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Abstract: Microparticles are small ($<1\ \mu\text{m}$), nonbiological particles that are used in many areas of daily life. As food additive they are used as anticaking agents or food colorants. The most common food-derived ingested compounds are aluminium silicate and titanium dioxide (TiO(2)), the latter being a white pigment used in toothpaste or sugar toppings. The increasing abundance of microparticles in the Western diet raises the question of the potential risks associated with gastrointestinal diseases such as Crohn's disease (CD). Accumulation of particles has been shown in cells of Peyer's patches, but it is not clear whether this also has pathological effects. NLRP3 is a member of the intracellular pattern recognition receptor family and it is part of the inflammasome, a multiprotein complex containing caspase-1 which activates the proinflammatory cytokines interleukin (IL)-1 and IL-18. With regard to recent findings identifying small particles such as asbestos and monosodium urate as NLRP3 activators, TiO(2) may be another potential target for inflammasome studies. We found that macrophage-like cells readily take up TiO(2) after 6 h. Incubation of cells with TiO(2) resulted in the assembly of NLRP3 with caspase-1. This inflammasome assembly correlated with secretion of IL-1. In intestinal epithelial cells, TiO(2) also was found to be ingested. The counting of particles localized intracellularly revealed a dose-dependent increase of TiO(2)-positive cells. This points to the fact that in humans with a leaky intestinal barrier (such as IBD patients), TiO(2) microparticles may be taken up by macrophages and intestinal epithelial cells, may activate the inflammasome and induce IL-1 and IL-18 secretion. This may aggravate inflammation in susceptible individuals.

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Microparticles and their impact on intestinal immunity

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Abstract

Microparticles are small (<1µm), non-biological particles that are used in many areas of daily life. As food additive they are used as anti-caking agents or food colorants. The most commonly food derived ingested compounds are aluminium silicate and titanium dioxide (TiO₂), the latter being a white pigment used in toothpaste or sugar toppings. The increasing abundance of microparticles in Western diet raises the question of potential risks associated with gastrointestinal diseases such as Crohn's disease (CD). Accumulation of particles has been shown in cells of Peyer's patches, but it is not clear whether this also has pathological effects. NLRP3 is a member of the intracellular pattern recognition receptor family and it is part of the inflammasome, a multiprotein complex containing caspase-1 which activates pro-inflammatory cytokines IL-1β and IL-18. With regard to recent findings identifying small particles such as asbestos and monosodium urate as NLRP3 activators, TiO₂ may be another potential target for inflammasome studies.

We found that macrophage like cells readily take up TiO₂ after 6 h. Incubation of cells with TiO₂ resulted in assembly of NLRP3 with caspase-1. This inflammasome assembly correlated with secretion of IL-1β. In intestinal epithelial cells, TiO₂ also was found to be taken up. Counting of particles localized intracellularly revealed a dose-dependent increase of TiO₂-positive cells. This points to the fact that in humans with a leaky barrier (such as IBD patients) TiO₂ microparticles may be taken up by macrophages and intestinal epithelial cells, may activate the inflammasome and induce IL-1β and IL-18 secretion. This may aggravate inflammation in susceptible individuals.

Key Words: inflammatory bowel disease; inflammasome; microparticles; interleukin-18

Introduction

Intestinal epithelial cells (IECs) form a single layer on the surface of the intestinal mucosa and the primary barrier against the content of the gut lumen. They protect the underlying tissue and the entire organism from potentially harmful microorganisms, particles or larger molecules. IECs secrete antimicrobial substances such as defensins [1,2] and communicate with immune cells of the intestinal mucosa via a high number of soluble mediators such as chemokines and cytokines. In patients with inflammatory bowel disease (IBD) the epithelial and mucosal barrier function is disturbed, finally leading to a chronic relapsing inflammation of the intestinal mucosa.

Both environmental triggers as well as a genetic susceptibility have been shown to contribute to the pathophysiology of IBD. More than 140 susceptibility loci for IBD have been identified mainly by GWAS studies [3], among them a number of genes related to innate immune functions including single nucleotide polymorphisms (SNPs) in so-called Pattern Recognition Receptors (PRR) which recognize microbial patterns (pathogen-associated molecular patterns, PAMP) but also other molecular signatures that may be a risk for health (so-called danger associated molecular patterns, DAMP).

In healthy individuals activation of PRRs and the subsequent pro-inflammatory signalling is tightly regulated. Among other members of the PRR family, NLRP3 (nucleotide-binding domain and leucine-rich repeat containing (NLR) family, pyrin domain-containing 3), has been shown to play a role in IBD aetiology. Alternative names for NLRP3 are NALP3, CIAS (cold-induced autoinflammatory syndrome-1), PYPAF (PYRIN domain-containing Apaf1-like) and cryopyrin. In biopsies of Crohn's disease (CD) patients, NLRP3 expression was found to be increased [4]. Besides

microbial patterns NLRP3 also recognizes “danger signals” or DAMPS such as ATP [5], asbestos [6] and uric acid crystals [7]. Upon activation signals, NLRP3 gets associated with a large multi-protein complex, the NLRP3-inflammasome, which activates caspase-1. Activated caspase-1 then finally cleaves pro-interleukin-1 β (IL-1 β) and pro-interleukin 18 (IL-18) (FIGURE 1).

Expression of all components of the NLRP3-inflammasome has been observed in professional immune cells such as macrophages or dendritic cells, but also found to a high level in IECs.

NLRP3 and susceptibility to IBD

Similar to NOD2, the first identified CD susceptibility gene, NLRP3 contains a “caspase-recruitment domain” (CARD) and – upon activation - assembles with caspase-1 to form the inflammasome [8]. The relevance of NLRP3 for CD pathogenesis is obvious from the finding that a gain-of-function SNP in NLRP3 and a correlating CARD8 loss-of-function SNP both result in a protective effect against CD [9,10]. NLRP3 gain of function mutations were previously associated with other inflammatory diseases and syndromes such as rheumatoid arthritis [11], Muckle-Wells Syndrome (MWS), familial cold autoinflammatory syndrome (FCAS) [12] and chronic infantile neurological cutaneous and articular syndrome (CINCA) [13]. All are characterized by elevated IL-1 β levels, indicating a NLRP3/inflammasome activation. Further, NLRP3 was shown to be upregulated in colon tissue after induction of acute or chronic colitis in mouse models. An elevated expression was also found in colon specimen of CD patients [4].

The inflammasome proteins belong to a family of NLRs, all sharing common domains that act as intracellular sensors for PAMPs and DAMPs. Similar to TLRs, the NLR family is evolutionary ancient and shares structural homologies with plant

resistance (R-) proteins. Both protein families have leucine-rich repeat regions (LRRs) for the recognition of bacterial motifs, an oligomerization domain and an effector domain [14] (FIGURE 2).

Upon activation, NLRP3 assembles into a multiprotein complex, recruiting other NLRP3 proteins, CARD8, ASC and caspase-1 (FIGURE 1). The recruitment of caspase-1 triggers its autocatalytic cleavage and activation. Subsequently, caspase-1 cleaves pro-IL-1 β or pro-IL-18; this is usually followed by the release of biologically active forms of these cytokines.

Table 1: Activators of the NLRP3 inflammasome (Table adapted from [15].)

		Reference
Microorganisms		
Sendai Virus	09/2006	[16]
Influenza virus	09/2006, 04/2009	[16,17]
Adenovirus	03/2008	[18]
<i>Staphylococcus aureus</i>	03/2006, 09/2009	[5,19]
<i>Listeria monocytogenes</i>	03/2006, 05/2008	[5,20]
<i>Sachharomyces cerevisiae</i>	06/2009	[21]
<i>Candida albicans</i>	08/2009	[22]
<i>Aspergillus fumigatus</i>	04/2010	[23]
Bacterial RNA or synthetic purine bases	03/2006	[24]
Bacterial pore forming toxins (aerolysin, maitotoxin, nigericin)	03/2006,09/2006, 11/2006	[5,25,26]
Malaria toxin	08/2009	[27]
Danger signals		
Extracellular ATP	03/2006	[5]
Hyalorunan	03/2009	[28]
Glucose	12/2009	[29]
Monosodium urate	03/2006	[7]
Amyloid-beta	08/2008	[30]
Silica	08/2008, 07/2008	[31,32]
Asbestos	07/2008, 05/2008	[6,32]
Alum	08/2008	[31]
Cholesterol crystals	04/2010	[33]

Food derived environmental factors in the pathogenesis of IBD?

IBD cannot be only attributed to genetic factors as monozygotic twins have only a concordance rate to develop IBD of 20-50% [34-37]. The increased incidence of IBD in the last 100 years, the higher incidence of IBD in countries with Western lifestyle, and the observed rise of IBD in countries recently undergoing “westernization” in Asia has led to several hypotheses explaining this phenomenon as summarized by Hanauer [38]. Hygiene, diet, social status and smoking are factors potentially influencing the risk for IBD [39]. Some childhood factors such as breastfeeding and childhood infections are likely to have protective functions,

whereas increased hygienic standards are discussed to increase the risk of IBD. Other factors taken up with typical Western diet are microparticles.

Microparticles are used as food additives in order to influence the colour, the consistency, or the appearance of a product. The most commonly used additives are titanium dioxide (TiO_2 , E171), or aluminium silicate (AlSi, E559). Both compounds are sub-micron sized (0.1-1 μm diameter) oxides of titanium, silicon or aluminium. Since they are inorganic, they are highly stable and resistant to degradation. According to current European legislation, there is no maximum TiO_2 level specified. It is recommended that TiO_2 should be used in amounts according to good manufacturing practice [40]. The international agency for research on cancer (IARC) published an overview on studies addressing the hazardous potential of TiO_2 in 2006. In animal studies, Inhalation was followed by development by tumours. *In vitro* studies with human alveolar macrophages revealed inhibited phagocytosis and DNA damage. Subsequently, TiO_2 was rated as possibly carcinogenic for humans (Group 2b) [41].

Different microparticles are used in food industry

TiO_2 is a white, crystalline powder which is widely used as food supplement in refined food. The pigment is used in toothpaste, confectionary, white colored sauces and dressings and non-dairy creamers [42]. In pharmaceutical industry it is used as pill coating. It is inert to most influences influence (FIGURE 3).

TiO_2 -iron oxide mixture

TiO_2 -Iron oxide mixture is a gold, mica powder. The powder is used as food colorant and as coating for pharmaceutical formulations.

Iron oxide

Iron oxide (Fe_2O_3) is a non soluble, brown and red pigment. It is applied as food colorant as well as in cosmetics. The product name of iron oxide is “Red luster” which is also used for coating for pharmaceutical formulations.

AlSi

AlSi, also known as Kaolin or Bolus alba is a versatile material. It is a natural white, fine grained material. The field of application includes paper manufacturing industry, powder fabrication, but it is also used in the food industry as carrier substance, bleaching product and parting agent as it prevents caking (FIGURE 3).

TiO_2 – AlSi mixture

TiO_2 with an additive of potassium - aluminum silicate is used as a whitener (FIGURE 3).

Sources of dietary microparticles

As the content of microparticles is not declared in many products and may vary the daily intake of microparticles is difficult to evaluate. The daily uptake is highly dependent on the individual diet. According to a study by the European Food Safety Authorities (EFSA), the estimated daily intake of TiO_2 is 70-80 mg/day [43]. Sources may be tablets, milk whitener, tooth paste and others. Other authors estimated the daily ingested amount of both TiO_2 and AlSi to 40 mg or 10^{12} particles per person/per day [44,45].

An overview of sources and estimated amounts of microparticles is given in Table 2.

Source	mg/person/day
TiO₂	
Food supplement tablet	37.5
Confectionary	24.4
Medicinal product tablet	15.0
Coffee whitener	0.52
Hard coated candies	0.32
Chewing gum	0.28
Marshmallows	0.27
Low fat or fat free dressings	0.22
AlSi	
Salt	1.30
Drinking chocolate powder	1.26
Chewing gum	0.92
Sugar, icing	0.30

Table 2: Sources of microparticles in food products and estimated daily intake.

Two most commonly microparticles, namely titanium dioxide (TiO₂) and aluminum silicate (AlSi) are listed. Table adapted from [43],[44].

Effects of TiO₂ on IEC

Microparticles may penetrate the intestinal barrier and accumulate in the mucosa. Early reports on dark, pigmented structures in human intestinal lymphoid aggregates date back to 1987 [46]. These structures were later found to contain high amounts of titanium [47]. TiO₂ particles have been shown to accumulate in M-cells of Peyer's patches and are passed on to underlying macrophages [48]. Interestingly, the sites of TiO₂ uptake correspond to regions where first signs of inflammation in CD manifest [49].

In vivo studies on the capacity of TiO₂ to penetrate the gastrointestinal tract revealed that TiO₂ can be found in systemic organs after oral exposition of 10 days [50-52]. In humans, upon one single ingestion of 22 and 45 mg TiO₂, Ti levels in blood rose after 0.5-2 h, and reached a maximum after 8-12 h [52].

Clinical studies also have addressed the influence on TiO_2 on CD patients. A pilot study performed in 2002 observed significant decreases in CD activity index [53] after 4 months of low- TiO_2 diet. However, a larger multi-centre double-blind trial was not able to confirm these results [54]. Under present living conditions a reduction or exclusion of dietary TiO_2 may be very difficult. With the recent discovery of TiO_2 being an activator of the NLRP3-inflammasome, the influence of TiO_2 on IEC in the healthy and chronically inflamed mucosa gained new interest. DCs derived from Nlrp3-deficient mice were shown to secrete less IL-1 β upon exposure to TiO_2 [55]. In human keratinocytes and murine macrophages, TiO_2 activated the NLRP3-inflammasome [56]. These data point towards a role of the inflammasome as direct or indirect sensor of TiO_2 .

Pathways of NLRP3 activation

It is still not clear how the NLRP3-inflammasome is activated in detail. Some authors suggest an initial priming event via the NF- κ B-pathway [57]. The so-called DAMPs comprise a group of inflammasome activators, and several pathways and mechanisms have been shown to be involved in a multi-step activation. Recently, Chen and Nuñez summarized the most important facts on DAMP-associated inflammasome activation [58]. Three separate pathways have been suggested. The first activation pathway requires high levels of extracellular ATP. ATP may be released by apoptotic/necrotic cells and trigger opening of the purinergic receptor P2X, ligand-gated ion channel 7 (P2RX7). This channel triggers the formation of pores in the cell membrane that allow efflux of K^+ ions. Inflammasome activation can be prevented by high concentrations of extracellular K^+ [32,59].

The second pathway may be relevant for uric acid crystals (MSU). MSU (similar to cholesterol crystals) induce inflammasome activation by a different mechanism [7,33]. MSU crystals are taken in lysosomes by cells such as macrophages. As they cannot be degraded, lysosomal damage occurs followed by release of the lysosomal protease cathepsin B which in turn triggers NLRP3 activation. The lysosomal rupture may cause the production of reactive oxygen species (ROS) [60,61]. In line with this, the activation of the inflammasome and subsequently caspase-1 in response to asbestos crystals was partially prevented when ROS synthesis was inhibited [6]. Release of ROS is a danger signal for surrounding cells, and NLRP3 has been shown to be activated by necrotic cells. In the third concept of inflammasome activation subsequently ROS is regarded to be the most important trigger. ROS may act both as extra- and intracellular danger signals.

Microparticles and inflammasome activation

Under *in vitro* conditions we found microparticles consisting of TiO₂ intracellularly in IEC and macrophages already at low concentrations (FIGURE 4). Their intracellular accumulation did not depend on endocytosis. TiO₂ aggregates were not restricted to a specific subcellular structure as observed by electron microscopy. The incubation of macrophages and IEC with low concentrations of TiO₂ resulted in assembly of the NLRP3-inflammasome, and correspondingly, the downstream effectors caspase-1, IL-1 β and IL-18 were activated and secreted. High doses of TiO₂ caused the generation of ROS in the tested cell types, as previously reported for other particulate structures [6]. In contrast to other findings with silica crystals [31], the uptake of TiO₂ did not lead to lysosomal rupture.

Increased levels of functionally active IL-18 in IEC from CD patients are indicative of an increased inflammasome activation. This is in line with findings of other groups reporting transcriptional upregulation of IL-18 and increased protein levels of mature IL-18 in mucosa of CD patients [62,63].

TiO₂ has mainly been subject of studies focusing on effects upon inhalation exposure so far. However, microparticles are also ingested on a daily basis in Western diet and its use as food additive is on the rise and not limited by food laws. The long-term impact of dietary microparticles such as TiO₂ and AlSi has not been studied, and the risk evaluation is inconclusive. Given that TiO₂ is also used in pharmaceutical pills, one can assume that local luminal concentrations of TiO₂ can reach substantial amounts. Depending on the properties of the pills, they disintegrate in different sections of the GI tract and may affect the intestinal epithelium in a very distinct and concentrated manner.

Further work needs to include other dietary microparticles such as AlSi and iron oxide and evaluate their capacity to activate the NLRP3-inflammasome and pro-inflammatory signalling in macrophages and IEC. Preliminary data show that for instance, iron oxide may be too large to penetrate the cells. An effect of TiO₂ on epithelial cells that has been reported by others was the reduction of transepithelial resistance [64,65].

Excluding microparticles from food could be a way to improve the wellbeing of IBD patients. Therefore, it would be worthwhile to initiate further studies with IBD patients to re-evaluate the impact of dietary microparticles on the IBD disease activity.

Conclusion

The immune function of IEC is a large field of research, and it has become evident that IEC play an important role as first line of defence, but also function as signal inducer for underlying immune cells. The tight regulation of pro- and antiinflammatory signalling is an important feature of IEC, which is out of balance in the state of chronic intestinal inflammations. The emerging role of genetic variations leading to an increased susceptibility to develop IBD is overwhelming, but the actions of many susceptibility genes are not fully understood.

The discovery of NLRP3 as platform for the recognition of various microorganisms and the more recent revelations on non-bacterial activators of NLRP3 has gained much attention in the past years. The identification of TiO₂ microparticles as activators of the NLRP3-inflammasome shows that not only immune cells, but also IEC are responsive to non-bacterial stimuli. The current data suggests that TiO₂ triggers inflammation, ROS and at high concentrations, also apoptosis. Since TiO₂ is consumed as white pigment in a number of foods and pharmaceutical pills, this finding may evoke questions about food safety, especially in the context of IBD. Further investigation in animal models but also in healthy individuals and IBD patients will help to re-evaluate the risk of microparticles in our food.

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Figure 1 NLRP3 inflammasome activation

Pathogen associated molecular patterns (PAMPs) and danger associated molecular patterns (DAMPs) such as reactive oxygen species (ROS) have been shown to activate the NLRP3 inflammasome. Upon activation, NLRP3 assembles to a multiprotein complex together with the adaptor protein ASC (apoptosis-like speck protein) and caspase-1. Caspase-1 undergoes autocatalytic activation, which enables the cleavage of its substrates pro-IL-1 β and pro-IL-18. The cleaved cytokines are then released by the cell to activate downstream targets.

Figure 2 Domains of the NLR family.

In the NLRP subfamily, the PYD domain enables interaction with other proteins, whereas NOD1, NOD2, IPAF and CIITA contain CARD domains for interaction. Furthermore, LRRs are found at the C-terminus of all NLR family members. NLR, nucleotide-binding domain and leucine-rich repeat containing; NALP=NLRP, NLR family, pyrin domain containing ; NOD; nucleotide binding oligomerization domain containing; IPAF, ice protease-activating factor; CIITA, MHC class II transactivator; PYD, pyrin domain; LRR, leucin rich repeats; FIIND domain with function to find; CARD, caspase recruitment domain family; AD, acid transactivation domain BIR, baculovirus inhibitor of apoptosis repeat. Figure from Tschopp et al.[66].

Figure 3 Different microparticles in light microscopy

The examined microparticles TiO_2 (A), a mixture of TiO_2 and iron oxide (B), iron oxide (C), AlSi (D) and a mixture of TiO_2 and AlSi, were diluted to $100 \mu\text{g/ml}$ and investigated with a light microscope. Scale bar: $100 \mu\text{m}$.

Figure 4 Eosin stained cells stimulated with TiO₂

TiO₂ particles could be found intracellularly. The most frequent uptake of particles could be detected in cells at the margin of the monolayer. Scale bars: 50 μ m (A), 40x, 50 μ m (B), scale bar 20 μ m (C).