fluorescence emission was subsequently detected at 585 nm with a flow cytometer.

## *Effect of HEMA on Human B Cell Immunoglobulin Production (II)*

### *Purification of Human CD19+ B Cells from Blood*

Blood cells from eight healthy blood donors were obtained from Sahlgrenska university hospital in Gothenburg, Sweden. Only blood cells that were donated on the day of the experiment were used in the study. Peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation (Ficoll-Paque Plus, GE Healthcare

Biosciences AB, Uppsala, Sweden). CD19+ B cells from each donor were then purified using magnetic beads coated with anti-human CD19 antibodies. CD19+ B cells from each donor were resuspended separately in enriched growth medium, *i.e.* D-MEM Glutamax-I (GIBCO®) supplemented with 10% heat inactivated fetal bovine serum, gentamycin (50 μg $mL^{-1}$), penicillin (100 Units $mL^{-1}$) and streptomycin (100 μg $mL^{-1}$).

*Pokeweed Stimulation and HEMA Exposure in vitro*

B cells were seeded in triplicate in 96-well plates at a concentration of $10^5$ cells $well^{-1}$. B cells were incubated (6 days, 37°C, humidified atmosphere, 5% $CO_2$) with 20 μg $mL^{-1}$ pokeweed mitogen (Lectin from *Phytolacca americana*) and various concentrations of HEMA (0 μmol $L^{-1}$, 15 μmol $L^{-1}$, 37.5 μmol $L^{-1}$, 75 μmol $L^{-1}$, 150 μmol $L^{-1}$, 750 μmol $L^{-1}$). Pokeweed is a lectin that stimulates B lymphocyte proliferation [158].

On the sixth day the plates were frozen.

*B Cell Proliferation in vitro – Measuring β-rays emitted from [$^3$H]thymidine*

For proliferation studies [$^3$H]thymidine was added on the fifth culture day to some of the plates (1 μCurie $well^{-1}$). Following subsequent freezing and thawing cell lysates were harvested (Harvester 96, Tomtec) onto glass fiber filters on top of which a melt-on scintillator (Meltilex® A, Wallac Oy) was applied. Incorporated [$^3$H]thymidine was then counted (Microbeta ® Trilux, PerkinElmer Sweden AB).

*Human IgA, IgG1 and IgM Production in vitro - ELISA*

After thawing the B cell cultures, the individual B cell supernatants were analyzed for the presence of IgA, IgG1 and IgM with an ELISA. MaxiSorp™ plates (Nunc A/S, Roskilde, Danmark) were treated as follows:

*IgA*

- Coated (overnight, 4 ºC) with goat anti-human IgA antibodies (Mabtech AB, Nacka Strand, Sweden), diluted 1:500 in PBS, pH 7.4
- Washed 2 times with PBS
- Blocked (1 hr, room temperature) with 0.1% BSA/PBS-Tween (0.05%)
- Washed 5 times with PBS-Tween (0.05%)
- Incubated (2 hrs, room temperature) with standard (human IgA, Mabtech AB) and B cell supernatants
- Washed 5 times with PBS-Tween (0.05%)
- Incubated (1 hr, room temperature) with goat anti-human IgA antibodies conjugated to ALP (Mabtech AB), diluted 1:1000 in 0.1% BSA/PBS-Tween (0.05%)
- Washed 5 times with PBS-Tween (0.05%)
- Developed in the dark with p-Nitrophenyl-phosphate dissolved in diethanolamine buffer, pH 9.8.

- Absorbance was read at 405 nm on a spectrophotometer (Spectra MAX 340, Molecular Devices, Sunnyvale, CA, USA)

*IgG1 and IgM*

- Coated (overnight, room temperature) with mouse anti-human IgG1 antibodies (clone 8c/6-39, Sigma-Aldrich AB) diluted 1:1000 in PBS, pH 7.2 or goat anti-human IgM antibodies (Chemicon International Inc., Temecula, CA, USA) diluted 1:500 in PBS, pH 7.2
- Blocked (1 hr, room temperature) with 1% BSA/PBS
- Incubated (2 hrs, room temperature) with standard (Human IgG1 (The Binding Site Limited, Birmingham, UK) or Beadlyte® Human IgM (Upstate, Lake Placid, NY, USA)) and B cell supernatants
- Incubated (2 hrs, room temperature) with HRP conjugated antibodies, either sheep anti-human IgG (The Binding Site Limited), diluted 1:1000 in 1% BSA/PBS, or goat anti-human IgM (Chemicon International Inc.), diluted 1:5000 in 1% BSA/PBS
- Developed (in the dark) with tetramethylbenzidine dissolved in 0.05 mol $L^{-1}$ citrate-phosphate buffer, pH 5.0 supplemented with dimethyl sulfoxide and 30% $H_2O_2$
- The reaction was stopped with 1 mol $L^{-1}$ $H_2SO_4$
- Absorbance was read at 450 nm on a spectrophotometer (Spectra MAX 340, Molecular Devices), subtracting with the background absorbance read at 540 nm
- Between each incubation the plates were washed 3 times in PBS-Tween (0.05%)

## *Effects of HEMA Exposure in vivo (III, IV)*

### *Animal Husbandry*

Male Balb/c mice (Charles River Laboratories, Sulzfeld, Germany) were housed under specific-pathogen-free conditions in individually ventilated cages at the Laboratory for Experimental Biomedicine at the University of Gothenburg. Food and water were provided *ad libitum*. Experimental protocols were approved by the Ethical Committee for Animal Experimentation in Gothenburg, Sweden (# 380-2008).

### *HEMA Exposure in vivo - Osmotic Pumps (III)*

Alzet ® miniature osmotic pumps (model 2006, Alzet, Cupertino, CA, USA) were loaded with either 0.9% filter-sterilized NaCl, 8.2 mol $L^{-1}$ HEMA or 183 μmol $L^{-1}$ HEMA and implanted subcutaneously at the lower back, under aseptic conditions (Fig. 6). Animals were sacrificed 40 days after pump implantation by terminal bleeding. Serum and spleens were collected.

### *OVA Exposure in vivo – Subcutaneous Injections (III)*

Animals were immunized 19 days after pump implantation with ovalbumin (OVA) (Sigma-Aldrich AB) dissolved in 100 mmol $L^{-1}$ bicarbonate buffer. The injection consisted of 50 μg OVA in 50 μl buffer. An identical injection was given as a booster on day 34 post implantation.

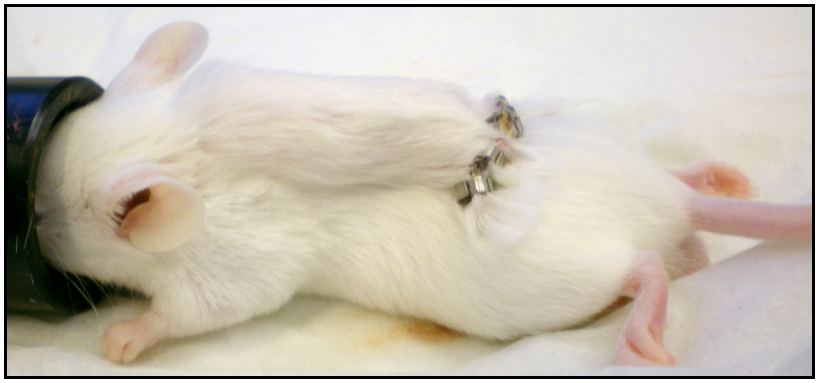

**Figure 6. Osmotic pumps filled with HEMA or saline were implanted subcutaneously into the back of mice and left *in situ* for 40 days.**

*HEMA and OVA Exposure in vivo – Subcutaneous Injections (IV)*

*Protocol 1*

Groups of mice were injected subcutaneously in the tail (50 µl/animal) with HEMA (20 µmol/animal) dissolved in bicarbonate buffer or bicarbonate buffer alone. After 3 weeks the animals received a second injection. Animals were sacrificed 6 days later, in order to study the short-term effects of HEMA exposure *in vivo* on cytokine production.

*Protocol 2*

Groups of mice were injected subcutaneously in the tail (50 µl/animal) with HEMA (20 µmol/animal) and OVA (50 µg/animal) in bicarbonate buffer, OVA (50 µg/animal) in bicarbonate buffer or bicarbonate buffer alone. After 3 weeks the animals received a booster injection. Animals were sacrificed 2 weeks later in order to study the effects of HEMA exposure *in vivo* on antibody production.

*Splenocyte Isolation (III, IV)*

Spleens were squeezed through a cell strainer with a pore size of 70 µm (BD Falcon, Bedford, MA, USA). Splenocytes were then isolated by density centrifugation, washed two times in PBS supplemented with penicillin (100 Units $mL^{-1}$) and streptomycin (100 µg $mL^{-1}$) and then resuspended in D-MEM Glutamax-I supplemented with 5% heat inactivated fetal bovine serum, gentamycin (50 µg $mL^{-1}$), penicillin (100 Units $mL^{-1}$) and streptomycin (100 µg $mL^{-1}$).

*Production of TNF-α, IL-2 and IL-6 in vitro – ELISA (III, IV)*

Mouse splenocytes were cultured for 2 days (37°C, humidified atmosphere, 5% $CO_2$). Supernatants were subsequently prepared by freezing and thawing. Supernatants were analyzed for the presence of TNF-α (DuoSet® # DY410, R&D Systems, Abingdon, UK), IL-2 (DuoSet® # DY402, R&D Systems) and IL-6 (DuoSet® # DY402, R&D Systems) by a sandwich ELISA. MaxiSorp™ plates (Nunc A/S) were treated as follows:

- Coated (overnight, room temperature) with goat anti-mouse TNF-α, rat anti-mouse IL-2 or rat anti-mouse IL-6 diluted 1:180 in PBS, pH 7.2
- Blocked (1 hr, room temperature) with 1% BSA/PBS
- Incubated (2 hrs, room temperature) with appropriate, recombinant standards and splenocyte supernatants
- Incubated (2 hrs, room temperature) with appropriate, biotinylated antibodies (goat anti-mouse TNF-α, goat anti-mouse IL-2, goat anti-mouse IL-6), diluted 1:180 in 0.1% BSA/PBS-Tween (0.05%), pH 7.2 (IL-2) or 1% BSA/PBS, pH 7.2 (TNF-α, IL-6)
- Incubated (20 min, room temperature, dark) with streptavidin-HRP
- Developed (20 min, room temperature, dark) with Tetramethylbenzidine dissolved in 0.05 mol $L^{-1}$ citrate-phosphate buffer, pH 5.0, supplemented with dimethyl sulfoxide and 30% $H_2O_2$
- The reaction was stopped with 1 mol $L^{-1}$ $H_2SO_4$.
- Absorbance was read at 450 nm on a spectrophotometer (Spectra MAX 340, Molecular Devices), subtracting the background absorbance read at 540 nm
- Between each incubation the plates were washed 3 times in PBS-Tween (0.05%)

*Detoxification of OVA using Polymixin B (III)*

Prior to initiating OVA stimulation experiments *in vitro* (see below), LPS was removed from the OVA solution by passing it twice through a column of polymixin B (DetoxiGel™AffinityPak™, Pierce, Rockford, IL, USA), according to the manufacturer's instructions. Polymixin B binds to the lipid A portion of LPS [159].

The LPS content of the OVA solution used for *in vitro* experiments was 28 endotoxin units $mL^{-1}$, as determined by the *Limulus* amebocyte lysate assay carried out at the Department of Clinical Bacteriology, the Sahlgrenska Academy. The *Limulus* amebocyte lysate assay relies on the activating effect of LPS on proteases participating in the coagulation cascade of the American horseshoe crab *Limulus polyphemus* [160]. In the *Limulus* amebocyte lysate assay any LPS present in a sample activates the *Limulus polyphemus* proteases. The activated enzymes then react with a color substrate. Next the absorbance of the color substrate is read with a spectrophotometer.

### *Con A and OVA Stimulation in vitro – Splenocyte Proliferation (III, IV)*

Concanavalin A (Con A) is a lectin that stimulates T lymphocyte proliferation by binding to carbohydrates on CD3 in the CD3/T cell receptor complex [161].

Splenocytes were seeded ($5x10^5$ cells *per* well) in triplicate (Nunclon Δ Surface F96 well, Nunc A/S) and then stimulated, either 4 days with 40 μg detoxified OVA (III) or 3 days with 1 μg Con A (Pharmacia AB, Uppsala, Sweden) (III, IV), at 37°C, in humidified atmosphere containing 5% $CO_2$. Unstimulated cells were cultured in parallel. Proliferation was analyzed by measuring [$^3$H]thymidine incorporation (see B Cell Proliferation *in vitro* – Measuring β-rays emitted from [$^3$H]thymidine).

### *IgA, IgG and IgM anti-OVA Antibody Activity in Serum – ELISA (III, IV)*

Serum was analyzed for the presence of IgA, IgG and IgM anti-OVA antibody activity with an ELISA. MaxiSorp™ plates (Nunc A/S) were treated as follows:

- Coated (overnight, 4 ºC) with OVA (0.5 μg $well^{-1}$) dissolved in PBS, pH 7.2
- Washed 3 times with PBS-Tween (0.05%)
- Incubated (overnight, 4 ºC) with serum samples and standard (pool of serum from all animals in the study)
- Washed 3 times with PBS-Tween (0.05%)
- Incubated (2 hrs, room temperature) with biotin-conjugated antibodies, either goat anti-mouse IgG (Jackson Immunoresearch Laboratories Inc., West Grove, PA, USA) diluted 1:100 000 in PBS-Tween (0.05%), goat anti-mouse IgM (Southern Biotech, Birmingham, AL, USA) diluted 1:20000 in PBS-Tween (0.05%) or goat anti-mouse IgA (Southern Biotech) diluted 1:20000 in PBS-Tween (0.05%)
- Washed 4 times with PBS-Tween (0.05%)
- Incubated (2 hrs, room temperature, dark) with ExtrAvidin-ALP (Sigma-Aldrich AB)
- Washed 4 times with PBS-Tween (0.05%)
- Developed (in the dark) with p-Nitrophenyl-phosphate (Sigma-Aldrich AB) dissolved in diethanolamine buffer, pH 9.8
- Absorbance was read at 405 nm on a spectrophotometer (Spectra MAX 340, Molecular Devices)

## *Statistical Analysis*

Data was analyzed statistically as follows:
ANOVA and Tukey's *post hoc* test (I)
Related samples Wilcoxon's signed ranks test (II)
ANOVA together with either Tamhane's or Bonferroni's *post hoc* test, Independent samples t test (III)
Independent samples t test (IV)
A $p$-value $< 0.05$ was considered to be statistically significant.

# Results

## *Exposing Granulocytes and Monocytes to HEMA in vitro - Consequences for Bactericidal Functions (I)*

HEMA has previously been shown to induce apoptosis by stimulating the production of ROS [162]. ROS production is important for the bactericidal function of phagocytes like neutrophils, also known as polymorphonuclear granulocytes, and monocytes/macrophages [94, 163]. Neutrophils, monocytes and macrophages are present in the oral cavity where they can come into contact with unpolymerized HEMA monomers [30, 34]. Our aim with study I was to examine the effect of HEMA exposure on the phagocytic and respiratory burst activity of neutrophils and monocytes. To achieve this we exposed human blood cells to 7.5 mmol $L^{-1}$ and 15 mmol $L^{-1}$ HEMA *in vitro* for 2 hrs. The HEMA concentrations that we used were chosen based on a previous study where Samuelsen *et al.* analyzed the effect of HEMA exposure on ROS production in rat submandibular salivary gland acinar cells [162]. Samuelsen *et al.* showed that 7.5 mmol $L^{-1}$ and 15 mmol $L^{-1}$ HEMA stimulates ROS production.

After incubating the blood cells with HEMA we examined the phagocytic and respiratory burst activity using flow cytometry, with *E. coli* as bait.

### *Phagocytic Activity*

The phagocytic activity of granulocytes and monocytes was detected by measuring the fluorescence of ingested FITC-conjugated *E. coli*. We could not demonstrate any HEMA dependent, significant differences in the number of granulocytes or monocytes capable of performing phagocytosis. Nor were there any HEMA dependent, significant differences in granulocyte or monocyte phagocytic efficiency.

### *Respiratory Burst Activity*

The respiratory burst activity was detected by measuring the conversion of DHR 123 to fluorescent R 123, occurring as a result of *E. coli* phagocytosis. The respiratory burst activity was significantly reduced in granulocytes exposed *in vitro* to 7.5 mmol $L^{-1}$ and 15 mmol $L^{-1}$ HEMA (MFI ± standard deviation = 1093 ± 262, $p = 0.000$ and MFI ± standard deviation = 616 ± 185, $p = 0.000$ respectively) compared to unexposed granulocytes (MFI ± standard deviation = 3338 ± 1393) (Fig. 7). However, HEMA had no significant effect on the respiratory burst activity of monocytes. Furthermore, there were no HEMA dependent, significant effects on the number of granulocytes or monocytes capable of performing a respiratory burst.

*Cell Membrane Integrity*

In order to examine if the different effects of HEMA on granulocyte and monocyte respiratory burst activity was due to differences in susceptibility to HEMA cytotoxicity, we also examined granulocyte and monocyte membrane permeability by measuring PI fluorescence. We could however not demonstrate any HEMA dependent changes in granulocyte or monocyte membrane permeability.

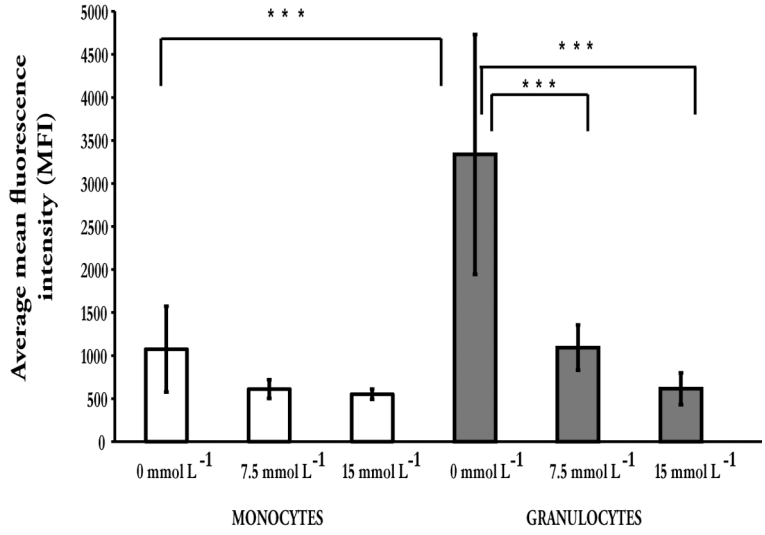


**Figure 7. Effect of HEMA on the respiratory burst activity of granulocytes and monocytes. Human whole blood was exposed *in vitro* to different concentrations of HEMA for 2 hrs. The production of hydrogen peroxide was assessed using DHR 123 as a substrate. The fluorescence of R 123 was detected with a flow cytometer. Data is presented as mean MFI ± standard deviation. n = 5 blood donors (0 mmol $L^{-1}$ HEMA, 7.5 mmol $L^{-1}$ HEMA) and n = 4 blood donors (15 mmol $L^{-1}$ HEMA). *** $p < 0.001$, ANOVA and Tukey's *post hoc* test.**

## *Exposing Human B cells to HEMA in vitro – Consequences for Immunoglobulin Production and B Cell Proliferation (II)*

HEMA has previously been shown to affect the activity of mouse B cells. For instance, HEMA exposure *in vivo* leads to autoantibody production as well as an increased production of IgE antibodies in mice [40, 153]. The aim of study II was to examine if HEMA can affect the activity of human B cells as well. Nespoli *et al.* have shown that stimulating peripheral blood B cells with pokeweed *in vitro* enables the detection of IgG and IgM immunoglobulins in B cell culture supernatants [164]. Further Agger *et al.* have reported that the maximum number of IgG+, IgM+ and IgA+ B cells is reached after 6 days of culture in the presence of pokeweed [165]. In addition we were unable to detect IgG1, IgM and IgA immunoglobulins in preliminary experiments where B cells were cultured in the absence of pokeweed. To study the effects of HEMA exposure on human B cell activity we therefore chose to stimulate

CD19+ B cells, purified from peripheral blood, with pokeweed for 6 days *in vitro,* in the presence of different concentrations of HEMA, followed by analysis of the immunoglobulin production.

*IgA, IgG1 and IgM Immunoglobulins*

The presence of IgA, IgG1 and IgM immunoglobulins in the B cell supernatants was analyzed by an ELISA. B cells exposed to 15 μmol $L^{-1}$ or 37.5 μmol $L^{-1}$ HEMA produced significantly more IgG1 immunoglobulins *in vitro* (median for the relative concentration = 122.8%, interquartile range = 80.8%, $p = 0.01$ and median for the relative concentration = 155.8 %, interquartile range = 81.7%, $p = 0.02$ respectively) compared to unexposed B cells (Fig. 8). B cells exposed to 750 μmol $L^{-1}$ HEMA on the other hand had a reduced IgG1 immunoglobulin production *in vitro* (median for the relative concentration = 26.3%, interquartile range = 41.4%, $p = 0.01$), compared to unexposed cells.

B cells exposed to 150 μmol $L^{-1}$ or 750 μmol $L^{-1}$ HEMA had a reduced IgM immunoglobulin production *in vitro* (median for the relative concentration = 71.2%, interquartile range = 32.7%, $p = 0.02$ and median for the relative concentration = 30.8%, interquartile range = 16.0%, $p = 0.01$ respectively), compared to unexposed cells. Furthermore, in B cell cultures containing 15 μmol $L^{-1}$, 37.5 μmol $L^{-1}$ and 75 μmol $L^{-1}$ HEMA, the sum of the relative concentrations of IgG1 immunoglobulins was significantly higher compared to the sum of the relative concentrations of IgM immunoglobulins ($p = 0.01$). We could not demonstrate any statistically significant changes in the production of IgA immunoglobulin in the cultures containing the different HEMA concentrations, compared to unexposed cultures.

*B Cell Proliferation*

B cell proliferation, detected by measuring incorporated [$^3$H]thymidine, was significantly reduced in B cell cultures containing 150 μmol $L^{-1}$ HEMA and 750 μmol $L^{-1}$ HEMA (median for the relative proliferation = 74.6%, interquartile range = 6.7%, $p = 0.02$ and median for the relative proliferation = 39%, interquartile range = 46.8%, $p = 0.02$ respectively). However we could not demonstrate a HEMA dependent, significant difference in B cell proliferation *in vitro*, in the B cell cultures containing 15 μmol $L^{-1}$, 37.5 μmol $L^{-1}$ or 75 μmol $L^{-1}$ HEMA.

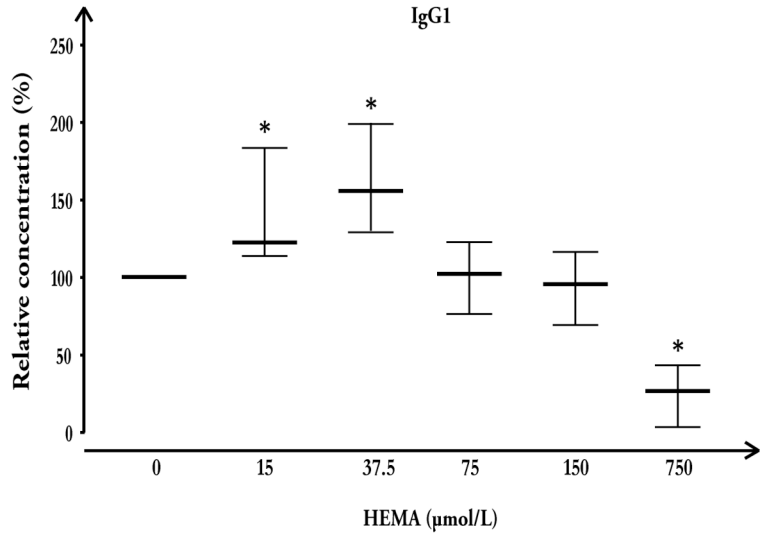


**Figure 8. Effect of HEMA exposure *in vitro* on B cell IgG1 production. CD19+ B cells were isolated from human blood and cultured for 6 days in the presence of different concentrations of HEMA. The presence of IgG1 immunoglobulin in the culture supernatants was then analyzed by an ELISA. Data is presented as the median and the interquartile range for the relative concentration of IgG1. n = 8 blood donors. * $p < 0.05$, Wilcoxon's signed-ranks test.**

## *Long-Term Exposure to HEMA in vivo – Consequences for General Health Status, Splenocyte Proliferation, Splenocyte Cytokine Production and Antibody Activity (III)*

Our aim with study III was to examine how long-term exposure to minute amounts of HEMA affects the general health and the immune system of mice. We strived to mimic the effect of exposure to low levels of HEMA monomers, as occurs when recently cured restorations leak or when a person working in a dental clinic inhales the air [166, 167]. To accomplish this we filled Alzet® miniature osmotic pumps with different concentrations of HEMA monomers and implanted the pumps subcutaneously into the back of mice. Mice receiving pumps filled with 0.9% NaCl served as controls. The osmotic pumps were designed to deliver a dose of 0.15 µl $hr^{-1}$. In order to study the effects of long-term HEMA exposure *in vivo* on the immune system we immunized the mice with OVA. Spleens and serum were collected 40 days after pump implantation.

### *Health Status*

Mice exposed to 161 µg of HEMA $hr^{-1}$ *in vivo* increased significantly less in body weight throughout the HEMA exposure period ($0.000 \leq p \leq 0.018$), compared to mice exposed to 1.35 µg of NaCl $hr^{-1}$ (control group). Mice exposed to 3.6 ng of HEMA $hr^{-1}$ *in vivo* increased significantly less in body weight in the beginning of the HEMA exposure period ($0.000 \leq p \leq 0.037$), compared to the control group.

*Splenocyte Proliferation in vitro*

The proliferation of splenocytes was measured by detecting incorporated [$^3$H]thymidine with a scintillation counter. Splenocytes from mice exposed to 3.6 ng of HEMA $hr^{-1}$ *in vivo* had a significantly lower Con A stimulated *in vitro* proliferation (mean counts per minute ± standard deviation = 2708 ± 2097) than splenocytes from both the control group (mean counts per minute ± standard deviation = 6191 ± 3701, $p = 0.036$) and the mice exposed to 161 μg of HEMA $hr^{-1}$ *in vivo* (mean counts per minute ± standard deviation = 6852 ± 4123, $p = 0.029$).

*Production of IL-2 and TNF-α by Splenocytes in vitro*

Splenocytes were cultured for 2 days. The concentration of the cytokines was then measured in splenocyte supernatants by an ELISA. Splenocytes from mice exposed to either 161 μg of HEMA $hr^{-1}$ or 3.6 ng of HEMA $hr^{-1}$ *in vivo* had a significantly lower IL-2 production *in vitro* (mean concentration of IL-2 ± standard deviation = 44.9 ± 7.2 pg $mL^{-1}$, $p = 0.019$ and mean concentration of IL-2 ± standard deviation = 33.5 ± 8.9 pg $mL^{-1}$, $p = 0.002$ respectively) compared to splenocytes from the control group (mean concentration of IL-2 ± standard deviation = 66.4 ± 19.8 pg $mL^{-1}$). We were unable to demonstrate a HEMA dependent, significant effect on TNF-α production *in vitro*.

*IgA, IgG and IgM anti-OVA Antibody Activity*

The anti-OVA antibody activity in serum was determined with an ELISA. Mice exposed to 3.6 ng of HEMA $hr^{-1}$ had a significantly lower serum IgA anti-OVA antibody activity (mean activity of anti-OVA IgA ± standard deviation = 37564 ± 3766 ELISA units, $p = 0.026$) than the control group (mean activity of anti-OVA IgA ± standard deviation = 50566 ± 13004 ELISA units) (Fig. 9). We could not demonstrate any significant differences in the activity of IgG or IgM anti-OVA antibodies in mice exposed *in vivo* to either 161 μg of HEMA $hr^{-1}$ or 3.6 ng of HEMA $hr^{-1}$, compared to the control group.

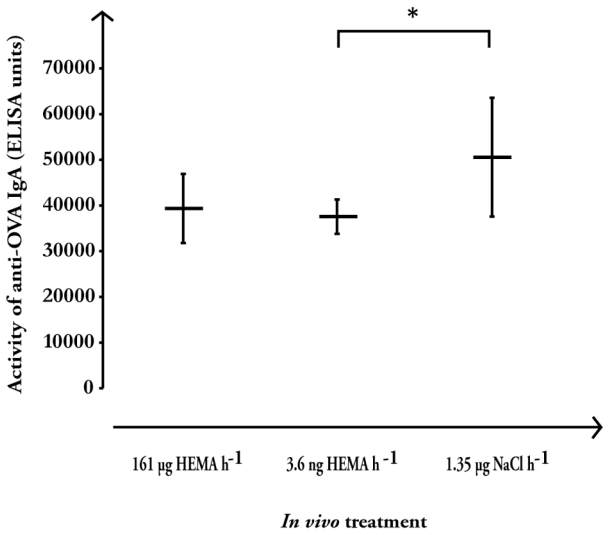


**Figure 9. Osmotic pumps filled with different concentrations of HEMA were implanted subcutaneously into the back of mice. Control animals received pumps filled with saline. The pumps were left *in situ* for 40 days, during which time the animals were injected with ovalbumin (OVA) in bicarbonate buffer on two occasions. Animals were sacrificed 6 days after the last OVA injection and serum was prepared. The anti-OVA IgA antibody activity in the serum was determined with an ELISA. Data is presented as mean ELISA units ± standard deviation. n = 6 animals (161 µg of HEMA $hr^{-1}$), n = 8 animals (3.6 ng of HEMA $hr^{-1}$) and n = 8 animals (1.35 µg of NaCl $hr^{-1}$). * $p < 0.05$, Independent samples t test.**

## *Subcutaneous HEMA Exposure - Consequences for the Immune System (IV)*

Previous studies have demonstrated the inflammatogenic and adjuvant properties of HEMA [153, 168]. For instance mice injected subcutaneously with HEMA dissolved in 100 mmol $L^{-1}$ bicarbonate buffer once a week for 3 weeks have a reduced splenocyte IL-10 production *in vitro*. Further the IL-6 production from draining lymph node cells, stimulated *in vitro* with anti-CD3 antibodies for 3 days, is higher when cells are harvested from mice painted on the ears with HEMA in acetone/olive oil, compared to control mice painted with only acetone/olive oil. Our aim with study IV was to further explore the specificity of the immunomodulatory properties of HEMA *in vivo*. Therefore we injected mice subcutaneously in the tail with HEMA in bicarbonate buffer or only bicarbonate buffer. The animals were sacrificed 6 days after receiving a second injection and splenocyte proliferation and inflammatory cytokine production *in vitro* was determined. Other groups received HEMA in bicarbonate buffer or OVA and HEMA in bicarbonate buffer. These animals were sacrificed 2 weeks after receiving a booster injection. The activity of IgG and IgM anti-OVA antibodies in blood was then determined.

*Splenocyte Proliferation in vitro*

Baseline splenocyte *in vitro* proliferation was higher for mice exposed two times to HEMA in bicarbonate buffer *in vivo* (mean counts per minute ± standard deviation = 712 ± 413, $p = 0.001$), than for control mice exposed to bicarbonate buffer alone (mean counts per minute ± standard deviation = 285 ± 136).

Mice exposed two times to HEMA in bicarbonate buffer *in vivo* also had a higher Con A stimulated *in vitro* proliferation of splenocytes (mean counts per minute ± standard deviation = 24631 ± 10686, $p = 0.016$), compared to control animals (mean counts per minute ± standard deviation = 12201 ± 9346).

*Production of TNF-α and IL-6 by Splenocytes in vitro*

Splenocytes from animals exposed two times *in vivo* to HEMA in bicarbonate buffer produced less TNF-α *in vitro* (mean concentration of TNF-α ± standard deviation = 233 ± 100 pg $mL^{-1}$, $p = 0.043$) than splenocytes from animals exposed to bicarbonate buffer alone (mean concentration of TNF-α ± standard deviation = 399 ± 185 pg $mL^{-1}$). Furthermore, the splenocyte TNF-α *in vitro* production in relation to the IL-6 *in vitro* production was significantly lower in the mice exposed to HEMA *in vivo* (mean for the ratio [TNF-α]/[IL-6] ± standard deviation = 2.6 ± 0.5, $p = 0.000$), compared to the animals that received only bicarbonate buffer (mean for the ratio [TNF-α]/[IL-6] ± standard deviation = 5.8 ± 1.4).

*IgG and IgM anti-OVA Antibody Activity*

Animals immunized with OVA in the presence of HEMA had an IgG anti-OVA antibody activity in blood that was 5.7 times higher (mean anti-OVA IgG activity ± standard deviation = 17350 ± 10381 ELISA units, $p = 0.01$), than the IgG anti-OVA antibody activity in blood from animals immunized with OVA alone (mean anti-OVA IgG activity ± standard deviation = 3042 ± 3041 ELISA units). Furthermore, animals immunized with OVA in the presence of HEMA had a significantly higher IgG anti-OVA antibody activity relative to the corresponding IgM anti-OVA antibody activity (mean for the ratio anti-OVA IgG activity/anti-OVA IgM activity ± standard deviation = 1.7 ± 1.0, $p = 0.012$), compared to animals that received only OVA (mean for the ratio anti-OVA IgG activity/anti-OVA IgM activity ± standard deviation = 0.3 ± 0.3) (Fig. 10).

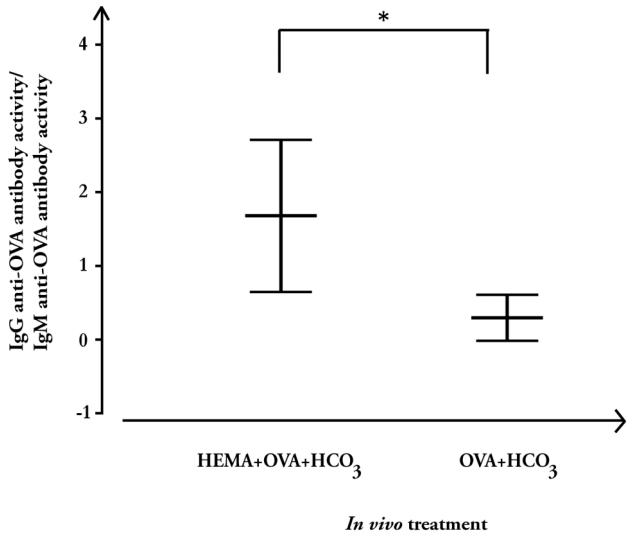


**Figure 10. Mice were injected two times, 3 weeks apart, with HEMA in bicarbonate buffer ($HCO_3$), alone or together with ovalbumin (OVA). Animals were sacrificed 2 weeks after the last injection. Blood was collected and serum was prepared. The anti-OVA IgG and anti-OVA IgM antibody activity was determined with an ELISA. Data is presented as the anti-OVA IgG antibody activity relative to the anti-OVA IgM antibody activity ± standard deviation. n = 7 animals (HEMA+OVA+$HCO_3$) and n = 8 animals (OVA+$HCO_3$).**
*** $p < 0.05$, Independent samples t test.**

# General Discussion

In the present thesis the immunomodulatory properties of HEMA were studied. Specific points that were addressed were the effect of HEMA exposure on the bactericidal functions of neutrophils and monocytes, the production of immunoglobulins, the activity of antibodies, the production of splenocyte cytokines and splenocyte proliferation.

We have as yet not been able to determine how HEMA acts on a molecular level *in vivo*. The proteins, lipids and/or nucleic acids that HEMA interacts with *in vivo* and how such and interaction can modulate specific physiological responses remains unclear. However based on the immunological responses shown to be susceptible to HEMA exposure, different mechanisms for the immunomodulatory properties of HEMA can be proposed. In the following discussion possible mechanisms behind the effects of HEMA exposure on the immune system will be suggested.

In study I we investigated the effect of HEMA exposure on neutrophil and monocyte phagocytosis and respiratory burst activity. We found that HEMA specifically inhibited the neutrophil respiratory burst activity. The respiratory burst is driven by the multi-subunit NADPH oxidase, an enzyme whose activation depends on the assembly of several subunits at the cell membrane [89]. It has been shown previously that HEMA decreases the formation of phosphatidic acid, a precursor of phospholipids [169]. One possibility for the impaired neutrophil respiratory burst could therefore be that HEMA disrupts the formation of certain phospholipids and hence the integrity of the phospholipid bilayer of the plasma membrane. This could affect the proper assembly of NADPH oxidase subunits at the plasma membrane and hence the optimal function of the NADPH oxidase. To study the effects of HEMA on cell membrane integrity we incubated the blood cells with PI. However we could not demonstrate an effect of HEMA on cell membrane integrity. A cell membrane associated activity that appeared insensitive to HEMA exposure was neutrophil and monocyte phagocytosis. In other words the HEMA dependent decrease in neutrophil respiratory burst that we show in study I was not due to an impaired phagocytic capacity.

Nocca *et al.* have, by exposing neutrophils and monocytes to the monomers diurethane dimethacrylate and 1,4-butanediol dimethacrylate, shown that the respiratory burst of neutrophils and monocytes differs in terms of sensitivity to methacrylate exposure [170]. It was demonstrated that both diurethane dimethacrylate and 1,4-butanediol dimethacrylate suppressed the respiratory burst of unstimulated neutrophils, however neither diurethane dimethacrylate nor 1,4-butanediol dimethacrylate was able to suppress the respiratory burst of unstimulated monocytes. In our experiments we could demonstrate an effect of HEMA exposure on neutrophil respiratory burst activity but not an effect of HEMA on monocyte respiratory burst

activity. This suggests that HEMA might be targeting some cellular structure that is expressed by neutrophils but not expressed by monocytes. Another possibility is that monocytes somehow manage to negate the effect of HEMA on the respiratory burst. Phagocytes respond with phagocytosis and a respiratory burst when bacteria are recognized by PRRs, *e.g.* TLRs, expressed on the cell surface of the phagocytes [66]. Expression of TLRs is higher in monocytes than in neutrophils. As such one possible scenario could be that HEMA binds to TLRs on both neutrophils and monocytes, thereby blocking TLR signaling. However due to the higher degree of TLR expression on the monocyte cell surface some monocyte TLRs remain unoccupied by HEMA and these "free" TLRs enable monocytes to maintain proper TLR signaling and a normal level of respiratory burst activity. However if HEMA does affect neutrophil TLR signaling, then the phagocytic activity of neutrophils would presumably be affected as well. The fact that we were unable to demonstrate an effect of HEMA on the phagocytic activity of neutrophils argues against an effect of HEMA on neutrophil TLR signaling.

The fact that we exposed whole blood, and not purified cells, to HEMA might be the reason for the apparent insensitivity of the monocyte respiratory burst to HEMA exposure. The cytokine IFNγ is produced by T cells and has the ability to stimulate macrophage ROS production [171, 172]. It is possible that in our experiments performed on whole blood HEMA dampened the respiratory burst of the monocytes as well as stimulated T cell IFNγ production. The IFNγ thus produced could counteract a negative effect of HEMA on monocyte respiratory burst activity, giving monocytes the appearance of insensitivity to HEMA exposure.

In summary, even if the phagocytic capacity is unaffected by HEMA the ability of neutrophils to kill ingested pathogens is impaired.

Phagocytes are not the only cells capable of expressing the NADPH oxidase and performing a respiratory burst. B cells express all the subunits of the NADPH oxidase (gp91$^{phox}$, p22$^{phox}$, p40$^{phox}$, p47$^{phox}$, p67$^{phox}$, Rac2) [173]. Furthermore ROS is produced by B cells following BCR stimulation, the ROS production of B cells being less than 1/10 of the phagocyte ROS production [174]. Previous studies have shown that the NADPH oxidase is involved in regulating antibody production. For instance Vené *et al.* demonstrated that blocking the NADPH oxidase activity of LPS stimulated B cells resulted in an impaired IgM production [175]. Further by immunizing mice with sheep red blood cells they showed that $H_2O_2$ and peroxynitrite is produced in spleen germinal centers.

HEMA, like ROS, has previously been shown to affect antibody production in mice [152, 153]. We wanted to investigate if HEMA could affect the activity of human B cells as well (study II). To achieve this we purified CD19+ B cells from fresh human blood and exposed them to HEMA, at different concentrations, in the presence of pokeweed

mitogen. We found that HEMA, at certain concentrations, specifically stimulated IgG1 production *in vitro*, while the IgM and IgA production *in vitro* was unaffected by the same HEMA concentrations. Further we showed that the HEMA dependent increase in IgG1 production *in vitro* was not due to an increase in B cell proliferation. B cells producing IgG1 have undergone the process of CSR [176]. CSR requires the action of the enzyme Activation Induced cytidine Deaminase (AID). AID initiates an intrachromosomal recombination event between two switch regions located in the Ig heavy chain locus. The presence of a donor switch region located upstream of the exon coding for the constant region of IgM and IgD ($C_\mu/C_\delta$) together with acceptor switch regions located upstream of $C_\alpha$, $C_\varepsilon$ and $C_\gamma$ enables the production of the different isotypes. CSR is promoted by CD40 and TLR signaling together with cytokines secreted from T helper cells and dendritic cells. CD40/TLR signalling stimulates AID transcription, while cytokines determine which isotype will be produced due to the presence of germline promoters upstream of each acceptor switch region. The germline promoters have binding sites for cytokine activated transcription factors. For instance the germline γ1 promoter have a binding site for the IL-4 induced transcription factor Stat-6. One possible explanation for the stimulatory effect of HEMA on IgG1 production *in vitro* could be that HEMA activates the IL-4 receptor on the B cell surface, thereby initiating a signaling cascade similar to that of true IL-4. This could lead to an increase in Stat-6 activated transcription of the germline γ1 promoter and ultimately an increased IgG1 production. Another cytokine that has been shown to stimulate IgG production by human blood B cells is IL-6 [177, 178]. Hence the stimulatory effect of HEMA on IgG1 production could be due to an effect of HEMA on IL-6 signaling. On the other hand previous experiments performed by Burdin *et al.* have shown that blocking the B cell IL-6 receptor leads to a decrease in both IgG and IgM production [179]. We could not demonstrate a stimulatory effect of HEMA on IgM production *in vitro*, which would argue against a stimulatory effect of HEMA on IL-6 signaling. However Burdin *et al.* analyzed the immunoglobulin production of B cells isolated from tonsils, not the immunoglobulin production of pokeweed stimulated blood B cells. Indeed it has been shown that IL-6 promotes the IgG production of pokeweed stimulated PBMC [180]. This finding might lend some support to the theory that an increased IL-6 signaling might be causing the HEMA stimulated increase in IgG1 production *in vitro* that we observed.

In summary, the results presented in study II suggest that HEMA can stimulate the production of human immunoglobulins of a particular isotype, while leaving the production of immunoglobulins of other isotypes unaffected.

We were also interested in studying how long-term exposure to HEMA would affect the immune system. We therefore filled osmotic pumps with either concentrated HEMA or diluted HEMA. We chose to dilute HEMA to a concentration that reflects the level of HEMA to which people working in a dental clinic are exposed. Next we implanted the pumps subcutaneously into the back of mice, leaving them *in situ* for 40

days. Control animals received pumps filled with saline. We show in study III that mice exposed to both high and low concentrations of HEMA initially gain less in weight than control animals, while mice exposed to concentrated HEMA have an impaired weight gain throughout the entire exposure period. A possible reason for the impaired weight gain could be the presence of an inflammation, where the inflammation affects the animal's food intake. In humans inflammatory reactions due to HEMA exposure, such as contact dermatitis, have been reported [3]. We could however not observe an inflammatory infiltrate in the connective tissue surrounding the outlet of the HEMA filled pumps (results not shown). However we only analyzed the tissue for inflammatory infiltrates 40 days after pump implantation. An inflammation could have been present during the first week of the experiment, an inflammation that even though it had been resolved early on had an impact on the subsequent weight gain of the animals.

Splenocytes from animals exposed *in vivo* to minute amounts of diluted HEMA for 40 days had a reduced proliferative response to Con A stimulation *in vitro*. Con A stimulates T cell proliferation by binding to carbohydrates on CD3 [161]. A possible reason for the decrease in Con A stimulated splenocyte proliferation observed following 40 days of HEMA exposure *in vivo* could thus be that HEMA affects the responsiveness of T cells by binding to CD3 *in vivo*.

We also show in study III that HEMA exposure *in vivo* has a negative effect on splenocyte IL-2 production *in vitro*. T cells proliferate and produce IL-2 when IL-2 binds to the IL-2 receptor on the T cell surface [181]. In other words IL-2 functions in an autocrine manner. Granchi *et al.* has previously shown that methacrylates can have a negative effect on the expression of the IL-2 receptor [182]. Granchi *et al.* exposed lymphocytes *in vitro* to polymethylmethacrylate, a treatment that resulted in a decreased expression of CD25, that is the α-chain of the IL-2 receptor. One possibility for the decrease in splenocyte IL-2 production *in vitro* following HEMA exposure *in vivo* could therefore be that HEMA decreases the expression of the IL-2 receptor *in vivo*, which would have consequences for both T cell proliferation as well as IL-2 production *in vitro*.

Long-term exposure to minute amounts of diluted HEMA *in vivo* also affected the antibody response of the animals in an isotype specific manner. That is long-term exposure to HEMA *in vivo* resulted in a decreased IgA anti-OVA antibody activity, while leaving the IgG and IgM anti-OVA antibody activity unaffected. IgA synthesis is regulated by the cytokine transforming growth factor (TGF)-β [183]. TGF-β promotes transcription from the germline Igα promoter by activating the transcription factors Smad3 and Smad4. It has previously been shown that exposure *in vitro* to a methacrylate can negatively affect TGF-β expression [184]. Hence a possible explanation for the HEMA dependent decrease in IgA anti-OVA antibody activity could be that HEMA binds to the TGF-β receptor *in vivo*, thereby hampering TGF-β signal

transduction and hence the production of IgA anti-OVA antibodies.

In summary, the results presented in study III suggest that long-term HEMA exposure *in vivo* can affect the production of antibodies in an isotype specific manner as well as certain T cell activities.

In study IV we analyzed further the specific effects of HEMA exposure *in vivo* by injecting HEMA subcutaneously into the tail of mice. We found that Con A stimulated splenocyte proliferation was two times higher in animals exposed to HEMA dissolved in bicarbonate buffer compared to animals only exposed to the buffer. In the skin there are different metabolizing enzymes, for instance cytochrome P450 [185]. In theory HEMA that has been injected subcutaneously could be metabolized by cytochrome P450 into a product that can prevent inhibitory regulation of T cell receptor signaling, thus enhancing the effect of stimulation with a mitogen like Con A. In a previous study it was shown that mitogen activated protein kinase p38 negatively regulates the proliferation of anti-CD3 stimulated CD4+ T cells [186]. Based on that study a possibility for the enhancing effect of HEMA on Con A stimulated splenocyte proliferation is that HEMA binds to p38, thereby preventing p38 from inhibiting Con A stimulated T cell proliferation.

Production of the cytokine TNF-α has previously been shown to be sensitive to HEMA exposure. For instance exposing human monocytes to HEMA decreases LPS stimulated TNF-α production *in vitro* [96]. We show in study IV that exposing mice to HEMA in bicarbonate buffer two times results in a reduced splenocyte TNF-α production *in vitro*. Macrophages are a major source of TNF-α [187]. Therefore one could argue that the reduction in splenocyte TNF-α production that we observed was due to an impairment of macrophage cytokine production as a result of previous HEMA exposure. However macrophages are also a major source of IL-6 [187]. That we were unable to demonstrate an effect of HEMA exposure *in vivo* on splenocyte IL-6 production *in vitro* might therefore argue against an impaired macrophage cytokine production. Alternatively HEMA might affect macrophage TNF-α production specifically.

The negative effect of HEMA exposure *in vivo* on splenocyte TNF-α production *in vitro* is contradictory to the observation made in study III where HEMA exposure *in vivo* had no demonstrable effect on splenocyte TNF-α production *in vitro*. One factor that differs between study III and study IV is that in the latter the mice were injected with OVA on two occasions. Furthermore LPS was not removed from the OVA solution used for immunizations. Hence the differing results might be due to a stimulatory effect of the LPS present in the OVA solution administered in study III. In other words HEMA might have had a negative effect on the TNF-α production in study III similar to that observed in study IV, but the LPS present in the OVA solution administered could have counteracted it.

We present results indicating that B cells producing antibodies of different isotypes respond differently to HEMA exposure. In study III HEMA specifically reduced IgA anti-OVA antibody production, while in study IV HEMA specifically increased IgG anti-OVA antibody production. Further in both study III and study IV the IgM anti-OVA antibody production was unaffected. In other words injecting HEMA subcutaneously on two occasions has a stimulatory effect on IgG anti-OVA antibody production while long-term exposure to minute amounts of HEMA has a negative effect on IgA anti-OVA antibody production. How HEMA can scrutinize between IgA+, IgG+ and IgM+ B cells is difficult to explain. Generally speaking protein production is regulated at different stages, from the assembly of transcription factor complexes at promoters, through transcription and translation to the stability and proper folding of a functional protein. HEMA could potentially interact with regulatory proteins at any stage in the process of antibody production. One possible explanation for the apparent scrutinizing ability of HEMA is that due to the different experimental setups in study III and study IV HEMA was degraded into different metabolites. These metabolites could have different propensities for affecting IgA or IgG anti-OVA antibody production by binding to specific amino acid sequences in the promoter region of IgA and IgG exons.

In summary studies III and IV suggest that HEMA exposure *in vivo* can modulate the immune system with a high degree of specificity, a specificity that is dependent on concentration and length of exposure.

In conclusion, the studies presented in this thesis demonstrate that HEMA can have suppressive as well as enhancing effects on the immune system. However the molecules with which HEMA interacts *in vivo*, that enable HEMA to exert its specific effects, remain to be discovered.

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# References


1. van Noort, R. Introduction to dental materials, Edn. 3rd (Mosby Elsevier, Edinburgh; New York; 2007).

2. Aalto-Korte, K., Alanko, K., Kuuliala, O. & Jolanki, R. Methacrylate and acrylate allergy in dental personnel. *Contact Dermatitis* **57**, 324-330 (2007).

3. Geukens, S. & Goossens, A. Occupational contact allergy to (meth)acrylates. *Contact Dermatitis* **44**, 153-159 (2001).

4. Goon, A.T., Isaksson, M., Zimerson, E., Goh, C.L. & Bruze, M. Contact allergy to (meth)acrylates in the dental series in southern Sweden: simultaneous positive patch test reaction patterns and possible screening allergens. *Contact Dermatitis* **55**, 219-226 (2006).

5. Kanerva, L. *et al.* Increase in occupational skin diseases of dental personnel. *Contact Dermatitis* **40**, 104-108 (1999).

6. Piirila, P. *et al.* Occupational respiratory hypersensitivity in dental personnel. *Int Arch Occup Environ Health* **75**, 209-216 (2002).

7. Piirila, P. *et al.* Occupational respiratory hypersensitivity caused by preparations containing acrylates in dental personnel. *Clin Exp Allergy* **28**, 1404-1411 (1998).

8. Alanko, K., Susitaival, P., Jolanki, R. & Kanerva, L. Occupational skin diseases among dental nurses. *Contact Dermatitis* **50**, 77-82 (2004).

9. Wallenhammar, L.M. *et al.* Contact allergy and hand eczema in Swedish dentists. *Contact Dermatitis* **43**, 192-199 (2000).

10. Wrangsjo, K., Swartling, C. & Meding, B. Occupational dermatitis in dental personnel: contact dermatitis with special reference to (meth)acrylates in 174 patients. *Contact Dermatitis* **45**, 158-163 (2001).

11. Lindstrom, M., Alanko, K., Keskinen, H. & Kanerva, L. Dentist's occupational asthma, rhinoconjunctivitis, and allergic contact dermatitis from methacrylates. *Allergy* **57**, 543-545 (2002).

12. Fonacier, L.S., Dreskin, S.C. & Leung, D.Y. Allergic skin diseases. *J Allergy Clin Immunol* **125**, S138-149 (2010).

13. Basketter, D., Dooms-Goossens, A., Karlberg, A.T. & Lepoittevin, J.P. The chemistry of contact allergy: why is a molecule allergenic? *Contact Dermatitis* **32**, 65-73 (1995).

14. Vocanson, M., Hennino, A., Rozieres, A., Poyet, G. & Nicolas, J.F. Effector and regulatory mechanisms in allergic contact dermatitis. *Allergy* **64**, 1699-1714 (2009).

15. Bour, H. *et al.* Major histocompatibility complex class I-restricted CD8+ T cells and class II-restricted CD4+ T cells, respectively, mediate and regulate contact sensitivity to dinitrofluorobenzene. *Eur J Immunol* **25**, 3006-3010 (1995).

16. Xu, H., DiIulio, N.A. & Fairchild, R.L. T cell populations primed by hapten sensitization in contact sensitivity are distinguished by polarized patterns of cytokine production: interferon gamma-producing (Tc1) effector CD8+ T cells and interleukin (Il) 4/Il-10-producing (Th2) negative regulatory CD4+ T cells. *J Exp Med* **183**, 1001-1012 (1996).

17. Traidl, C. *et al.* Disparate cytotoxic activity of nickel-specific CD8+ and CD4+ T cell subsets against keratinocytes. *J Immunol* **165**, 3058-3064 (2000).

18. Tsuji, R.F. *et al.* B cell-dependent T cell responses: IgM antibodies are required to elicit contact sensitivity. *J Exp Med* **196**, 1277-1290 (2002).

19. Biedermann, T. *et al.* Mast cells control neutrophil recruitment during T cell-mediated delayed-type hypersensitivity reactions through tumor necrosis factor and macrophage inflammatory protein 2. *J Exp Med* **192**, 1441-1452 (2000).

20. Gorbachev, A.V. & Fairchild, R.L. CD4+CD25+ regulatory T cells utilize FasL as a mechanism to restrict DC priming functions in cutaneous immune responses. *Eur J Immunol* **40**, 2006-2015 (2010).

21. Grimbaldeston, M.A., Nakae, S., Kalesnikoff, J., Tsai, M. & Galli, S.J. Mast cell-derived interleukin 10 limits skin pathology in contact dermatitis and chronic irradiation with ultraviolet B. *Nat Immunol* **8**, 1095-1104 (2007).

22. Van Landuyt, K.L. *et al.* Systematic review of the chemical composition of contemporary dental adhesives. *Biomaterials* **28**, 3757-3785 (2007).

23. Doi, J., Itota, T., Torii, Y., Nakabo, S. & Yoshiyama, M. Effect of 2-hydroxyethyl methacrylate pre-treatment on micro-tensile bond strength of resin composite to demineralized dentin. *J Oral Rehabil* **31**, 1061-1067 (2004).

24. Nakabayashi, N. & Takarada, K. Effect of HEMA on bonding to dentin. *Dent Mater* **8**, 125-130 (1992).

25. Allam, J.P. *et al.* Characterization of dendritic cells from human oral mucosa: a new Langerhans' cell type with high constitutive FcepsilonRI expression. *J Allergy Clin Immunol* **112**, 141-148 (2003).

26. Hasseus, B., Jontell, M., Bergenholtz, G. & Dahlgren, U.I. Langerhans cells from human oral epithelium are more effective at stimulating allogeneic T cells *in vitro* than Langerhans cells from skin. *Clin Exp Immunol* **136**, 483-489 (2004).

27. Attstrom, R. Presence of leukocytes in crevices of healthy and chronically inflamed gingivae. *J Periodontal Res* **5**, 42-47 (1970).

28. Wilton, J.M., Renggli, H.H. & Lehner, T. The isolation and identification of mononuclear cells from the gingival crevice in man. *J Periodontal Res* **11**, 262-268 (1976).

29. Delima, A.J. & Van Dyke, T.E. Origin and function of the cellular components in gingival crevice fluid. *Periodontol 2000* **31**, 55-76 (2003).

30. Schroeder, H.E. Transmigration and infiltration of leucocytes in human junctional epithelium. *Helv Odontol Acta* **17**, 6-18 (1973).

31. Jontell, M., Gunraj, M.N. & Bergenholtz, G. Immunocompetent cells in the normal dental pulp. *J Dent Res* **66**, 1149-1153 (1987).

32. Okiji, T., Jontell, M., Belichenko, P., Bergenholtz, G. & Dahlstrom, A. Perivascular dendritic cells of the human dental pulp. *Acta Physiol Scand* **159**, 163-169 (1997).

33. Geurtsen, W., Spahl, W., Muller, K. & Leyhausen, G. Aqueous extracts from dentin adhesives contain cytotoxic chemicals. *J Biomed Mater Res* **48**, 772-777 (1999).

34. Michelsen, V.B., Moe, G., Strom, M.B., Jensen, E. & Lygre, H. Quantitative analysis of TEGDMA and HEMA eluted into saliva from two dental composites by use of GC/MS and tailor-made internal standards. *Dent Mater* **24**, 724-731 (2008).

35. Gerzina, T.M. & Hume, W.R. Effect of hydrostatic pressure on the diffusion of monomers through dentin *in vitro*. *J Dent Res* **74**, 369-373 (1995).

36. Gerzina, T.M. & Hume, W.R. Diffusion of monomers from bonding resin-resin composite combinations through dentine *in vitro*. *J Dent* **24**, 125-128 (1996).

37. Kawahara, T. *et al.* Leachability of plasticizer and residual monomer from commercial temporary restorative resins. *J Dent* **32**, 277-283 (2004).

38. Rathke, A., Alt, A., Gambin, N. & Haller, B. Dentin diffusion of HEMA released from etch-and-rinse and self-etch bonding systems. *Eur J Oral Sci* **115**, 510-516 (2007).

39. Samuelsen, J.T. *et al.* Role of thiol-complex formation in 2-hydroxyethyl-methacrylate-induced toxicity *in vitro*. *J Biomed Mater Res A* **96**, 395-401 (2010).

40. Sandberg, E., Bergenholtz, G., Eklund, C. & Dahlgren, U.I. HEMA bound to self-protein promotes auto-antibody production in mice. *J Dent Res* **81**, 633-636 (2002).

41. Mogensen, T.H. Pathogen recognition and inflammatory signaling in innate immune defenses. *Clin Microbiol Rev* **22**, 240-273 (2009).

42. Girardin, S.E. *et al.* Nod1 detects a unique muropeptide from gram-negative bacterial peptidoglycan. *Science* **300**, 1584-1587 (2003).

43. Girardin, S.E. *et al.* Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. *J Biol Chem* **278**, 8869-8872 (2003).

44. Kato, H. *et al.* Length-dependent recognition of double-stranded ribonucleic acids by retinoic acid-inducible gene-I and melanoma differentiation-associated gene 5. *J Exp Med* **205**, 1601-1610 (2008).

45. Kerrigan, A.M. & Brown, G.D. C-type lectins and phagocytosis. *Immunobiology* **214**, 562-575 (2009).

46. Peiser, L., Gough, P.J., Kodama, T. & Gordon, S. Macrophage class A scavenger receptor-mediated phagocytosis of *Escherichia coli*: role of cell heterogeneity, microbial strain, and culture conditions *in vitro*. *Infect Immun* **68**, 1953-1963 (2000).

47. Schroder, K. & Tschopp, J. The inflammasomes. *Cell* **140**, 821-832 (2010).

48. Thomas, C.A. *et al.* Protection from lethal gram-positive infection by macrophage scavenger receptor-dependent phagocytosis. *J Exp Med* **191**, 147-156 (2000).

49. Allen, L.A. & Aderem, A. Molecular definition of distinct cytoskeletal structures involved in complement- and Fc receptor-mediated phagocytosis in macrophages. *J Exp Med* **184**, 627-637 (1996).

50. Kaplan, G. Differences in the mode of phagocytosis with Fc and C3 receptors in macrophages. *Scand J Immunol* **6**, 797-807 (1977).

51. Bain, B.J. Blood cells: A practical guide, Edn. 3rd. (Blackwell Science, Oxford; 2002).

52. Whitelaw, D.M. Observations on human monocyte kinetics after pulse labeling. *Cell Tissue Kinet* **5**, 311-317 (1972).

53. Passlick, B., Flieger, D. & Ziegler-Heitbrock, H.W. Identification and characterization of a novel monocyte subpopulation in human peripheral blood. *Blood* **74**, 2527-2534 (1989).

54. Serbina, N.V., Jia, T., Hohl, T.M. & Pamer, E.G. Monocyte-mediated defense against microbial pathogens. *Annu Rev Immunol* **26**, 421-452 (2008).

55. Belge, K.U. *et al.* The proinflammatory CD14+CD16+DR++ monocytes are a major source of TNF. *J Immunol* **168**, 3536-3542 (2002).

56. Fingerle, G. *et al.* The novel subset of CD14+/CD16+ blood monocytes is expanded in sepsis patients. *Blood* **82**, 3170-3176 (1993).

57. Thieblemont, N., Weiss, L., Sadeghi, H.M., Estcourt, C. & Haeffner-Cavaillon, N. CD14lowCD16high: a cytokine-producing monocyte subset which expands during human immunodeficiency virus infection. *Eur J Immunol* **25**, 3418-3424 (1995).

58. Ziegler-Heitbrock, H.W. *et al.* The novel subset of CD14+/CD16+ blood monocytes exhibits features of tissue macrophages. *Eur J Immunol* **23**, 2053-2058 (1993).

59. Ginhoux, F. *et al.* Langerhans cells arise from monocytes *in vivo*. *Nat Immunol* **7**, 265-273 (2006).

60. Landsman, L., Varol, C. & Jung, S. Distinct differentiation potential of blood monocyte subsets in the lung. *J Immunol* **178**, 2000-2007 (2007).

61. Leon, B. *et al.* Dendritic cell differentiation potential of mouse monocytes: monocytes represent immediate precursors of CD8- and CD8+ splenic dendritic cells. *Blood* **103**, 2668-2676 (2004).

62. Randolph, G.J., Inaba, K., Robbiani, D.F., Steinman, R.M. & Muller, W.A. Differentiation of phagocytic monocytes into lymph node dendritic cells *in vivo*. *Immunity* **11**, 753-761 (1999).

63. Varol, C. *et al.* Monocytes give rise to mucosal, but not splenic, conventional dendritic cells. *J Exp Med* **204**, 171-180 (2007).

64. Cowburn, A.S., Condliffe, A.M., Farahi, N., Summers, C. & Chilvers, E.R. Advances in neutrophil biology: clinical implications. *Chest* **134**, 606-612 (2008).

65. Borregaard, N. Neutrophils, from marrow to microbes. *Immunity* **33**, 657-670 (2010).

66. Dale, D.C., Boxer, L. & Liles, W.C. The phagocytes: neutrophils and monocytes. *Blood* **112**, 935-945 (2008).

67. Faurschou, M. & Borregaard, N. Neutrophil granules and secretory vesicles in inflammation. *Microbes Infect* **5**, 1317-1327 (2003).

68. Detmers, P.A. *et al.* Endotoxin receptors (CD14) are found with CD16 (Fc gamma RIII) in an intracellular compartment of neutrophils that contains alkaline phosphatase. *J Immunol* **155**, 2085-2095 (1995).

69. Sengelov, H., Boulay, F., Kjeldsen, L. & Borregaard, N. Subcellular localization and translocation of the receptor for N-formylmethionyl-leucyl-phenylalanine in human neutrophils. *Biochem J* **299 ( Pt 2)**, 473-479 (1994).

70. Nathan, C. Neutrophils and immunity: challenges and opportunities. *Nat Rev Immunol* **6**, 173-182 (2006).

71. Fleming, A. Lysozyme: President's Address. *Proc R Soc Med* **26**, 71-84 (1932).

72. Turner, J., Cho, Y., Dinh, N.N., Waring, A.J. & Lehrer, R.I. Activities of LL-37, a cathelin-associated antimicrobial peptide of human neutrophils. *Antimicrob Agents Chemother* **42**, 2206-2214 (1998).

73. Wiesner, J. & Vilcinskas, A. Antimicrobial peptides: the ancient arm of the human immune system. *Virulence* **1**, 440-464 (2010).

74. Wimley, W.C., Selsted, M.E. & White, S.H. Interactions between human defensins and lipid bilayers: evidence for formation of multimeric pores. *Protein Sci* **3**, 1362-1373 (1994).

75. Papayannopoulos, V., Metzler, K.D., Hakkim, A. & Zychlinsky, A. Neutrophil elastase and myeloperoxidase regulate the formation of neutrophil extracellular traps. *J Cell Biol* **191**, 677-691 (2010).

76. Chertov, O. *et al.* Identification of defensin-1, defensin-2, and CAP37/azurocidin as T-cell chemoattractant proteins released from interleukin-8-stimulated neutrophils. *J Biol Chem* **271**, 2935-2940 (1996).

77. Chertov, O. *et al.* Identification of human neutrophil-derived cathepsin G and azurocidin/CAP37 as chemoattractants for mononuclear cells and neutrophils. *J Exp Med* **186**, 739-747 (1997).

78. De, Y. *et al.* LL-37, the neutrophil granule- and epithelial cell-derived cathelicidin, utilizes formyl peptide receptor-like 1 (FPRL1) as a receptor to chemoattract human peripheral blood neutrophils, monocytes, and T cells. *J Exp Med* **192**, 1069-1074 (2000).

79. Fadok, V.A. *et al.* A receptor for phosphatidylserine-specific clearance of apoptotic cells. *Nature* **405**, 85-90 (2000).

80. Nakanishi, Y., Henson, P.M. & Shiratsuchi, A. Pattern recognition in phagocytic clearance of altered self. *Adv Exp Med Biol* **653**, 129-138 (2009).

81. Flannagan, R.S., Cosio, G. & Grinstein, S. Antimicrobial mechanisms of phagocytes and bacterial evasion strategies. *Nat Rev Microbiol* **7**, 355-366 (2009).

82. Desjardins, M., Huber, L.A., Parton, R.G. & Griffiths, G. Biogenesis of phagolysosomes proceeds through a sequential series of interactions with the endocytic apparatus. *J Cell Biol* **124**, 677-688 (1994).

83. Cech, P. & Lehrer, R.I. Phagolysosomal pH of human neutrophils. *Blood* **63**, 88-95 (1984).

84. Jensen, M.S. & Bainton, D.F. Temporal changes in pH within the phagocytic vacuole of the polymorphonuclear neutrophilic leukocyte. *J Cell Biol* **56**, 379-388 (1973).

85. Segal, A.W., Dorling, J. & Coade, S. Kinetics of fusion of the cytoplasmic granules with phagocytic vacuoles in human polymorphonuclear leukocytes. Biochemical and morphological studies. *J Cell Biol* **85**, 42-59 (1980).

86. Lam, G.Y., Huang, J. & Brumell, J.H. The many roles of NOX2 NADPH oxidase-derived ROS in immunity. *Semin Immunopathol* **32**, 415-430 (2010).

87. Abo, A., Webb, M.R., Grogan, A. & Segal, A.W. Activation of NADPH oxidase involves the dissociation of p21rac from its inhibitory GDP/GTP exchange protein (rhoGDI) followed by its translocation to the plasma membrane. *Biochem J* **298 Pt 3**, 585-591 (1994).

88. Borregaard, N., Heiple, J.M., Simons, E.R. & Clark, R.A. Subcellular localization of the b-cytochrome component of the human neutrophil microbicidal oxidase: translocation during activation. *J Cell Biol* **97**, 52-61 (1983).

89. DeLeo, F.R., Allen, L.A., Apicella, M. & Nauseef, W.M. NADPH oxidase activation and assembly during phagocytosis. *J Immunol* **163**, 6732-6740 (1999).

90. El-Benna, J., Dang, P.M. & Gougerot-Pocidalo, M.A. Priming of the neutrophil NADPH oxidase activation: role of p47phox phosphorylation and NOX2 mobilization to the plasma membrane. *Semin Immunopathol* **30**, 279-289 (2008).

91. Busetto, S., Trevisan, E., Decleva, E., Dri, P. & Menegazzi, R. Chloride movements in human neutrophils during phagocytosis: characterization and relationship to granule release. *J Immunol* **179**, 4110-4124 (2007).

92. Menegazzi, R. *et al.* Triggering of chloride ion efflux from human neutrophils as a novel function of leukocyte beta 2 integrins: relationship with spreading and activation of the respiratory burst. *J Immunol* **162**, 423-434 (1999).

93. Fang, F.C. Antimicrobial reactive oxygen and nitrogen species: concepts and controversies. *Nat Rev Microbiol* **2**, 820-832 (2004).

94. Nocca, G. *et al.* Alteration of monocytic cell oxidative burst caused by methacrylic monomers present in dental materials: a chemiluminescence study. *Luminescence* **21**, 202-206 (2006).

95. Heil, T.L., Volkmann, K.R., Wataha, J.C. & Lockwood, P.E. Human peripheral blood monocytes versus THP-1 monocytes for *in vitro* biocompatibility testing of dental material components. *J Oral Rehabil* **29**, 401-407 (2002).

96. Noda, M. *et al.* Sublethal, 2-week exposures of dental material components alter TNF-alpha secretion of THP-1 monocytes. *Dent Mater* **19**, 101-105 (2003).

97. Rakich, D.R., Wataha, J.C., Lefebvre, C.A. & Weller, R.N. Effect of dentin bonding agents on the secretion of inflammatory mediators from macrophages. *J Endod* **25**, 114-117 (1999).

98. Bouillaguet, S. *et al.* Effect of sub-lethal concentrations of HEMA (2-hydroxyethyl methacrylate) on THP-1 human monocyte-macrophages, *in vitro*. *Dent Mater* **16**, 213-217 (2000).

99. Noda, M. *et al.* Components of dentinal adhesives modulate heat shock protein 72 expression in heat-stressed THP-1 human monocytes at sublethal concentrations. *J Dent Res* **81**, 265-269 (2002).

100. Nocca, G. *et al.* Alterations of energy metabolism and glutathione levels of HL-60 cells induced by methacrylates present in composite resins. *J Dent* **35**, 187-194 (2007).

101. Noda, M. *et al.* Dental adhesive compounds alter glutathione levels but not glutathione redox balance in human THP-1 monocytic cells. *J Biomed Mater Res B Appl Biomater* **73**, 308-314 (2005).

102. Mantellini, M.G. *et al.* Adhesive resin and the hydrophilic monomer HEMA induce VEGF expression on dental pulp cells and macrophages. *Dent Mater* **22**, 434-440 (2006).

103. Smetana, K., Jr., Bartunkova, J., Gabius, H.J., Cebecauer, M. & Vancova, E. Effect of phagocytosis of pHEMA particles and of heat-killed *Candida albicans* on expression of carbohydrate-binding sites such as endogenous lectins in phagocytes. *Biomaterials* **17**, 741-744 (1996).

104. Lee, D.H., Kim, N.R., Lim, B.S., Lee, Y.K. & Yang, H.C. Effects of TEGDMA and HEMA on the expression of COX-2 and iNOS in cultured murine macrophage cells. *Dent Mater* **25**, 240-246 (2009).

105. Becher, R. *et al.* Pattern of cell death after *in vitro* exposure to GDMA, TEGDMA, HEMA and two compomer extracts. *Dent Mater* **22**, 630-640 (2006).

106. Paranjpe, A., Bordador, L.C., Wang, M.Y., Hume, W.R. & Jewett, A. Resin monomer 2-hydroxyethyl methacrylate (HEMA) is a potent inducer of apoptotic cell death in human and mouse cells. *J Dent Res* **84**, 172-177 (2005).

107. Abbas, A.K., Lichtman, A.H. & Pillai, S. Cellular and Molecular Immunology, Edn. 6th. (Saunders, Elsevier, Philadelphia; 2007).

108. LeBien, T.W. & Tedder, T.F. B lymphocytes: how they develop and function. *Blood* **112**, 1570-1580 (2008).

109. Pillai, S. & Cariappa, A. The follicular versus marginal zone B lymphocyte cell fate decision. *Nat Rev Immunol* **9**, 767-777 (2009).

110. Martin, F., Oliver, A.M. & Kearney, J.F. Marginal zone and B1 B cells unite in the early response against T-independent blood-borne particulate antigens. *Immunity* **14**, 617-629 (2001).

111. Allman, D. & Pillai, S. Peripheral B cell subsets. *Curr Opin Immunol* **20**, 149-157 (2008).

112. Cariappa, A. *et al.* Perisinusoidal B cells in the bone marrow participate in T-independent responses to blood-borne microbes. *Immunity* **23**, 397-407 (2005).

113. Batista, F.D. & Harwood, N.E. The who, how and where of antigen presentation to B cells. *Nat Rev Immunol* **9**, 15-27 (2009).

114. Junt, T. *et al.* Subcapsular sinus macrophages in lymph nodes clear lymph-borne viruses and present them to antiviral B cells. *Nature* **450**, 110-114 (2007).

115. Qi, H., Egen, J.G., Huang, A.Y. & Germain, R.N. Extrafollicular activation of lymph node B cells by antigen-bearing dendritic cells. *Science* **312**, 1672-1676 (2006).

116. Wykes, M., Pombo, A., Jenkins, C. & MacPherson, G.G. Dendritic cells interact directly with naive B lymphocytes to transfer antigen and initiate class switching in a primary T-dependent response. *J Immunol* **161**, 1313-1319 (1998).

117. Gonzalez, S.F., Pitcher, L.A., Mempel, T., Schuerpf, F. & Carroll, M.C. B cell acquisition of antigen *in vivo*. *Curr Opin Immunol* **21**, 251-257 (2009).

118. Suzuki, K., Grigorova, I., Phan, T.G., Kelly, L.M. & Cyster, J.G. Visualizing B cell capture of cognate antigen from follicular dendritic cells. *J Exp Med* **206**, 1485-1493 (2009).

119. Pape, K.A., Catron, D.M., Itano, A.A. & Jenkins, M.K. The humoral immune response is initiated in lymph nodes by B cells that acquire soluble antigen directly in the follicles. *Immunity* **26**, 491-502 (2007).

120. Hewitt, S.L., Chaumeil, J. & Skok, J.A. Chromosome dynamics and the regulation of V(D)J recombination. *Immunol Rev* **237**, 43-54 (2010).

121. Klein, U. & Dalla-Favera, R. Germinal centres: role in B-cell physiology and malignancy. *Nat Rev Immunol* **8**, 22-33 (2008).

122. Allen, C.D., Okada, T., Tang, H.L. & Cyster, J.G. Imaging of germinal center selection events during affinity maturation. *Science* **315**, 528-531 (2007).

123. Blink, E.J. *et al.* Early appearance of germinal center-derived memory B cells and plasma cells in blood after primary immunization. *J Exp Med* **201**, 545-554 (2005).

124. Kurosaki, T., Aiba, Y., Kometani, K., Moriyama, S. & Takahashi, Y. Unique properties of memory B cells of different isotypes. *Immunol Rev* **237**, 104-116 (2010).

125. Manz, R.A., Lohning, M., Cassese, G., Thiel, A. & Radbruch, A. Survival of long-lived plasma cells is independent of antigen. *Int Immunol* **10**, 1703-1711 (1998).

126. Oracki, S.A., Walker, J.A., Hibbs, M.L., Corcoran, L.M. & Tarlinton, D.M. Plasma cell development and survival. *Immunol Rev* **237**, 140-159 (2010).

127. Schroeder, H.W., Jr. & Cavacini, L. Structure and function of immunoglobulins. *J Allergy Clin Immunol* **125**, S41-52 (2010).

128. Grey, H.M. & Kunkel, H.G. Heavy-chain subclasses of human gamma-G-globulin. Peptide and immunochemical relationships. *Biochemistry* **6**, 2326-2334 (1967).

129. Mestas, J. & Hughes, C.C. Of mice and not men: differences between mouse and human immunology. *J Immunol* **172**, 2731-2738 (2004).

130. Preud'homme, J.L. *et al.* Structural and functional properties of membrane and secreted IgD. *Mol Immunol* **37**, 871-887 (2000).

131. Milstein, C. Chemical structure of light chains. *Proc R Soc Lond B Biol Sci* **166**, 138-149 (1966).

132. Valentine, R.C. & Green, N.M. Electron microscopy of an antibody-hapten complex. *J Mol Biol* **27**, 615-617 (1967).

133. Edelman, G.M. & Poulik, M.D. Studies on structural units of the gamma-globulins. *J Exp Med* **113**, 861-884 (1961).

134. Fleischman, J.B., Porter, R.R. & Press, E.M. THE ARRANGEMENT OF THE PEPTIDE CHAINS IN GAMMA-GLOBULIN. *Biochem J* **88**, 220-228 (1963).

135. Press, E.M., Givol, D., Piggot, P.J., Porter, R.R. & Wilkinson, J.M. The chemical structure of the heavy chains of rabbit and human immunoglobulin G (IgG). *Proc R Soc Lond B Biol Sci* **166**, 150-158 (1966).

136. Porter, R.R. Separation and isolation of fractions of rabbit gamma-globulin containing the antibody and antigenic combining sites. *Nature* **182**, 670-671 (1958).

137. Braden, B.C. *et al.* Structure and thermodynamics of antigen recognition by antibodies. *Ann N Y Acad Sci* **764**, 315-327 (1995).

138. Xu, J.L. & Davis, M.M. Diversity in the CDR3 region of V(H) is sufficient for most antibody specificities. *Immunity* **13**, 37-45 (2000).

139. Lux, A., Aschermann, S., Biburger, M. & Nimmerjahn, F. The pro and anti-inflammatory activities of immunoglobulin G. *Ann Rheum Dis* **69 Suppl 1**, i92-96 (2010).

140. Peng, Y., Kowalewski, R., Kim, S. & Elkon, K.B. The role of IgM antibodies in the recognition and clearance of apoptotic cells. *Mol Immunol* **42**, 781-787 (2005).

141. Ravetch, J.V. & Bolland, S. IgG Fc receptors. *Annu Rev Immunol* **19**, 275-290 (2001).

142. Monteiro, R.C. Role of IgA and IgA fc receptors in inflammation. *J Clin Immunol* **30**, 1-9 (2010).

143. Wilton, J.M. Suppression by IgA of IgG-mediated phagocytosis by human polymorphonuclear leucocytes. *Clin Exp Immunol* **34**, 423-428 (1978).

144. Barral, P. *et al.* B cell receptor-mediated uptake of CD1d-restricted antigen augments antibody responses by recruiting invariant NKT cell help *in vivo*. *Proc Natl Acad Sci U S A* **105**, 8345-8350 (2008).

145. Fehr, T. *et al.* T-cell independent IgM and enduring protective IgG antibodies induced by chimeric measles viruses. *Nat Med* **4**, 945-948 (1998).

146. Minguet, S. *et al.* Enhanced B-cell activation mediated by TLR4 and BCR crosstalk. *Eur J Immunol* **38**, 2475-2487 (2008).

147. Posadas, S.J. & Pichler, W.J. Delayed drug hypersensitivity reactions - new concepts. *Clin Exp Allergy* **37**, 989-999 (2007).

148. Shimizu, T. *et al.* T cells specific to hapten carrier but not to carrier alone assist in the production of anti-hapten and anti-carrier antibodies. *Int Immunol* **19**, 1157-1164 (2007).

149. Ueda, A., Chandswang, N. & Ovary, Z. The action of interleukin-4 on antigen-specific IgG1 and IgE production by interaction of *in vivo* primed B cells and carrier-specific cloned Th2 cells. *Cell Immunol* **128**, 31-40 (1990).

150. Palm, N.W. & Medzhitov, R. Immunostimulatory activity of haptenated proteins. *Proc Natl Acad Sci U S A* **106**, 4782-4787 (2009).

151. Li, Q., Rodriguez, L.G., Farnsworth, D.F. & Gildersleeve, J.C. Effects of hapten density on the induced antibody repertoire. *Chembiochem* **11**, 1686-1691 (2010).

152. Sandberg, E., Bergenholtz, G., Kahu, H. & Dahlgren, U.I. Low HEMA conjugation induces high autoantibody titer in mice. *J Dent Res* **84**, 537-541 (2005).

153. Sandberg, E., Kahu, H. & Dahlgren, U.I. Inflammatogenic and adjuvant properties of HEMA in mice. *Eur J Oral Sci* **113**, 410-416 (2005).

154. Crow, J.P. Dichlorodihydrofluorescein and dihydrorhodamine 123 are sensitive indicators of peroxynitrite *in vitro*: implications for intracellular measurement of reactive nitrogen and oxygen species. *Nitric Oxide* **1**, 145-157 (1997).

155. Rothe, G., Oser, A. & Valet, G. Dihydrorhodamine 123: a new flow cytometric indicator for respiratory burst activity in neutrophil granulocytes. *Naturwissenschaften* **75**, 354-355 (1988).

156. King, M.A. Detection of dead cells and measurement of cell killing by flow cytometry. *J Immunol Methods* **243**, 155-166 (2000).

157. Krysko, D.V., Vanden Berghe, T., D'Herde, K. & Vandenabeele, P. Apoptosis and necrosis: detection, discrimination and phagocytosis. *Methods* **44**, 205-221 (2008).

158. Mellstedt, H. *In vitro* activation of human T and B lymphocytes by pokeweed mitogen. *Clin Exp Immunol* **19**, 75-82 (1975).

159. Morrison, D.C. & Jacobs, D.M. Binding of polymyxin B to the lipid A portion of bacterial lipopolysaccharides. *Immunochemistry* **13**, 813-818 (1976).

160. Nakamura, T., Morita, T. & Iwanaga, S. Lipopolysaccharide-sensitive serine-protease zymogen (factor C) found in *Limulus* hemocytes. Isolation and characterization. *Eur J Biochem* **154**, 511-521 (1986).

161. Kanellopoulos, J.M., De Petris, S., Leca, G. & Crumpton, M.J. The mitogenic lectin from *Phaseolus vulgaris* does not recognize the T3 antigen of human T lymphocytes. *Eur J Immunol* **15**, 479-486 (1985).

162. Samuelsen, J.T., Dahl, J.E., Karlsson, S., Morisbak, E. & Becher, R. Apoptosis induced by the monomers HEMA and TEGDMA involves formation of ROS and differential activation of the MAP-kinases p38, JNK and ERK. *Dent Mater* **23**, 34-39 (2007).

163. Segal, A.W. How neutrophils kill microbes. *Annu Rev Immunol* **23**, 197-223 (2005).

164. Nespoli, L., Vitiello, A., Maccario, R., Lanzavecchia, A. & Ugazio, A.G. Polyclonal activation of human B lymphocytes *in vitro* by pokeweed mitogen: a simple technique for the simultaneous assessment of cell proliferation, generation of plasma cells, plaque-forming cells and immunoglobulin production. *Scand J Immunol* **8**, 489-496 (1978).

165. Agger, R., Petersen, J., Dinesen, B., Wiik, A. & Andersen, V. Production and secretion of immunoglobulins by *in vitro*-activated human B lymphocytes. *Allergy* **37**, 179-185 (1982).

166. Hagberg, S., Ljungkvist, G., Andreasson, H., Karlsson, S. & Barregard, L. Exposure to volatile methacrylates in dental personnel. *J Occup Environ Hyg* **2**, 302-306 (2005).

167. Henriks-Eckerman, M.L., Alanko, K., Jolanki, R., Kerosuo, H. & Kanerva, L. Exposure to airborne methacrylates and natural rubber latex allergens in dental clinics. *J Environ Monit* **3**, 302-305 (2001).

168. Sandberg, E. & Dahlgren, U.I. Application of HEMA on intact mouse skin--effects on the immune system. *Contact Dermatitis* **54**, 186-191 (2006).

169. Schuster, G.S., Caughman, G.B. & Rueggeberg, F.A. Changes in cell phospholipid metabolism *in vitro* in the presence of HEMA and its degradation products. *Dent Mater* **16**, 297-302 (2000).

170. Nocca, G. *et al.* Effect of methacrylic monomers on phagocytes reactive oxygen species: a possible BDDMA modulating action. *Luminescence* **23**, 54-57 (2008).

171. Cassatella, M.A., Della Bianca, V., Berton, G. & Rossi, F. Activation by gamma interferon of human macrophage capability to produce toxic oxygen molecules is accompanied by decreased Km of the superoxide-generating NADPH oxidase. *Biochem Biophys Res Commun* **132**, 908-914 (1985).

172. Doherty, T.M. T-cell regulation of macrophage function. *Curr Opin Immunol* **7**, 400-404 (1995).

173. Jones, O.T.G., Jones, S.A. & Wood, J.D. Expression of components of the superoxide generating NADPH oxidase by human leucocytes and other cells *Protoplasma* **184**, 79-85 (1995).

174. Capasso, M. *et al.* HVCN1 modulates BCR signal strength via regulation of BCR-dependent generation of reactive oxygen species. *Nat Immunol* **11**, 265-272 (2010).

175. Vene, R. *et al.* Redox remodeling allows and controls B-cell activation and differentiation. *Antioxid Redox Signal* **13**, 1145-1155 (2010).

176. Stavnezer, J., Guikema, J.E. & Schrader, C.E. Mechanism and regulation of class switch recombination. *Annu Rev Immunol* **26**, 261-292 (2008).

177. Brieva, J.A., Roldan, E., Rodriguez, C. & Navas, G. Human tonsil, blood and bone marrow *in vivo*-induced B cells capable of spontaneous and high-rate immunoglobulin secretion *in vitro*: differences in the requirements for factors and for adherent and bone marrow stromal cells, as well as distinctive adhesion molecule expression. *Eur J Immunol* **24**, 362-366 (1994).

178. Rieckmann, P., D'Alessandro, F., Nordan, R.P., Fauci, A.S. & Kehrl, J.H. IL-6 and tumor necrosis factor-alpha. Autocrine and paracrine cytokines involved in B cell function. *J Immunol* **146**, 3462-3468 (1991).

179. Burdin, N. *et al.* Endogenous IL-6 and IL-10 contribute to the differentiation of CD40-activated human B lymphocytes. *J Immunol* **154**, 2533-2544 (1995).

180. Muraguchi, A. *et al.* The essential role of B cell stimulatory factor 2 (BSF-2/IL-6) for the terminal differentiation of B cells. *J Exp Med* **167**, 332-344 (1988).

181. Letourneau, S., Krieg, C., Pantaleo, G. & Boyman, O. IL-2- and CD25-dependent immunoregulatory mechanisms in the homeostasis of T-cell subsets. *J Allergy Clin Immunol* **123**, 758-762 (2009).

182. Granchi, D. *et al.* Effects of bone cement extracts on the cell-mediated immune response. *Biomaterials* **23**, 1033-1041 (2002).

183. Park, S.R., Lee, J.H. & Kim, P.H. Smad3 and Smad4 mediate transforming growth factor-beta1-induced IgA expression in murine B lymphocytes. *Eur J Immunol* **31**, 1706-1715 (2001).

184. Ma, G.K. *et al.* Polymethylmethacrylate particle exposure causes changes in p38 MAPK and TGF-beta signaling in differentiating MC3T3-E1 cells. *J Biomed Mater Res A* **94**, 234-240 (2010).

185. Smith Pease, C.K., Basketter, D.A. & Patlewicz, G.Y. Contact allergy: the role of skin chemistry and metabolism. *Clin Exp Dermatol* **28**, 177-183 (2003).

186. Li, C. *et al.* Activation of p38 mitogen-activated protein kinase is critical step for acquisition of effector function in cytokine-activated T cells, but acts as a negative regulator in T cells activated through the T-cell receptor. *Immunology* **132**, 104-110 (2011).

187. Commins, S.P., Borish, L. & Steinke, J.W. Immunologic messenger molecules: cytokines, interferons, and chemokines. *J Allergy Clin Immunol* **125**, S53-72 (2010).