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The role of pathogen-associated molecular patterns in inflammatory responses against alginate based microcapsules

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ABSTRACT

Alginate-based microcapsules are used for immunoisolation of cells to release therapeutics on a minute-to-minute basis. Unfortunately, alginate-based microcapsules are suffering from varying degrees of success, which is usually attributed to differences in tissue responses. This results in failure of the therapeutic cells. In the present study we show that commercial, crude alginates may contain pathogen-associated molecular patterns (PAMPs), which are recognized by the sensors of the innate immune system. Known sensors are Toll-like receptors (TLRs), NOD receptors, and C-type lectins. By using cell-lines with a non-functional adaptor molecule essential in Toll-like receptor signaling, *i.e.* MyD88, we were able to show that alginates signal mainly *via* MyD88. This was found for low-G, intermediate-G, and high-G alginates applied in calcium-beads, barium-beads as well as in alginate–PLL–alginate capsules. These alginates did stimulate TLRs 2, 5, 8, and 9 but not TLR4 (LPS receptor). Upon implantation in rats these alginates provoked a strong inflammatory response resulting in fibrosis of the capsules. Analysis demonstrated that commercial alginates contain the PAMPs peptidoglycan, lipoteichoic acid, and flagellin. By applying purification procedures, these PAMPs were largely removed. This was associated with deletion of the inflammatory tissue responses as confirmed by an implantation experiment in rats. Our data also show that alginate itself does not provoke TLR mediated responses. We were able to unravel the sensor mechanism by which contaminants in alginates may provoke inflammatory responses.

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1. Introduction

Immunoisolation by encapsulation of therapeutic cells has been proposed as a potential therapy for a variety of diseases [1–4] in which regulation of metabolites on a minute-to-minute basis is required. The most commonly applied procedure for immunoisolation is encapsulation of tissues in alginate-based capsules as originally described by Lim and Sun [5]. Alginate, the main component of the capsule, is a polysaccharide composed of different amounts of mannuronic acid (M-chains) and guluronic acid (G-chains) linked in blocks (MM-blocks, GG-blocks and MG-blocks). Binding the molecules with multi-valent cations, such as Ca^{2+} or Ba^{2+}, forms alginate beads [6]. These beads can subsequently be coated with polyaminoacids such as poly-L-lysine to form capsules [7]. Capsules with specific physical and chemical properties [1,8] can be obtained by varying the type and concentration of the cations and the polyaminoacids. During recent years, important advances have been made with this technology. New capsule types have been developed and tested [9,10]. Human trials have been started which have shown the principle applicability of microencapsulation for the treatment of thyroid disorders [11] and diabetes type 1 [12,13]. Although successful, these studies have also shown a major hurdle that has to be overcome. Graft survival was never permanent and varied considerably from several days to months [14]. This variation in survival rate is considered to be the consequence of differences in the tissue responses (*i.e.* biocompatibility) against the applied capsules.

Many have pointed towards impurities in alginate as the major cause of the variations in success of the capsules [15–17]. Purification of alginate has been reported to reduce or delete the responses but many groups have difficulties in reproducibly producing ultrapure alginates [6,18,19]. Surprisingly, in spite of a decade of intensive research, not many have reported on the molecules that have to be removed from the alginate. Also up to now, there are to the best of our knowledge no reports on the mechanisms by which impurities contribute to inflammatory responses. We hypothesize that pathogen-associated molecular patterns (PAMPs) might be impurities present in the alginates and a dominant factor in inducing inflammatory responses following implantation of encapsulated tissues or cells. PAMPs are small molecular motifs found on groups of pathogens. PAMPs are recognized by Toll-like receptors (TLRs) and other pattern recognition receptors (PRRs) on cells of the innate immune system [20]. PAMPs activate the innate immune response with the aim to protect the host from infections by deletion of pathogenic bacteria. Lipopolysaccharide (LPS), an endotoxin found on the bacterial cell membrane, is a classical example of a PAMP. LPS binds to TLR4 and subsequently activates the immune system [21]. There are many more examples of PAMPs that bind to

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different types of PRRs that might be involved in responses against alginate-based capsules.

To test the hypothesis of the involvement of PAMPs in responses against alginate based capsules a number of specific experiments were performed. First we implanted capsules composed of three different types of alginates in rats to study the tissue response against the capsules. Next we studied whether these different types of alginates were recognized by PRRs and whether these PRRs were TLRs followed by the identification of which TLRs are involved. In these experiments, next to the alginates in solution, alginates were also tested in three commonly applied encapsulation systems *i.e.* calcium-beads, barium-beads, and alginate–poly-L-lysine–alginate (APA) capsules. Finally we determined which PAMPs could be found in alginates and have to be held responsible for the responses against alginates. This was done *in vitro* and *in vivo*. Our results provide new insight in the mechanisms of responses against alginate based capsules and contribute to a better understanding of which components should be removed from alginate to reproducibly produce ultra-pure alginates and prevent inflammatory responses against alginate-based capsules.

2. Materials and methods

2.1. Graft recipients

Male inbred Albino Oxford (AO/G) rats served as recipients of capsules and were obtained from the Central Animal Laboratory of Groningen. Their body weights ranged from 300 to 350 g. NIH guidelines for the care and use of laboratory animals have been observed. All experiments were approved by the Groningen ethical commission.

2.2. Chemicals

Alginates of different compositions were obtained from ISP Alginates Ltd UK. Three types of alginates have been applied: low-G (21% G-chains, 79% M-chains, 15% GG-chains, 14% GM-chains, 57% MM-chains) [22], 2) intermediate-G (44% G-chains, 56% M-chains, 23% GG-chains, 21% GM-chains, 37% MM-chains) and 3) high-G (67% G-chains, 33% M-chains, 54% GG-chains, 13% GM-chains, 21% MM-chains). Alginates were being applied in its crude form and purified form. The composition of alginate was studied by nuclear magnetic resonance (NMR).

A 0.1% solution of poly-L-lysine (PLL) (poly-L-lysine-HCl, M_w 22 kDa, Sigma-Aldrich, The Netherlands) was sterilized by 0.2 μm filtration (Corning®, NY, USA).

QUANTI-Blue™ (InvivoGen, Toulouse, France) is a medium with a colorimetric enzyme used to detect activity of any alkaline phosphatase. QUANTI-Blue™ medium turns purple-blue color in the presence of alkaline phosphatase (SEAP) and can be quantified using a spectrophotometer at 620–655 nm.

2.3. Purification of the alginates

For purification, the 3 types of crude sodium alginate were dissolved at 4 °C in a 1 mM sodium EGTA solution to a 1% solution under constant stirring. Subsequently the solutions were filtered over successively 5.0, 1.2, 0.8, and 0.45 μm filters (Whatman®, Dassel, Germany). During this filtration step all visible aggregates were removed.

Next, the pH of the solution was lowered to 3.5 by addition of 2 N HCl + 20 mM NaCl. The solution was kept on ice to prevent hydrolysis of alginate. The next step was slow lowering of the pH from 3.5 to 2, which was associated with gradual precipitation of alginate as alginic acid [23]. Routinely, the solutions were brought at a pH of 2.0 and subsequently filtered over a Buchner funnel (pore size 1.5 mm) to wash out non-precipitated contaminants. To extend the washout of non-precipitated contaminants, the precipitate was brought in 0.01 N HCl + 20 mM NaCl, vigorously shaken, and filtered again over the Buchner funnel. This washing procedure was performed three times.

Then, proteins were removed by extraction with chloroform/butanol [24]. The alginic acid was suspended in 100 ml of 0.01 N HCl + 20 mM NaCl and supplemented with 20 ml chloroform and 5 ml 1-butanol. The mixture was vigorously shaken for 30 min and filtered over the Buchner funnel. This chloroform/butanol extraction was performed three times. Next, the alginic acid was brought in water and slowly dissolved by gradually raising the pH to 7.0 by slow addition of 0.5 N NaOH + 20 mM NaCl over a period of at least 1 h. The alginate solution obtained was subjected to a chloroform/butanol extraction to remove those proteins which can only be dissolved in chloroform/butanol at neutral pH [24]. The solution was vigorously shaken in a mixture of chloroform (20 ml at each 100 ml alginate solution) and 1-butanol (5 ml at each 100 ml alginate solution) for 30 min. The mixture was centrifuged for 3–5 min at 1800 rpm, which induced the formation of a separate chloroform/butanol phase, and was removed by aspiration. The extraction was repeated once.

The last step was precipitation of the alginate with ethanol [23]. To each 100 ml of alginate solution we added 200 ml absolute ethanol. After an incubation period of 10 min all alginate had precipitated. The alginate was filtered over the Buchner funnel and washed two times with absolute ethanol. Subsequently, the alginate was washed three times with diethyl ether. Finally, the alginate was freeze-dried overnight.

2.4. Production of microcapsules

Purified or crude alginates were dissolved at 4 °C in Krebs–Ringer–Hepes (KRH) with an appropriate osmolarity. In order to produce capsules with a similar mechanical stability we tested low-G, intermediate-G and high-G capsules from alginate solution with a different concentration (2.9, 4, and 3% respectively). For production of beads/capsules we could not apply solutions higher than the concentration indicated as this produced solutions with a viscosity above 4 cps, which is above the 0.2 μm filtration limit. This latter filtration step is required for sterilization of alginate.

The alginate solution was converted into droplets using an air-driven droplet generator as previously described [15]. Three types of beads/capsules were produced: (i) calcium-beads were formed by collecting alginate droplets in a 100 mM $CaCl_2$ (10 mM HEPES, 2 mM KCl) solution for at least 5 min. (ii) Barium-beads were formed by collecting alginate droplets in a 10 mM $BaCl_2$ solution for at least 5 min. After gelification, calcium and barium beads were washed with Krebs–Ringer–Hepes (KRH) and stored for further processing. (iii) Alginate–poly-L-lysine–alginate (APA) capsules were formed by collecting alginate droplets in 100 mM $CaCl_2$ (10 mM HEPES, 2 mM KCl) solution. After 5 to 10 min of gelification the calcium–alginate beads were suspended for 1 min in KRH containing 2.5 mM $CaCl_2$. A poly-L-lysine (PLL) membrane was formed by suspending the alginate beads in 0.1% PLL solution for 10 min. Non-bound PLL was removed by three successive washings during 3 min with calcium-free Krebs–Ringer–Hepes containing 135 mM NaCl. The outer alginate-layer was subsequently applied by 5 min incubation in ten times diluted alginate solution. The diameters of capsules and beads were measured with a dissection microscope (Leica $M77_5$, Germany) equipped with an ocular micrometer with an accuracy of 25 μm. The capsules had a diameter of 650 μm. All procedures were performed under sterile conditions [25].

2.5. Implantation and explantation of empty capsules

Capsules were injected into the peritoneal cavity with a 16 G cannula *via* a small incision (3 mm) in the linea alba. The abdomen was closed with a two-layer suture. The implanted volume was always 2.0 ml as assessed in a syringe with appropriate measure.

To study the tissue response we did subject the animals to laparotomy. The microcapsules were retrieved by peritoneal lavage. Peritoneal lavage was performed by infusing 5 ml KRH through a 3 mm midline incision into the peritoneal cavity and subsequent aspiration of the KRH

containing the capsules. Microcapsules were either freely floating and non-adherent, or adherent to the surface of abdominal organs. First, non-adherent microcapsules were retrieved by peritoneal lavage, and brought into a syringe with appropriate measures for quantification of the retrieval rate [26]. Subsequently, the microcapsules adherent to the surface of abdominal organs, were excised and processed for histology.

All surgical procedures were performed under fluothane/oxygen anesthesia.

2.6. Microscopy

To assess the integrity of capsules before implantation, samples of capsules were meticulously inspected for the presence of irregularities or broken parts in the capsule membranes by using a dissection microscope.

To detect physical imperfections and to assess the composition and degree of overgrowth after implantation, samples of adherent capsules recovered by excision and samples of non-adherent capsules were fixed in pre-cooled 2% paraformaldehyde, buffered with 0.05 M phosphate in saline (pH 7.4), and processed for glycol methacrylate (GMA) embedding [27]. Sections were prepared at 2 μm and stained with Romanowsky–Giemsa stain for detecting imperfections in the capsule membrane and for quantifying the composition of the overgrowth and determining the number of capsules with and without overgrowth. Different cell-types in the overgrowth were assessed by identifying cells in the capsular overgrowth with the morphological characteristics of monocytes/macrophages, lymphocytes, granulocytes, fibroblasts, basophiles, erythrocytes, and multinucleated giant cells.

2.7. Screening for immunostimulation and identification of pattern recognition receptor activation

To determine the immunostimulatory capacity, alginate molecules, beads, or capsules were co-incubated with different cell lines (InvivoGen, Toulouse, France) expressing pattern recognition receptors under the control of a reporter gene. A first screening was performed on THP1-XBlue™-MD2-CD14. This cell-line is derived from the human monocytic THP-1 cell line and expresses all Toll-like receptors (TLRs) as well as other PRRs. This cell-line carries an NF-κB/AP-1 inducible SEAP (secreted embryonic alkaline phosphatase) reporter gene and extra inserts for co-signaling molecules CD14 and MD2 to facilitate TLR-mediated responses.

To study whether the signaling is dependent on TLRs and not on other pattern recognition receptors we applied a THP1 cell expressing only a truncated, non-functional form of the TLR adapter MyD88 (MyD88) (THP1-XBlue™-defMyD). MyD88 is an essential messenger in the cascade from TLR activation towards NF-κB activation (see also Fig. 2).

THP1-XBlue™-MD2-CD14 and -defMyD88 cells were suspended in fresh RPMI 1640 culture medium containing 2 mM L-glutamine, 1.5 g/l sodium bicarbonate, 4.5 g/l, 10 mM HEPES and 1.0 mM sodium pyruvate, and supplemented with 10% fetal bovine serum (deactivated phosphatases), 100 μg/ml Normocin™, Pen-Strep (50 U/ml–50 μg/ml) at 1×10^6 cells/ml and plated in 96-well plates. Each well was stimulated with alginate molecules, beads, or capsules (at least $n = 20$) and cultured overnight at 37 °C and 5% CO_2. As positive controls, lipopolysaccharide from *Escherichia coli* K12 strain (LPS-EK Ultrapure 10 μg/ml, InvivoGen, Toulouse, France) was used for the THP1-XBlue™-MD2-CD14 cell line and L-Ala-γ-D-Glu-mDAP (Tri-DAP 10 μg/ml, InvivoGen, Toulouse, France) for the THP1-XBlue™-defMyD88 cell line. In both cell lines RPMI 1640 culture medium was used as a negative control. Production of SEAP was quantified by using QUANTI-Blue™ (InvivoGen, Toulouse, France) a medium for detection and quantification of alkaline phosphatase. QUANTI-Blue™ medium change to a purple-blue color in the presence of SEAP. An aliquot of QUANTI-Blue™ (200 μl) was brought in a new flat bottom 96-well plate with 20 μl of supernatant from the stimulated cell-lines for 45 min at 37 °C and SEAP activity, representing activation of NF-κB/AP-1, was measured at a wavelength of 650 nm on a VersaMax microplate reader (Molecular Devices GmbH, Biberach an der Riss, Germany) using SoftMax Pro Data Acquisition & Analysis Software.

To identify the specific pattern recognition receptors that are being activated by alginates, human embryonic kidney (HEK)-cells overexpressing a specific TLR or NOD were used. The HEK-Blue™ cell-lines are produced by co-transfection of the human TLRs (2, 4, 5, 7, 8, 9) and human NODs (1 and 2) into the HEK 293 cells. Human TLR2 was also transfected with the CD14 co-receptor gene, and human TLR4 was also transfected with MD-2 and CD14 co-receptor genes. They are designed for identifying the activation of specific human Toll-like receptors (TLRs) 2, 4, 5, 7, 8 or 9 and Nod-like receptors (NODs) 1 or 2 combined with AP-1 and a NF-κB reporter construct.

HEK-Blue™ cells were suspended in DMEM culture medium (4.5 g/l glucose, 10% (v/v) fetal bovine serum (deactivated phosphatases), Pen-Strep (50 U/ml–50 μg/ml), 100 μg/ml Normocin™ and 2 mM L-glutamine) at 280,000 cells/ml (hTLRs 2 and NOD 1), 140,000 cells/ml (hTLRs 4, 5 and NOD 2), 220,000 cells/ml (hTLRs 7, 8), and 450,000 cells/ml (hTLR 9), and plated in 96-well plates according to standard protocols. Each well was stimulated with alginate molecules, alginate beads or capsules and cultured overnight at 37 °C and 5% CO_2. DMEM culture medium was used as a negative control and TLR signaling was always confirmed using the appropriate TLR or NOD ligand (Table 1).

NF-κB activation was assessed by measuring SEAP activity using QUANTI-Blue™ (InvivoGen, Toulouse, France). Experiments were repeated at least 5 times.

2.8. Measurement of specific pathogen-associated molecular patterns (PAMPs)

Peptidoglycan (PG; TLR2 ligand) was quantified with a human peptidoglycan ELISA kit (BMassay, Beijing, China). According to the manufacturer's protocol, 0.1 ml of alginate solution (0.3% w/v) or standards (10 ng/ml and half dilutions until 0.156 ng/ml), by duplicates, was added to pre-coated wells with PG polyclonal antibodies and incubated for 90 min. After washing the plates, we added 0.1 ml of biotinylated anti-human PG antibody solution for 60 min. The plates were washed and subsequently 0.1 ml of Avidin–Biotin–Peroxidase complex (ABC) was added to the plates and incubated for 30 min. After a final wash 0.09 ml of 3,3′, 5,5′-tetramethylbenzidine (TMB) substrate was added. When shades of blue could be observed in the

Table 1
Human Toll- and NOD-like receptors, its ligands, and the specific positive controls that are applied [44–46].

TLR/NOD	Ligating pathogen-associated molecular patterns (PAMPs)	Applied positive control for specific HEK-cell lines
TLR2	Lipoproteins, triacyl and diacyl lipoproteins, lipoteichoic acid, zymosan, peptidoglycan, bacterial porines, viric hemagglutinin	Synthetic diacylated lipoprotein (FSL-1)
TLR4	Lipopolysaccharides, viral envelope proteins	Ultrapure lipopolysaccharide from *E. coli* K12 strain (LPS-EK Ultrapure)
TLR5	Bacterial flagellin	Recombinant flagellin from *Salmonella typhimurium* (RecFLA-ST)
TLR7	ssRNA	9-Benzyl-8 hydroxyadenine (CL264)
TLR8	ssRNA	20-Mer phosphorothioate protected single-stranded RNA oligonucleotide (ssRNA 40)
TLR9	Unmethylated dsDNA, hemozoin	Unmethylated CpG dinucleotides (ODN 2006)
NOD1	Peptidoglycan from Gram-negative bacteria	L-Ala-γ-D-Glu-mDAP (Tri-DAP)
NOD2	Muramyl dipeptide from both Gram-positive and negative bacteria	Synthetic derivative of muramyl dipeptide (L18-MDP)

standard wells, 0.1 ml of TMB stop solution was added. The plate was read in a spectrophotometer at a relative optical density of 450 nm within 30 min after adding the stop solution.

The presence of lipoteichoic acid (LTA; TLR2 ligand) was measured using a Lipoteichoic Acid Elisa Kit (Antibodies-online, Atlanta, GA, United States). This ELISA is based on the competitive binding enzyme immunoassay technique, the samples were analyzed in duplicate, 0.05 ml of alginate solution (0.3% w/v) or standard (20 ng/ml and half dilutions until 0.31 ng/ml) and 0.05 ml of detection solution were added to the microtiter plate pre-coated with a monoclonal antibody specific for LTA. Existent LTA in the sample will compete with a fixed amount of biotin-labeled LTA for sites in the pre-coated plates and incubated for 60 min. Excess conjugate and unbound sample or standard are washed and 0.1 ml of Avidin conjugated to Horseradish Peroxidase (HPR) was added and incubated for 45 min. After the washing process 0.09 ml of TMB substrate is added to each well and incubated for 15–30 min. The enzyme–substrate reaction is terminated by the addition of 0.05 ml of sulfuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 nm.

The content of lipopolysaccharides (LPS; TLR 4 ligand) was measured using a Human Lipopolysaccharides ELISA Kit (Cusabio, Wuhan, China). For this assay the quantitative sandwich enzyme immunoassay technique was employed, 0.1 ml of alginate solution (0.3% w/v) or standard (400 pg/ml and half dilutions until 6.25 pg/ml) was added in the wells pre-coated with an antibody specific for LPS and incubated for 2 h, after washing and removal of unbound substances, 0.1 ml of biotin-conjugated antibody specific for LPS was added to the wells and the plate was incubated for 60 min. After washing 0.1 ml of avidin-HRP was added to the wells and incubated for 60 min. Following a washing step for removing the unbounded avidin-enzyme reagent 0.09 ml of TMB substrate was added to each well and incubated for 15–30 min, the color reaction is stopped by adding 0.05 ml of stop solution and the optical density is measured at 450 nm within 5 min after the reaction was stopped. All the incubations were performed at 37 °C and 5% CO_2.

Quantification of flagellin (TLR5 ligand) and unmethylated dsDNA (TLR9 ligand) was done with a Human Flagellin ELISA Kit and a Human CpG oligodeoxynucleotide (CpG-ODN) ELISA Kit (BMassay, Beijing, China). In the assay 0.1 ml of the alginate solution (0.3% w/v) or standard (duplicates) was added into the pre-coated wells with human Flagellin (5000 pg/ml and half dilutions until 78.125 ng/ml) or human CpG-ODN polyclonal antibodies (500 ng/ml and half dilutions until 7.8 ng/ml) and incubated for 90 min. After washing the plates 0.1 ml of biotinylated anti-Human flagellin or CpG-ODN antibody solution was added and incubated for 60 min. The plates were washed afterwards. Subsequently 0.1 ml of ABC complex was added to the plates and incubated for 30 min. After a final wash 0.09 ml of TMB substrate was added. When shades of blue could be observed in the standard wells, 0.1 ml of TMB stop solution was added. The plates were read in a spectrophotometer at a relative optical density of 450 nm within 30 min after adding the stop solution. All the incubations were performed at 37 °C and 5% CO_2.

For detection of single stranded RNA (ssRNA) (TLR 8 ligand) we used the NanoDrop 1000 Spectrophotometer V3.8.1 (Thermo Scientific, United States) [28]. For that purpose 1 μl of alginate solution (0.3% w/v) was added without any dilution, on top of the fiber optic cable. A second fiber optic cable is brought in contact with the sample. A pulsed xenon flash lamp provides the light source and a spectrometer with a linear charged-coupled Device (CCD) array was used to analyze the light after passing through the sample applying a wavelength of 230 nm.

2.9. Statistical analysis

Results are expressed as mean ± SD. Statistical comparisons were made with the Mann–Whitney *U* test. A p value < 0.05 was considered statistically significant.

3. Results

Commercially available alginates, independent of their composition, provoked a strong inflammatory response. When rat-recipients of empty capsule-grafts were sacrificed at one month after implantation we found that all capsules were adherent to the abdominal organs and overgrown by thick layers of fibroblasts, giant-cells, and macrophages (Fig. 1). This was the same for low-, intermediate-, or high-G alginates. Also it was independent of the capsule type as the same was observed for barium-beads, calcium-beads, and alginate–PLL capsules. This confirms previous findings of our lab and that of other groups [6,7,15,18,29]. The cause and mechanism of this strong response, however, is still largely unknown. It is hypothesized that activation of pattern recognition receptors (PRRs) may be involved. To study the involvement of PRRs we applied a cell-line carrying many PRRs to study the activation of NF-κB by these crude alginates. This cell-line is of a monocytic background, the THP1 cell. We applied this cell-line in two forms. In the first form the cell is equipped with the full machinery to allow activation of NF-κB *via* PRRs, while in the second form a crucial messenger for TLR induced activation, *i.e.* MyD88, is not functional. Thus, activation of NF-κB in the first cell-line and absence of activation in the MyD88 deficient cell-line imply that the activation is mostly dependent on TLRs that signal *via* MyD88 (Fig. 2).

As shown in Fig. 3a, all three types of commercially available alginates provoke a strong NF-κB response in the THP1 cells with the full machinery for NF-κB activation. Notably the activation of the THP1 cell is highly alginate type dependent. Intermediate-G alginate provokes a statistically significant lower response when compared with low- and high-G alginates ($p < 0.05$). All differences were statistically significant when compared with the unstimulated cell lines ($p < 0.001$). The response was completely absent in the THP1 cell-line with the non-functional MyD88 (Fig. 3b).

As it may be argued that different results may be obtained when alginates are cross-linked in a network, it was questioned whether the same phenomena occurred when true capsules instead of alginate solutions are used. This set of experiments was performed using three commonly applied systems with different binding kinetics to alginate; *i.e.* barium-beads, calcium-beads [30,31], and alginate–poly-L-lysine–alginate (APA) capsules [32]. As shown in Fig. 4a all three systems gave an activation of NF-κB. This was TLR dependent as the response was absent in MyD88 deficient THP1 cells (Fig. 4b). The results

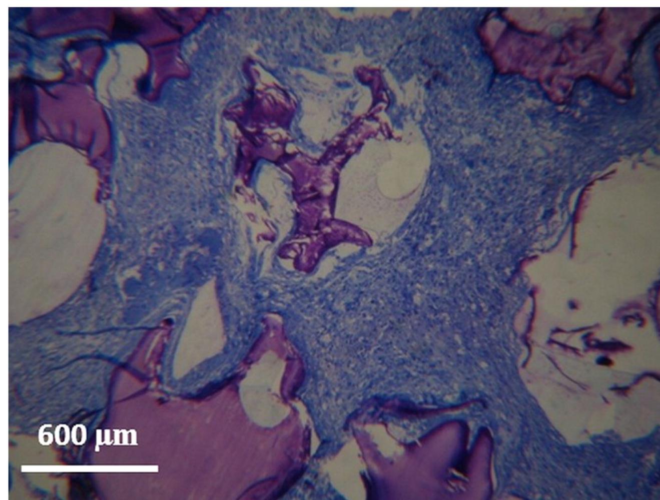

Fig. 1. Alginate poly-L-lysine capsules prepared from commercially available alginate one month after implantation in AO-rats. Explantation of empty capsules that adhered to the abdominal organs from the rat-recipients; all capsules are overgrown with fibroblast and macrophages. This was observed with low-, intermediate-, and high-G alginates applied in barium-beads, calcium-beads, and alginate–PLL capsules (GMA-embedded histological section, Romanowsky–Giemsa stain, original magnification ×800).

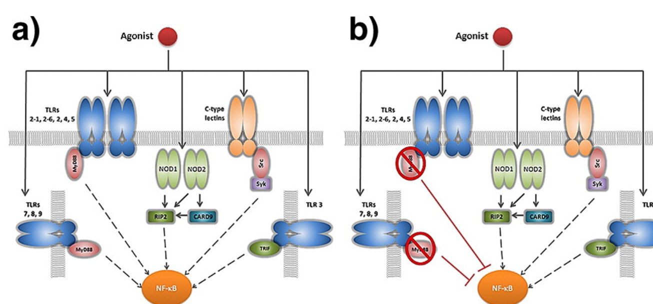

Fig. 2. Strategy to determine which family of pattern recognition receptors (PRRs) is involved in activation of NF-κB by biomaterials, *i.e.* Toll-like receptors (TLRs) or other PRRs such as C-type lectins and NOD-like receptors (NODs); a) We applied THP1 cells containing the full machinery to activate NF-κB *via* all PRRs on the cell. To subsequently determine whether it is TLRs or C-type lectins that are responsible for the NF-κB activation we apply a cell-line expressing a non-functional form of the TLR adapter MyD88 (b). This MyD88 is essential in TLR induced activation of NF-κB. A NF-κB activation in the THP1 with the full machinery and an absence in a non-functional MyD88 THP1 imply that the activation is MyD88 dependent.

shown in Fig. 4 applied to intermediate-G alginates, and similar results were observed for low-G and high-G alginates (data not shown).

3.1. Alginate stimulated human TLRs 2, 5, 8, and 9

As most TLRs (2, 4, 5, 6, 7, 8, 9) signal *via* MyD88, we decided to study which of the MyD88 dependent TLRs are involved in the immune activation. Intracellular NOD receptors that signal in a MyD88 independent fashion are expressed in low levels on THP1 cells. For this reason we also investigate whether NOD receptors were involved in the observed response. To this end we applied human embryonic kidney cells (HEKs) transfected with specific TLRs or NODs coupled to a NF-κB reporter gene. Fig. 5 shows the percentage of activation of all specific HEK cells expressing either TLRs 2, 4, 5, 7, 8, or 9 or NOD1 or 2 co-incubated with calcium beads prepared from intermediate-G alginate and compared with its own control of unstimulated cells. Similar results but somewhat lower in magnitude were obtained with solved alginates (data not shown). We set a threshold to consider a significant elevation of activation of NF-κB when this was above 0.25 (arbitrary units). This is the maximum value that the negative controls, *i.e.* media, can reach. Fig. 5 shows that the calcium beads do activate hTLRs 2, 5, 8, and 9, but not hTLR4 and 7 nor hNOD1 and 2.

3.2. Purification abolished TLR activation

Next it was investigated whether impurities or alginate itself activates TLRs. The purified alginates were subsequently tested for NF-κB activation in THP1 cells. As shown in Fig. 6, we found a profound reduction in the NF-κB activation when purified alginates were applied instead of the crude alginates. This was the same for low-, intermediate-, and high-G alginates and the three different encapsulation systems applied. This made us conclude that alginates as such do not activate PRRs.

3.3. Alginate contained the PAMPs peptidoglycan, lipoteichoic acid, and flagellin

Next we investigated whether any of the known ligands of TLRs 2, 5, 8, and 9, *i.e.* cell-wall components (TLR2), flagellin (TLR5), single stranded RNA (TLR8), and unmethylated CpG (TLR9) are present in commercially available crude alginate. Besides, as LPS is considered the most important endotoxin contributing to responses against alginates [33] we investigate the presence of lipopolysaccharide (LPS) in crude alginate. As shown in Fig. 7, we found peptidoglycan (PG) and lipoteichoic acid (LTA) in alginate but no LPS. Both of them are ligands for TLR2. Also we found flagellin, which is a ligand for TLR5. However, we were not able to find any of the common ligands for TLR8 and 9 *i.e.* single stranded RNA and unmethylated CpG. As shown in Fig. 7, these molecules were absent in the purified alginate, except for flagellin which was only reduced in high-G alginate. We could not completely remove it from high-G alginate with our purification procedure (Fig. 7c).

3.4. Implantation of barium beads produced from purified alginates

To confirm that purified alginates lacking PAMPs do not provoke a response, we implanted purified alginates as barium-beads in AO rats. We could not apply calcium-beads as we found that these were unstable with alginates with lower G content. Barium connects both guluronic and mannuronic chains and is therefore not associated with effects of alginate type on tissue responses. Barium beads (Table 3) remained largely free of any tissue response and only a few beads provoked a

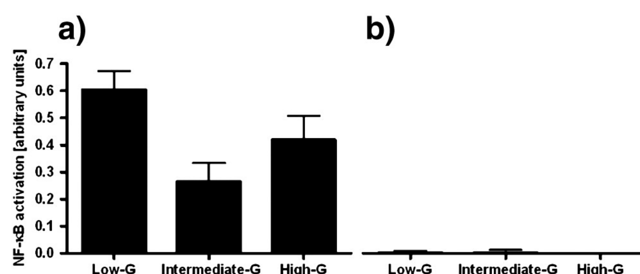

Fig. 3. Commercially available alginate stimulates monocytic THP1 cells in a MyD88 dependent fashion. NF-κB activation by different types of alginates in monocytic THP1 cells without (a) and with (b) a non-functional MyD88. Activation of NF-κB mediated by low-G, intermediate-G, and high-G alginates in a) THP1 and b) THP1 MyD88-deficient cell lines. Values are presented as mean ± SD (n = 20). Intermediate-G alginate gave the lowest response when compared to low- and high-G alginate. The three kinds of alginates have a statistically significant response when compared with unstimulated (0.14 ± 0.02 for (a) and 0.04 ± 0.02 for (b)) cell-lines ($p < 0.001$). This response was almost gone in MyD88 cell-lines illustrating the MyD88 dependency of the response. LPS (10 μg/ml) was used as positive control for THP1 cell line, and induced a NF-κB activation of 2.06 ± 0.14. For THP-1 cell line with a non-functional MyD88, Tri-DAP (10 μg/ml) was used as positive control, and induced a NF-κB activation of 0.075 ± 0.01.

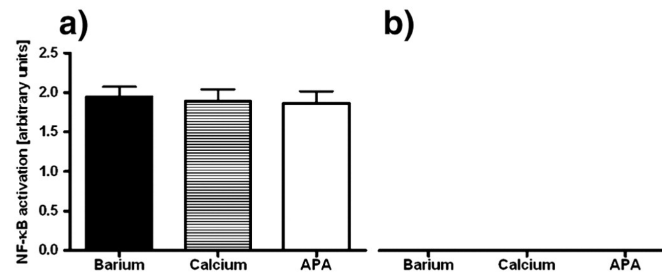

Fig. 4. Calcium-beads, barium-beads, and alginate–poly-L-lysine–alginate (APA) capsules stimulate NF-κB production by THP1 cells in a MyD88 dependent fashion. NF-κB activation mediated *via* Toll-like receptors (TLRs) in commonly applied systems for encapsulation of cells (barium beads, calcium beads and APA capsules) using intermediate-G alginate in a) THP1 (the three kinds of systems have a statistically significant response when compared with unstimulated cell-lines (0.05 ± 0.0003 for (a) and 0.04 ± 0.0002 for (b); $p < 0.001$) and b) THP1 MyD88-deficient cell lines. Values are presented as mean ± SD ($n = 20$). LPS (10 μg/ml) was used as positive control and induced a NF-κB activation of 2.06 ± 0.14. For THP1 cell line with a non-functional MyD88, Tri-DAP (10 μg/ml) was used as positive control, and induced a NF-κB activation of 0.06 ± 0.008.

response but this should be attributed to mechanical imperfections [34] rather than PAMP contamination.

4. Discussion

To the best of our knowledge this is the first study addressing the role of PAMPs in alginate as initiator of immune responses and not the alginate itself. Many impurities in alginate have been suggested to contribute to inflammatory responses such as polyphenols and LPS but up to now there have not been detailed studies on the source specificity of these contaminations nor have there been any studies on the mechanisms of how such impurities contribute to the strong responses that have often been observed against alginate-based biomaterials [25,32,35]. Remarkably our study could not demonstrate the presence of LPS in any alginate source.

Many have shown that alginate based microcapsules activate the innate immune system. It has been shown by us and others [3,36,37] that alginate can provoke release of typical proinflammatory, innate cytokines such as IL-1β, TNF-α and IL-6 from both murine and human macrophages and monocytes. The innate immune function is composed of a sensor and effector system. The sensors of innate immune cells regulate downstream cellular effector functions and finally the immune activation [38,39]. These innate sensors are pattern-recognition receptors (PRRs), of which the Toll-like receptors (TLRs) are the best studied [40]. However, other PRRs (*e.g.* C-type lectin receptors, NOD-like receptors, and inflammasomes) are equally important sensor pathways [41,42]. To determine whether the sensor activation occurs *via* the well-known TLR or other PRRs we applied the THP1 cell-line which is equipped with most of the known PRRs. We combined this with THP1 cells that have a specific non-functionality in an essential downstream regulatory transcription factor for TLRs 2, 4, 5, 6, 7, 8, and 9. This strategy allowed us to conclude that the sensor function activation of alginate based capsules is mainly TLR dependent. This strategy (Fig. 2) is applied for alginate but may be applicable for other (bio)materials as well.

In line with the finding that alginates activated TLR2 and 5, our study on the specific components in alginates which have to be held responsible for the TLR activation demonstrated that alginates contain significant amounts of the TLR2 agonists peptidoglycan and lipoteichoic acid

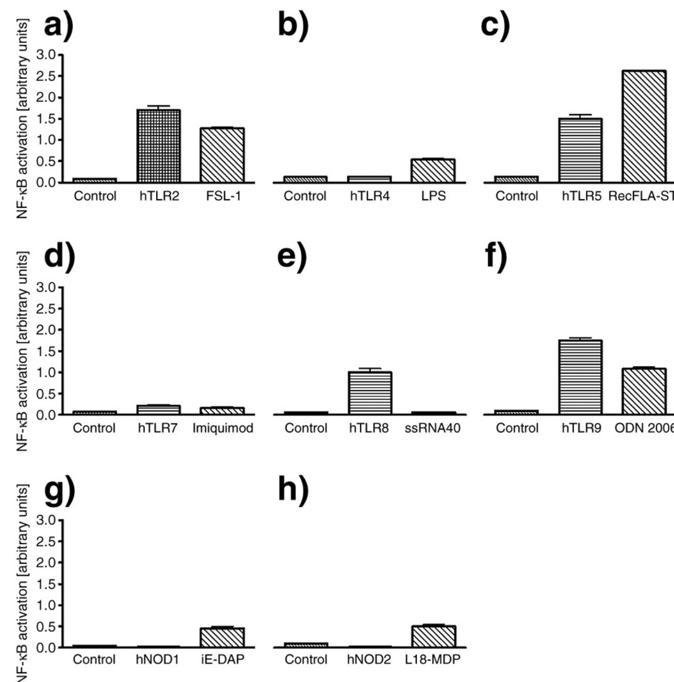

Fig. 5. NF-κB activation of specific Toll-like receptors (TLRs) by calcium beads produced from intermediate-G alginate. Calcium beads were coincubated with HEK-Blue™ cells carrying specific TLRs. Values are presented as mean ± SD ($n = 5$). Specific positive controls were used for TLRs with different levels of NF-κB activation (arbitrary units): a) TLR2 (FSL-1, 1 μg/ml, 2.07 ± 0.29); b) TLR4 (LPS-EK Ultrapure, 100 ng/ml, 0.166 ± 0.007); c) TLR5 (RecFLA-ST, 100 ng/ml, 0.96 ± 0.36); d) TLR7 (Imiquimod (R837), 50 μg/ml, 0.055 ± 0.003); e) TLR8 (ssRNA 40, 50 μg/ml, 0.062 ± 0.004); f) TLR9 (ODN2006, 100 μg/ml, 1.84 ± 0.18); g) NOD1 (Tri-DAP, 10 μg/ml, 0.054 ± 0.01); h) NOD2 (L18-MDP, 100 ng/ml, 0.061 ± 0.01). For all the cell lines, DMEM 1640 growth media was used as negative control with different levels of NF-κB activation (arbitrary units). In order to be considered as a biological relevant response we set a threshold of more than 0.25 NF-κB activation of unstimulated cells. As shown, alginate beads activate hTLRs 2, 5, 8, and 9.

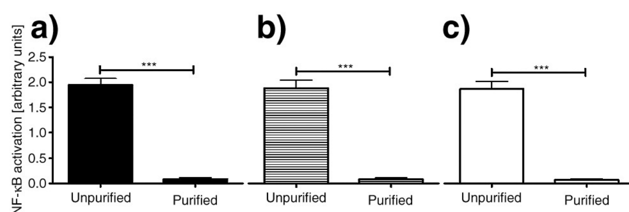

Fig. 6. Pathogen-associated molecular patterns (PAMPs) and not alginate as such activate THP1 cells in a MyD88 dependent manner. Effects of purification of intermediate-G alginate on NF-kB activation in commonly applied systems for encapsulation; a) barium beads, b) calcium beads and c) APA capsules. Values are presented as mean ± SD (n = 20); $p < 0.001$ (***). LPS (10 μg/ml) was used as positive control and induced a NF-κB activation of 2.21 ± 0.07. For all the set of experiments RPMI 1640 culture medium was used as negative control for unstimulated cells (0.05 ± 0.001).

and the TLR 5 ligand flagellin. These PAMPs are probably introduced during extraction of alginates from algae. PAMPs are found on pathogens but also on less infectious bacteria that are found in production plants and laboratories [43]. PAMPs can be found in different forms and compositions. In Table 2 we listed all the PAMPs we have screened for and on which innate immune cell type PRR specific for these PAMPs can be found. All these PAMPs have one common feature; they are strong activators of the host innate immune system even when present in very small amounts. Although alginates also activated TLR8 and 9, we were not able to identify the ligand for activation of TLR8 and 9 in the alginate. We screened for single stranded RNA (TLR8 ligand) and unmethylated DNA (TLR9 ligand). A possible explanation is that crude alginate contains a PAMP that has not been identified yet [21,44–47]. A remarkable finding is that we did not find LPS which has been found to be present by others and which has been considered to be a major cause of the inflammatory responses against alginate-based capsules [33]. However, this is in line with the fact that our alginates did not activate TLR4. Going through literature we found only one explanation for this discrepancy. Most groups measured endotoxin by the Limulus amebocyte lysate (LAL) assay and not specifically LPS. It is known, but not commonly recognized that the endotoxins lipoteichoic acid (LTA) and peptidoglycan (PG) result in positive LAL-assays [48]. We therefore have drawn the conclusion that at least in our studies PG and LTA have caused positive LAL results and not LPS. We therefore recommend not only to test for endotoxins by the LAL assays but also to specifically identify the specific endotoxin responsible for the positive signals. This is essential for designing specific strategies to remove the specific contamination.

Not only the quantity but also the type of PAMPs present in alginate was alginate type dependent. This is probably due to differences in contaminations of the alginate during the extraction processes rather than by differences in PAMP content of the algae-alginate source. Most PAMPs such as flagellin can be found on bacterial species such as *E. coli*, *Salmonella typhimurium*, *Caulobacter crescentus*, *Vibrio alginolyticus*, and *Campylobacter jejuni*. Most of these organisms are pathogenic but are generally found in nature and in biofilms in production plants [49]. Notably also in standard laboratory settings these kinds of PAMPs can be found in or on bacteria [50]. We even found in some occasions PAMPs in alginates that were purified. This introduction of new PAMPs should be attributed to bacterial contaminations during

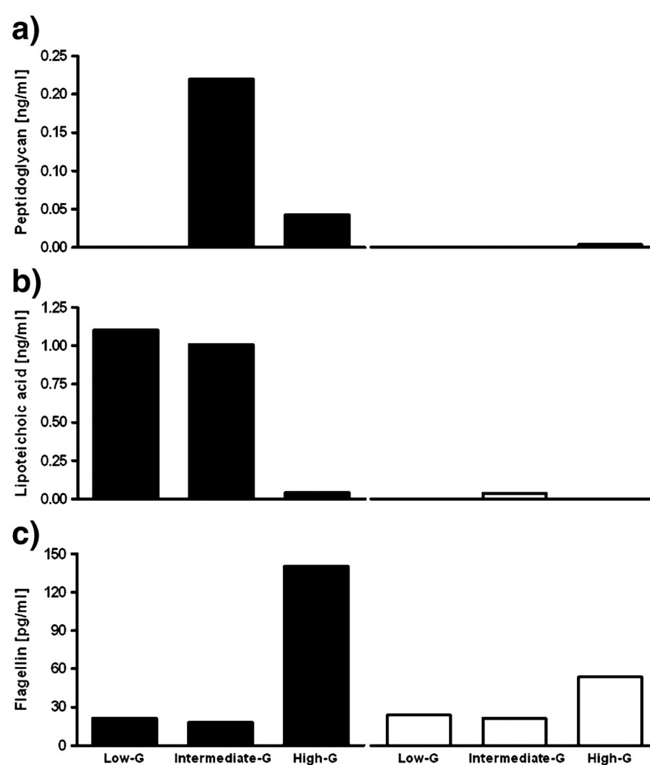

Fig. 7. Measurement of pathogen-associated molecular patterns found in low-G, intermediate-G, and high-G alginates. a) Peptidoglycan (PG; TLR2 ligand) in unpurified (left) and purified (right) alginates; b) lipoteichoic acid (LTA; TLR2 ligand) in unpurified (left) and purified (right) alginates; c) flagellin (TLR5-ligand) in unpurified (left) and purified (right) alginates. Single stranded RNA (ssRNA; TLR8 ligand), unmethylated CpG (CpG-ODN; TLR9 ligand), and lipopolysaccharides (LPS; TLR4 ligand) were not detected in unpurified and purified alginate solutions (0.3% w/v). All experiments are performed in triplicate.

Table 2
PAMPs that have been screened in this project and specific PRRs for recognizing these PAMPs [21,47].

Pathogen-associated molecular patterns (PAMPs)	Pattern recognition receptor (PRR)
Lipopolysaccharides (LPS)	TLR4
Peptidoglycan (PG)	TLR2, NOD1, NOD2
Lipoteichoic acid (LTA)	TLR2
Flagellin	TLR5, NAIP5, NAIP6, NLRC4
Single-stranded RNA (ssRNA)	TLR8
Unmethylated DNA (Cpg-ODN)	TLR9

purification process or during storage. As shown in the present study most of the PAMPs can be removed by the chemical procedure that we applied. This procedure is used by many and also used for alginates that are applied for human application [6]. Our data however also demonstrate that testing on efficacy of purification is always required as our procedure worked for most alginates except for high-G alginate. After purification we still found flagellin in high-G alginate. We feel that it is important to emphasize this lack of efficacy as it might contribute to the lab-to-lab variations that have been reported for alginate-based capsules [18,19]. It should be noted however that in spite of the presence of flagellin in the high-G alginate we did not find a pronounced inflammatory response against the barium-beads prepared of purified high-G alginate.

Alginates were being tested in different capsule configurations. This was done because it might be argued that contaminating PAMPs might be caught in the crosslinking networks by which immune activation might be avoided. Also it was done to exclude the suggestion that PLL can cause TLR activation. Surprisingly we not only found a similarly strong activation by calcium-beads, barium-beads, and alginate poly-L-lysine capsules but also a two-fold stronger activation than by the crude unbound alginates. This observation should be explained by the fact that in our assays with the solved molecules more steric hindrance by the alginate molecules is to be expected. This might interfere with adequate binding to the PRRs. When the alginates are complexed with cations the PAMPs can more easily diffuse out of the network and reach the PRRs on the cells.

The fact that all encapsulation systems provoked similar NF-κB activation suggests that the proinflammatory molecules diffuse out of the alginate-networks and that neither the surface nor the structure of the pores in the capsules has an influence on the responses. It has been published that alginates may also contain proteins and other contaminants [51]. To the best of our knowledge this is the first report in which strong immune activators such as peptidoglycan, lipoteichoic acid, and flagellin have been demonstrated. These molecules can bind on TLR on immune cells or be taken up by phagocytic immune cells and induce responses *via* intracellular TLRs.

During recent years a number of studies have addressed the role of PRRs in responses against biomaterials. Most studies have focused on the role of TLR4 in foreign body response [52,53]. Auquit-Auckbur et al. [53] demonstrated that the foreign body responses against silicon were significantly less in TLR4$^{-/-}$ mice than in wild-type mice. There were less granulocytes and macrophages present in the infiltrates as well as less vascular endothelial growth factor (VEGF) and transforming growth factor (TGF-β). Similar studies have been performed with polymethylmethacrylate (PMMA) [54,55]. Pearl et al. [54] applied inhibitors of MyD88 pathway to study the involvement of TLRs in responses against PMMA microparticles. They were able to demonstrate the essence of TLR dependent pathways but could not pinpoint the exact TLR and its ligand that was responsible for the responses. Tamaki et al. [56] also demonstrated an upregulation of TLR2, 4, and 9 in patients with rheumatoid arthritis having an implant. In any of these studies the presence of PAMPs as the cause of the TLR activation has been investigated. On the basis of the current findings we feel that it is advisable to study those PAMPs coming from biofilms [57] or the so-called danger associated molecular patterns (DAMPs) [58] in the biomaterial constructs or from dying cells that are responsible for the responses. DAMPs are intracellular components from eukaryotic cells that also can be found everywhere where dying cells are present. It may be found on the borders of implants or in devices and provoke strong immune responses. We hope our work will contribute to a deeper study of PAMPs and DAMPs in controlled release devices such as encapsulated cellular grafts.

On the basis of our findings we suggest the following sequence of events. Alginate may be contaminated with remnants of bacteria that serve as PAMP. PAMPs in alginate act predominantly as ligands for TLR2, 5, 8, and 9. In inflammatory cells this leads to downstream activation of NF-κB, resulting in cytokine release and an initial innate immune activation. This response may vary per type of alginate as the consequence of differences in presence/absence of molecules such as peptidoglycan, lipoteichoic acid, and flagellin. This leads to chemotaxis of immune cell elements and attraction of cell types such as basophiles, macrophages, and granulocytes as shown in our previous studies [7,35]. This finally results in an undesired surrounding of the capsules by macrophages and fibroblasts (Fig. 8). All these responses can be avoided by applying a purification procedure aiming on eliminating PAMPs from alginate.

Table 3
Recovery rate and percentage of overgrown barium beads manufactured from purified low-G, intermediate-G, and high-G alginates. One month after implantation in the peritoneal cavity. Non-purified alginates could not be retrieved, were adherent to the abdominal organs, and heavily overgrown.

Alginate type	n	% recovery	% overgrowth
Low-G	4	92.4 ± 1.5	2.0 ± 0.5
Intermediate-G	6	89.3 ± 4.5	1.3 ± 0.8
High-G	4	93.5 ± 5.6	2.0 ± 0.8

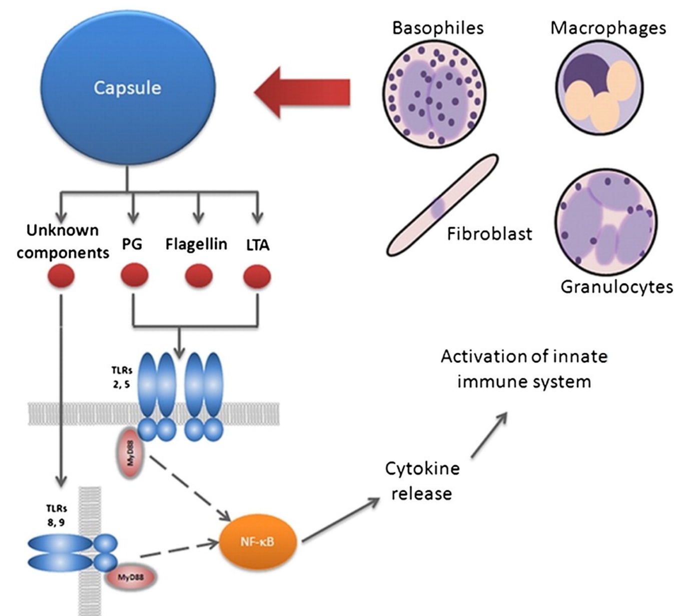

Fig. 8. Pathogen-associated molecular patterns in alginates (*e.g.* lipoteichoic acid, peptidoglycans and flagellin) can trigger the activation of innate immune cells by stimulating the sensory system in a TLR dependent fashion. This stimulation induces an intracellular cascade in inflammatory cells with activation of NF-κB, cytokine release, and initial activation of innate immune system and recruitment of immune cells that can adhere to the capsule. Thus, this activation is reduced if these PAMPs are removed from the preparation.

5. Conclusions

In this paper we show that PAMPs in alginate should be held responsible for proinflammatory responses in the host. Crude alginates contain PAMPs such as peptidoglycan, lipoteichoic acid, and flagellin. The PAMPs predominantly activate TLR2, 5, 8, and 9. We designed a strategy to determine in biomaterials which pattern recognition receptors have to be held responsible as sensor for innate immune cells and a strategy to identify specific PAMPs in biomaterials. By applying this technology platform we were able to unravel the sensor mechanism by which alginates may provoke inflammatory responses. We demonstrate that not alginate itself but PAMPs in the alginate provoke the responses.

Acknowledgments

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