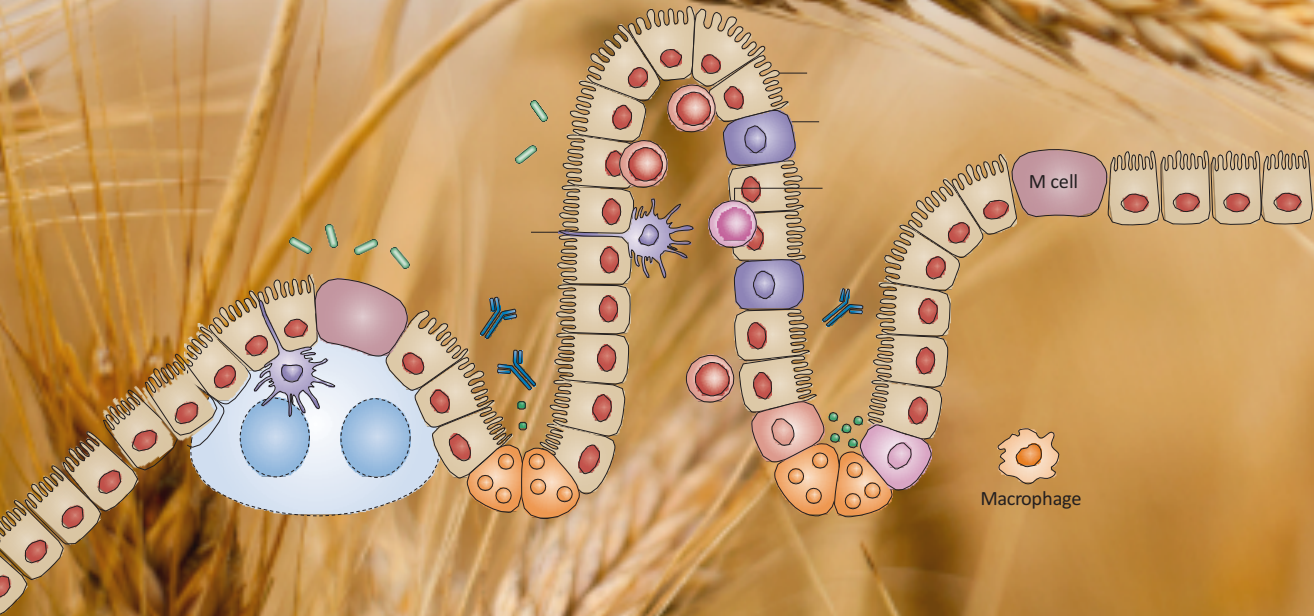# Non-digestible polysaccharides to support the intestinal immune barrier: *in vitro* models to unravel molecular mechanisms

Yongfu Tang

M cell

Macrophage

Propositions:

1. To support the immune system by dietary fibers, one should eat whole grain food.
   (this thesis)

2. Fibers steer macrophages.
    (this thesis)

3. Artificial intelligence is smarter than humans.

4. Plants have an adaptive immune system.
   (Spoel *et al.*, *Nature Reviews Immunology.* 2012. 12(2): 89-100)

5. Amyloid-ß peptide protects the brain.
   (Kumar *et al.*, *Science Translational Medicine.* 2016. 8(340): 340ra72)

6. To be yourself, select food smartly, otherwise you will be controlled by microbiota.

7. A guaranteed result of research is that it leads to new scientific questions.

Propositions belonging to the thesis, entitled:

Non-digestible polysaccharides to support the intestinal immune barrier: in vitro models to unravel molecular mechanisms

Yongfu Tang

Wageningen, 18 December 2017

Non-digestible polysaccharides to support the intestinal immune barrier: *in vitro* models to unravel molecular mechanisms

Yongfu Tang

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Non-digestible polysaccharides to support the intestinal immune barrier: *in vitro* models to unravel molecular mechanisms

Yongfu Tang

For my family

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Chapter 1

General introduction
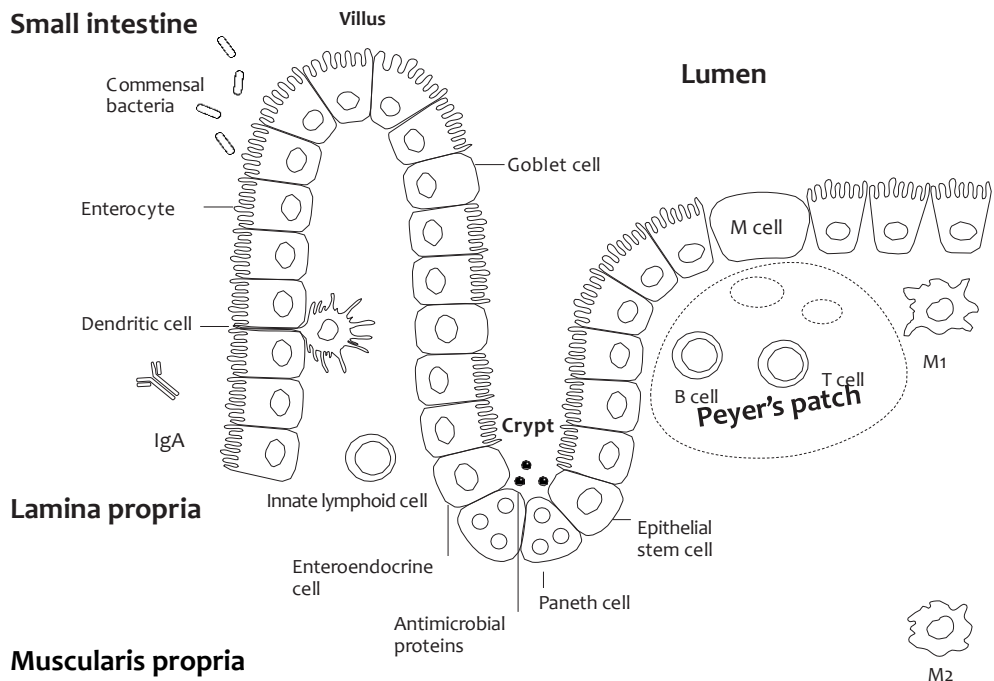
Intestinal immune barrier

The intestine has an intestinal immune barrier which is composed of, firstly, the epithelial barrier that is formed by a single layer of intestinal epithelial cells (IECs) composed of various cell lineages, such as enterocytes, enteroendocrine cells, Paneth cells, goblet cells, and Microfold cells (M cells), all differentiated from a common intestinal epithelial stem cells [1]. IECs provide a physical protective barrier for luminal factors harmful/undesired for the body and are involved in regulation of inflammatory processes [2]. IECs can regulate inflammation in response to antigens by secreting cytokines that attract and/or activate immune cells. IECs not only function as barrier but also absorb nutrients and other compounds from the intestinal lumen. This leads to the passage of an enormous number of antigens into the body. These antigens can be derived from commensal microbiota, pathogens, or ingested foods. To respond to these antigens, there is a large body of immune cells present in the intestines that have a gatekeeper function and form a second part of the intestinal immune barrier (Figure 1). These can be found in the lamina propria of the intestine, but also in Peyers paches (PPs), mesenteric lymph nodes (MLNs), colon patches, and appendix.

The intestinal immune system includes B cells, T cells, monocytes, macrophages, dendritic cells, and recently discovered innate lymphoid cells (ILCs) [3]. The intestinal homeostatic balance between tolerance and inflammation, requires complex interactions between intestinal microbiota, IECs, and intestinal immune cells. Intestinal homeostasis is jeopardized upon imbalance of tolerance and inflammation, and can lead to intestinal dysfunction. As IECs and macrophages are in the first line of immune defense, their response towards food components is pivotal to analyze the immunomodulatory effects of foods.

Intestinal epithelial cells

The intestinal epithelium is the largest surface of the human body. Often, its surface is described as approximately 300 m^2, but recent estimates are more in the order of 30 m^2 [4]. The principal function of IECs is that of absorptive enterocytes, to process and absorb nutrients to serve metabolic and digestive functions. Besides that, it also serves as a physical barrier that aids in maintaining intestinal homeostasis. IECs form an essential part of immunity, by maintaining the integrity, and through secretion of mucins by goblet cells, antimicrobial proteins by Paneth cells, and transcytosis of IgA after secretion by plasma cells [5]. IECs also express Toll-like receptors (TLRs) that respond to LPS, LPA, or flagellin which are pathogen-associated molecular patterns (PAMPs) from

microorganisms [6]. TLR2 and TLR4 are reported to be limitedly expressed by IECs. However, when intestinal damage induces inflammatory stress, TLR2 stimulation effectively preserves tight junction integrity and controls intestinal inflammation [7]. Mucosal inflammation and apoptosis in colitis were suppressed by oral TLR2 ligand treatment which resulted in restoring tight junction-associated barrier [7]. In addition, it was shown that TLR2 could be activated by a dietary fibre (Inulin β 2→1-fructans) [8].

Small intestine
Villus
Lumen
Commensal bacteria
Goblet cell
M cell
Enterocyte
Dendritic cell
M1
B cell
T cell
Peyer's patch
IgA
Crypt
Lamina propria
Innate lymphoid cell
Epithelial stem cell
Enteroendocrine cell
Paneth cell
Antimicrobial proteins
Muscularis propria
M2

Figure 1. The intestinal immune barrier is composed of an epithelial barrier formed by various cell types with immune cells residing in the lamina propria, Peyer's path, mesenteric lymph nodes, colon patches, and appendix (Adapted and modified from [9]).

M cells as specialized IECs, sample antigens and interact with microbiota to communicate with immune cells. These immune cells are concentrated in Peyer's patches. In addition, IECs recognize and integrate signals from the luminal side to intestinal immune cells [10] by secreting cytokines and chemokines that in turn modulate cytokine production by immune cells [11, 12]. Cytokines produced by the IECs in a steady-status function as anti-inflammatory and include TSLP, TGFβ, IL8, CCL20, CXCL10, IL18, IL33, and IL37. Those anti-

inflammatory cytokines are important to maintain immune tolerance, wound healing and also to recruit monocytes, neutrophils, eosinophils, lymphocytes, T cells, B cells, macrophages, and dendritic cells [11, 13, 14]. Specifically the role of TSLP in maintaining immune homeostasis by IEC that attenuate inflammation status and promote anti-inflammation is important, since TSLP secretion could inhibit IL12 production by DCs and activates Th2 polarizing cells [15].

Intestinal macrophages

Macrophages are described as one of the largest populations of cells which function in maintaining intestinal immune barrier [16]. Macrophages can be differentiated from monocytes in response to antigens presented by IECs or other immune cells [17]. In the steady state, these signals will be anti-inflammatory, produced by the IECs that are in close contact with the macrophages and resident Treg cells. This will lead to tolerant macrophages. In inflammatory status, those anti-inflammatory signals are reduced and inflammatory signals are produced which lead to inflammatory macrophages. Macrophages work as effector cells of the intestinal innate immune system. The contact between macrophages and IECs includes numerous immunoregulatory signals. This contact is in part mediated by the interaction of IECs and microbiota [18]. Through those interactions, macrophages direct the appropriate innate and adaptive immune cell responses and limited intestinal inflammation [19].

Two extreme phenotypes have been described for macrophages, and termed as classical macrophages (inflammatory macrophages or M1) [20] or alternative macrophages (tolerant macrophages or M2) [21]. Based on the most recent insights, an even larger variation of macrophage phenotypes has been described with an almost infinite continuum between the two most extremes. Therefore, it was suggested to indicate specific conditions that are used to polarize macrophages when describing macrophages subsets [22].

M1 are known to be formed from monocytes by exposure to IFNγ, LPS, or cytokines as TNFα, and are characterised by high expression of IL12, IL1β, TNFα, and IL6. This M1 inflammatory phenotype can be identified by specific markers such as IDO1, CXCL9, CXCL10, and CXCL11 [23]. M2 can be differentiated from monocytes after IL4 or IL13 stimulation and express a characteristic low level of IL12 and high levels of IL10 and scavenger receptors and receptors for mannose and galactose [21].

Beside the M1/M2 classification on a linear scale, a colour wheel of macrophages activation was introduced which was based on defined fundamental functions of

macrophages such as host defence, wound healing, and immune regulation [20]. In example, resolution-phase macrophages were defined as intermediate between M1 and M2, displaying predominantly an M2 phenotype, with weaker antibacterial activity than M1 macrophages, but expressing even higher levels of M1 markers than M1 (i.e., COX-2 and iNOS) [24]. These resolution-phase macrophages are supposed to have a role in mediating ILCs repopulation and restoring tissue homeostasis.

Based on their tissue localisation, intestinal macrophages were grouped as lamina propria macrophages (LpMs) and muscularis propria macrophages (MMs) [25, 26]. Those two types of intestinal macrophages were distinguished by morphological features and cell dynamics in distinct microenvironments in the intestinal tissue. LpMs show M1 features with highly expressed proinflammatory genes, such as IL1B, IL12B, and CCL24, and increased CD80 and increased oxidative burst. MMs typically show M2 features, with highly expressed Retnla, MRC1, CD163, and IL10, which are tissue protective and wound healing-related genes.

Non-digestible polysaccharide

Non-digestible polysaccharides (NDP), represent a class of food fibres composed of polymers of carbohydrates that are resistant to digestion in the stomach and small intestine but often are fermentable by the microbiota in the large intestine (Box 1) [27]. NDPs are typically long chain which can be made up of over a hundred thousand monomeric units but also can be shorter forming oligomers of only 10 sugar units. Beside differences in degree of polymerisation, they can have a complex composition of different types of sugars with variable linkages and different degrees of branching [28, 29]. NDPs have a proven physiological benefit to the intestine and immune related health, which will be discussed in more detail in next paragraphs.

Health effects of NDPs

NDPs initially received interest because for their function to increase stool bulk and improve laxation [30]. Later, also other physiological aspects of NDPs were demonstrated, both *in vitro* and *in vivo*. Increased intake of NDPs is associated with reduced incidence of cardiovascular disease [31] and colon cancer [32, 33]. Besides that, human trials have shown biological activity of specific NDPs on cholesterol lowering [34], serum glucose control [35], immunomodulation [36, 37], anticancer activity [38], and towards mitigation of inflammatory bowel disease [39].

Box 1 Dietary fibres classification

Dietary fibre has been recognized as having health benefits. However, often information on e.g. source, characterisation, etc. is lacking. Classification of dietary fibre was attempted based on their role in the plant, the type of polysaccharides, the ability to stimulate gastrointestinal activity, site of digestion, or products of digestion; however such classifications are still under debate [40]. The solubility or fermentability in an *in vitro* system were most commonly used to classify dietary components. Based on such criteria, fibres were classified as non-starch polysaccharide, non-digestible polysaccharides, or fermentable polysaccharides.

Non-starch polysaccharides (NSPs). NSPs include all the plant polysaccharides other than starch. They are the key components of the cell walls of various grains and cover a great variety of biological functions and chemical structures. The major polysaccharides of NSP are cellulose, pectin, β-glucans, pentosans, heteroxylans, and xyloglucan which cannot be hydrolysed by the endogenous enzymes of humans and monogastric animals [41].

Non-digestible polysaccharides (NDPs). Indigestible polysaccharides (fibre components) are all fibres that are resistant to digestion in the small intestine and that are fermentable in the large intestine. These polysaccharides are typically long polymeric carbohydrate chains containing up to several hundred thousand monomeric units. The polysaccharides differ by the number and type of monomeric units linked together, the order in the chain, the types of linkages between the various monomers, the presence of branch points in the backbone of the molecule, and those having acidic groups present (for example, uronic acids in pectins) [40].

Fermentable polysaccharides. Most fibre types are at least partially fermented, and, generally, well fermented fibres are soluble in water, while partially or poorly fermented fibres are insoluble. They can be fermented by intestinal microbiota into SCFA as major product [42].

These health effects of NDPs might link to the intestinal immune barrier modulation through NDPs consumption. Cytokines that are produced by IECs or immune cells have a crucial role in the pathogenesis of intestinal disease [43] and NDPs appear to have the potency to modulate these [44]. As an example, beneficial effects of NDPs on the treatment and prevention of IBD and direct effects on inflammation have been reported [44]. β-Glucans from either oat or shiitake inhibited pro-inflammatory factors such as TNFα, IL1β, IL6 and iNOS in colonic tissues in DSS-treated colitis mice [45, 46]. NDPs can

mediate the intestinal immune barrier through multiple ways such as changing fermentation products and microbiota composition, or activation of pattern recognition receptors (PRRs) (Box 2) and inducing cytokines expression and cellular responses.

Direct Interaction between NDP and intestinal immune barrier

The direct interaction of NDP with intestinal immunity was demonstrated by using fluorescent labelled β-glucans *in vivo* [47]. Those particles might adhere to and be transported by epithelial cells including M cells [48]. Orally administered β-glucans can also be taken up by macrophages and transported to other body tissue as spleen, lymph nodes, and bone marrow [47]. Macrophages subsequently can process these glucans and secrete β-1,3-glucan fragments that activate complement receptor 3 (CR3) resulting in enhanced granulocyte activity [47].

By direct interaction with IECs, NDPs demonstrated their ability to increase cytokine and chemokine gene expression and protein production in IECs (Caco-2 cells) [49]. This illustrates that NDPs could modulate immune responses via the IECs. Such effects are in some case linked to MW as low MW of cereal β-glucan (40 kDa) increased IL8 secretion both in a dose and time dependent manner, in contrast to high MW cereal β-glucan (123-359 kDa) [50]. Short chain NDPs (α3-sialyllactose and fructooligosaccharides) can reduce the production of proinflammatory cytokines as IL12 and TNFα in IECs (Caco-2 cells) via activation of PPARγ and peptidoglycan recognition protein 3 [51].

NDPs directly activate immune cells by increasing cell numbers and cytokines secretion in the intestine. NDPs have been shown to trigger innate immune responses by activating antigen presenting cells of the intestinal immune system [52, 53], increasing the number of lymphocytes and IFNγ production [54], and increasing the regulatory T cell population in the intestine [55, 56]. Oral intake of NDP decreased DSS-induced weight loss and intestinal inflammation in mice. This specific NDP was a multi-fiber mixture including insoluble and soluble polysaccharides [56]. Another well-described NDP are human-milk oligosaccharides (HMO) which are non-digestible and are expected to have an important role in early life. The immune effects of HMO are related to both changing microbiota composition and direct interaction with immune cells. HMO can skew the Th1/Th2 balance [57] and affect Th2-dominated immune response to peanut allergen in allergic individuals [58]. HMO can be recognized by CD209 which is expressed on macrophages and dendritic cells [59].

Box 2 Pattern recognition receptors (PRRs)

PRRs are receptors expressed on a variety of cells, of which mainly immune cells including macrophages and DCs, but also IECs. PRRs include the Toll-like receptors (TLRs), C-type lectin receptors (CLRs), Nod-like receptors (NLRs), and RIG-I-like receptors (RLRs) families. These receptors interact with evolutionary conserved structures on pathogens termed pathogen-associated molecular patterns (PAMPs).

Toll-like receptors (TLRs): The TLR family has 13 members in humans. TLRs can be expressed on the cell surface (TLR1, TLR2, TLR4, and TLR5), or intracellularly in endosomes (TLR3, TLR4, TLR7, TLR8, and TLR9). TLRs on intestinal epithelial cells can be activated by ligation of bacterial-derived structures such as lipopolysaccharides or lipoteichoic acid to induce antimicrobial peptide expression, IgA secretion and cell proliferation by the intestinal immune barrier [9].

C-type lectin receptors (CLRs): CLRs and their signalling pathways are essential for immune activation. Some of these CLRs were already reported respond to NDPs such as dectin-1, dectin-2, dectin-3, Mincle, and DC-SIGN. These CLRs could be activated by carbohydrate structures that are located in cell walls such as β-glucan and mannan [60-62]. CLR activation modulates both innate and adaptive responses. The response to CLR activation includes phagocytosis, cytokines secretion, and also helper T cells activation.

Nod-like receptor (NLR): NLR proteins are intracellular LRR (leucine-rich repeat)-containing proteins that can form an inflammasome upon binding of PAMPs. The formation of inflammasomes can also be induced by non-microbial danger signals such as microbial-derived products and cellular metabolic stress. NLRs can induce the production of pro-inflammatory cytokines such as IL1β and IL18 from pro-IL1β and pro-IL18 [63].

RIG-I-like receptor (RLR): The RLRs include RIG-I, MDA5, and LGP2 which detect a virus infection through viral RNA and initiate innate immune activation and inflammation to control infection. The RLRs cooperate in signalling crosstalk networks with TLRs to modulate both innate and adaptive immune response [64].

The direct activation of immune cells by NDPs might be mediated via PRR activation. For example, β-1,3-Glucans are well described for their interaction with dectin-1. These glucans are important components of cell walls from nearly all fungi, and are recognized by dectin-1 (CLEC7A), a c-type lectin-like receptor. Interaction between β-glucans and dectin-1 strongly influences intestinal immune barrier and health. Absence of dectin-1 in

colitis mice correlated to increased susceptibility to fungi [65]. The activation of dectin-1 by insoluble β-glucans initiates an innate immune response via phagocytosis and it could be blocked by soluble β-glucans [66]. Beside β-glucans from yeast, β-glucans from the shiitake mushroom also showed to inhibit IL8 gene expression and activate NFκB in DSS-induced colitis [67]. This could also be mediated via dectin-1 activation as α-Mannans, that are found in β-glucan preparations from shiitake, were shown to activate both dectin-2 and dectin-3 resulting in an inflammatory response [68] and also induced Th17 cell differentiation [69]. Inulin is another NDPs that can activate intestinal immunity through direct interaction with intestinal immune cells. Inulin β 2→1-fructans directly activated NF-κB/AP-1 for PBMCs via mainly TLR2 *in vitro* [70]. These direct effects related to chain length as shorter chain fructans induced a regulatory balance (IL10/IL12 ratio) more towards IL10 compared to longer chain fructans. In addition, β 2→1-fructans were also shown to modulate the immune system in conventional mice [71]. The immunomodulatory effects were again shown to be DP-dependent as both short chain and long chain β 2→1-fructans increased the number of Th1 cells in PPs, while only short-chain β 2→1-fructans increased Treg and CD11b⁻CD103⁻DCs in MLNs. This effect is likely not mediated by fermentation products and microbiota as no change in microbiota composition and SCFA production were reported. Microbiota-independent effects were also observed in germ-free mice, where β 2→1-fructans had no effects on Th1 cells, and only short chain β 2→1-fructans induced lower CD80 expression on CD11b⁻CD103⁻DCs in PPs [71].

Indirect interaction between NDP and intestinal immune barrier

Both via technological advances and because of an enormous increase in the understanding of the role of the microbiota, also the importance of microbial fermentation of NDPs and the formation of short chain fatty acids (SCFAs) from these have drawn attention [72, 73]. Fermentation of NDPs by intestinal microbiota results in changes of microbiota composition and SCFA production in both cecum and colon [74, 75]. Pectin supplementation modulated gut microbiota due to rapid fermentation, which in turn contributes to a shift in fermentation of cereal arabinoxylans [74]. Pectin structure and origin are important for shifting the fermentation site closer to distal parts of the large intestine in pig and for changing the composition of microbiota [75]. These types of responses to NDP could be source and composition independent since various NDPs were found to induce similar mucosa responses through PPARγ in mice [76]. The microbiota composition is very relevant for intestinal homeostasis. It has been reported that transplanted microbiota from obese female to germ-free mice increased total body

and fat mass, as well as induced obesity-associated metabolic phenotypes, compared to transplanted microbiota from lean female to germ-free mice [77]. The SCFA are considered as immunomodulatory products that are ligands of FFAR1, FFAR2, and GPR109A. Those GPCRs are broadly expressed on immune cells such as macrophages, dendritic cells, neutrophils, and eosinophils [78-80]. SCFA can target PPARγ and stimulate ANGPTL4 synthesis in vitro, and these effects were confirmed by feeding inulin to mice which subsequently showed an activation of PPARγ target genes and pathways in colon tissue [81]. By activating PPARγ and ANGPTL4, NDPs could regulate the lipid metabolism and protect against severe inflammation through SCFA production [82].
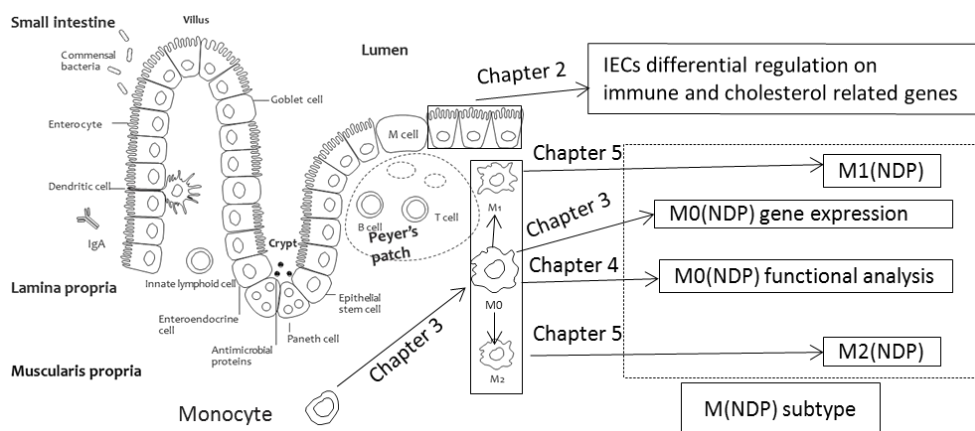
Figure 2. Overview of the structure of this thesis.

Outline of this thesis

This thesis focuses on the question to what extent and by which mechanisms NDPs directly interact with the intestinal immune barrier, and how the macrophages subsequently respond towards other immune cells (monocytes as an example) which could contribute to the observed intestinal immune support for NDPs. Although some of the NDPs immunomodulatory effects were already reported, this information is still limited regarding their direct interaction with the intestinal immune barrier. To investigate this, we chose five NDPs known to interact with the intestinal immune barrier to link our data to other findings (Figure 2). We selected Caco-2 and macrophages to represent the IEC and immune component of the intestinal immune barrier. Next to investigating the interaction of NDPs with those Caco-2 and macrophages separately, we

also applied NDPs on a co-culture of Caco-2 and macrophages. Besides this, we wanted to developed analysis strategies that could classify NDPs based on their bioactive activity.

In this thesis, research is based on 5 different NDPs with varying structure, monosaccharide composition, branch chain connection, and sources (**chapter 2**). These NDPs are Wellmune Soluble (a glucan from yeast), RG-I (a pectin from apple), OBG90 (a glucan from oat), LCES (lentinan containing extract from shiitake), and Naxus (rich in arabinoxylan from wheat).

As a first exploration of the potential direct effect of NDPs, research was based on intestinal epithelial cells, Caco-2 cells grown in transwell system for 21-days to resemble small intestine. The Caco-2 were exposed to various NDPs for 6 h. Cell layer integrity, whole-genome gene expression, and chemokine secretion were analysed and compared to characterize the result of the direct exposure (**chapter 2**).

Macrophages are amongst the most prevalent immune cells in the intestine. Therefore, as a next step we analysed the direct response of macrophages to NDPs (**chapter 3**). To this end we generated human macrophages from primary monocytes *in vitro* and established polarising cultures to acquire M1-like and M2-like macrophages to mimic inflammatory and tolerant macrophages in intestine, respectively. We did so using gene expression markers described in literature and found through microarray analysis. After stimulation with NDPs for 18 h, gene expression was analysed by qPCR and whole-genome gene expression of macrophages was analysed by microarray. This revealed that NDPs can stimulate macrophages towards an alternative subset macrophages (M_{NDP}). Subsequently the potential function of this M_{NDP} was studied by functional assays like phagocytosis capacity and antigen processing capacity. Specifically, the analysis of secreted cytokines and the effects on migration of immune cells revealed some of its potential function (**chapter 4**). Furthermore, it was studied whether NDPs can also modulate phenotype and function of M1 or M2 polarized cells towards this alternative subset M_{NDP}. This was to reveal the macrophage plasticity and NDPs immunomodulatory capacity which could be important *in vivo* as NDPs might encounter both types of macrophages in the intestine and should not over-stimulate in a certain stage (**chapter 5**).

Finally, in **chapter 6**, results from this thesis are discussed in relation to each other, to current literature and a general perspective in the research towards revealing immune support of NDPs by current and future models are given.

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Chapter 2

Gene expression analysis characterizes Caco-2 responses to non-digestible polysaccharides: a first step to a systematic comparison of dietary fibres

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Abstract

Scope: Dietary non-digestible polysaccharides (NDPs) have shown immunomodulatory activity following oral intake. The role of direct interaction with small intestinal epithelial cells, which are the first point of direct contact with NDPs, in this process is unclear. Here the transcriptional changes and cellular responses of small intestinal-like cells in response to different types of NDPs have been studied.

Methods and results: Polarized Caco-2 cells were exposed to NDPs (500 µg/ml) at the luminal side for 6 h. The NDPs reduced translocation of FITC-Dextran of 4 kDa across the epithelial layer, potentially through physical interference. Microarray analysis revealed unique gene expression characteristics in Caco-2 cells upon exposure to different NDPs. An arabinoxylan preparation from wheat and a lentinan containing extract from shiitake mushrooms induced transcriptional upregulation of the NF-κB pathway and production of CXCL10. Besides these immune-related changes by some NDPs, we also observed changes in transcriptional levels of some cell surface receptors (like TLR2, CD14 and GPCRs) and intracellular pathways, amongst which the cholesterol biosynthesis pathway.

Conclusion: NDP preparations are a direct trigger for intestinal epithelial cells to produce chemokines, via the NF-κB pathway. This may support communication towards the innate and adaptive immune cells in gut-associated lymphoid tissue.

Keywords: Arabinoxylan / β-Glucan / Microarray / NF-κB pathway / Oat

Abbreviations: FID, flame ionization detector; **IECs,** intestinal epithelial cells; **LCES,** lentinan containing extracted from shiitake; **MW,** molecular weight; **NDP,** non-digestible polysaccharide; **PRRs,** pathogen recognition receptors; **TEER,** trans-epithelial electrical resistance

1 Introduction

Polysaccharides are long chains of monosaccharide units bound together by glycosidic linkages. As the units, linkages, chain lengths and branching can vary, an almost infinite variation of polysaccharides can be found in nature, or be engineered [1, 2]. Dietary fibres or non-digestible polysaccharides (NDPs) are not hydrolysed by the endogenous enzymes in the small intestine of humans [3]. NDPs found in common foods, e.g. β-glucans, arabinoxylans, pectins, cellulose and chitin are, on a quantitative basis, nutritionally important dietary fibres and gained a lot of interest because of health effects as found by epidemiological research. Increased intake of dietary fibres is correlated with reduced incidences of cardiovascular disease [4] and colon cancer [5, 6] among others. Besides that, human trials have shown biological activity of specific NDPs on lowering of cholesterol [7], control of serum glucose [8], immunomodulation [9, 10], anticancer activity [11] and towards mitigation of inflammatory bowel disease [12].

Part of the bioactivity of these orally applied NDPs will result from effects on our gut and immune system. Indeed, receptors have been identified on different human gut and immune cells that detect the presence of NDPs and increase both the innate and adaptive host defence. An example are β-glucans found in the cell wall of yeast, fungi and bacteria, which can interact with the dectin-1 receptor [13, 14] and the complement receptor [15] as studied in immune cells (*i.e.* dendritic cells, macrophages, monocytes and lymphocytes) both *in vitro* and *in vivo* [16, 17]. Recently we reported that some NPDs can steer the polarisation of macrophages to a subset that can be distinguished from both inflammatory and tolerant macrophage subsets [18]. A less studied field is how β-glucans and other NDPs interact directly with small intestinal epithelial cells and whether the response of these cells contributes to the biological activity and maintenance of health.

Intestinal epithelial cells (IECs) form a physical barrier in the intestine. The enterocytes are the dominant lineage within the IECs (90% of total cells) [19] which can be mimicked *in vitro* by the use of Caco-2 cells. These cells can be differentiated towards polarised small intestinal-like cells [20], and express receptors such as pathogen recognition receptors (PRRs) [21, 22], G-protein coupled receptors, the low density lipid receptor [23], leptin receptor [24], and aryl hydrocarbon receptor [25]. Caco-2 cells have shown to be responsive to many food compounds [26, 27] although direct effects of NDPs have only been studied to a limited degree.

In this study, we exposed transwell-grown Caco-2 cells to five NDP preparations and analysed the effects on cell layer integrity, gene expression and chemokine secretion. This study can be used as a basis to build hypotheses on direct activity of NDPs towards

enterocytes which can be used as part of the classification of NDPs on the basis of their specific bioactivity.

2 Materials and Methods

2.1 Sources, preparation and handling of non-digestible polysaccharides

A water soluble β beta 1,3/1,6 glucan isolated from the yeast *Saccharomyces cerevisiae* (F3020 lot 12111-016) was provided by BioThera (Eagan, MN, USA). We will refer to this sample as Wellmune Soluble (Wellmune in tables and figures). A preparation of oat water soluble 1,3/1,4 beta-glucan was provided by Swedish Oat Fiber AB (Bua, Sweden) [28]. This sample will be referred to as OBG90. A polysaccharide from wheat flour rich in arabinoxylan (LC-AX 120305), was provided by BioActor (Maastricht, The Netherlands). This sample will be referred to as Naxus.

Shiitake mushrooms were grinded, boiled in water for 8 h, filtered over cheesecloth, and the supernatant ethanol precipitated after which the pellet was freeze dried and powdered. This water soluble, ethanol insoluble fraction contained mainly shiitake-derived beta 1,3/1,6 glucans (lentinan) and a minor amount of alfa 1,4/1,6 glucan, identified by susceptibility to hydrolysis by Termamyl (Novozymes). This sample is termed LCES (lentinan containing extract from shiitake).

A concentrate of rhamnogalacturonan I was prepared from apple juice. Apple juice was prepared under inert atmosphere at the INRA (Paris, France) experimental facility in Le Rheu. Apple juice was treated with pectin methyl esterase (30 UI/L, Rapidase SPE, DSM) and polygalacturonase (20 UI/L, PG, Megazyme) at 4 °C for 12 h in order to limitedly hydrolyse pectin. Sample was then concentrated by cross flow ultrafiltration using a ceramic membrane (0.5 m², diameter 25 mm, cut-off 8 kDa). A diafiltration step was further applied (dilution factor 1000), and the sample was finally centrifuged (2800 x g, 30 min, 4 °C) and freeze dried. This sample will be labelled as RG-I.

To improve solubilisation and to control the concentration in cell culture medium, NDPs were suspended in MQ (typically 0.5 mg/mL), boiled for 30 min, aliquoted, freeze dried and stored at -20 °C until use. Boiling did not result in breakdown of the samples as the molecular weight (MW), analysed by SEC-RI before and after the treatment, did not change (data not shown). Before use, the samples were re-suspended in DMEM containing 10% FBS at 37 °C.

Table 1. Characterization of the NDP samples.

		Wellmune	RG-I	OBG90	LCES	Naxus
Source		Yeast	Apple	Oat	Shiitake	Wheat
mono sugar composition	Rha	0.5	4.4	0.5	0.3	0.2
	Fuc	0.0	0.7	0.0	0.9	0.0
	Ara	0.0	23.5	0.6	1.8	24.5
	Xyl	0.0	1.8	0.7	0.4	29.5
	Man	0.0	0.3	0.0	4.6	1.2
	Gal	0.0	18.5	0.0	4.0	11.8
	Glc	93.1	1.4	88.2	59.0	9.2
	UA	1.0	29.7	1.1	1.6	1.0
Total sugar	percentage	94.6	80.3	91.1	72.6	77.4
Main basal structure		A β1,3 Glc backbone with short (4-6) side chains via β1-6 linkages	Rhamno-galacturonan I with branched L-arabinan, type II arabinogalactan and probably linear L-arabinan side chains	1,3/1,4 linked β-glucan	β1,3 Glc backbone and β1,6 linkages; Glc ramifications together with some starch β-1,4- and 1,4,6-glucose linkages	Arabinoxylans and additional arabinogalactan peptides together with some 1,3/1,4-linked β-glucans and β(1,4)-mannans
MW	kDa	~17	~95 and ~14	~600	~21000	~95
Protein	percentage	0.5	10.0		16.2	7.7
Starch	percentage	0.8	0.3	0.5	8.6	0.6
Solubility		Soluble	Soluble	Soluble (after preheating)	Partly insoluble	Soluble
Endotoxin levels	ng/mg NDP preparation	<0.01	~10.0	~5.0	~100	~10.0

Abbreviations: Rha, Fuc, Ara, Xyl, Man, Gal, Glc, UA: rhamnose, fucose, arabinose, xylose, mannose, galactose, glucose, uronic acid; total sugar: sum of the non-starch sugars.

2.2 Chemical analysis

Constituent neutral sugar analysis was based on acidic hydrolysis, and derivation into alditol acetates and GC as described [29]. In short, acid hydrolysis was performed with 1 M sulfuric acid (2 h, 100 °C) and subsequent derivation was performed as published [30]. Alditolacetates were separated on a DB 225 capillary column (J&W Scientific, Folsorn, CA, USA; temperature 205 °C, carrier gas H_2). Standard sugars solution and inositol as internal standard were used for calibration.

Secondly, uronic acids in hydrolysates were quantified using an automated metahydroxydiphenyl colorimetric acid method [31].

Chemical linkages between monomers were determined by permethylation analysis. Polysaccharides were dissolved in DMSO and methylated using the procedure described by Anumula and Taylor [32]. Methylated carbohydrates were hydrolysed with 2 M trifluoroacetic acid and converted to their alditol acetates. The partially methylated alditol acetates were analysed by GC/MS (TRACE-GC-ISQ, Therm, Breda, Netherlands) on a non-polar thermo scientific™ TraceGOLD™ TG-1MS GC Column (30 m x 0.25 mm x 0.25 µm), carrier gas H2 at 1.5 ml/min. The sample was injected at 240 °C. The oven temperature was maintained for 5 min at 60 °C and increased up to 315 °C (3 °C/min), where it was maintained for 2 min. The flow rate was set at 1.5 ml/min. The ion source temperature of the electron impact (EI) mass spectrometer was 230 °C. Masses were acquired with a scan range from m/z 100 to 500. Identification of partially methylated alditol acetates was based on their retention time and confirmed by mass spectra fragmentation. Quantitative detection was performed at 220 °C with a flame ionization detector (FID).

Starch quantification was performed according to McCleary et al. [33] using enzymatic digestion by α-amylase and amyloglucosidase and measurement of glucose released by HPAEC (CarboPac PA1 column, Thermo, isocratic mode, NaOH 100 mM). Standard glucose solutions and rhamnose as internal standard were used for calibration. MW analysis was carried out on an HPSEC system with on-line laser light scattering detection allowing absolute MW determination, and viscosity detection allowing determination of intrinsic viscosity. Solution at approximately 5 mg/ml (except for LCES: 0.5 mg/ml) were prepared in MilliQ water and filtered through a 0.45 µm filter (Millex-HV, PVDF) prior to injection. HPSEC was carried out at room temperature on a system consisting of a Shodex OH SB-G guard column (Shodex, Tokyo, Japan) and two Shodex OH-Pak columns SB-805HQ and SB-804HQ. Injection volume was 50 µL and columns were eluted at 0.7 ml/min with 50 mM sodium nitrate. A Viscotek tri-SEC model 270 was used for light scattering and differential pressure detection, and a Viscotek VE 3580 RI detector was
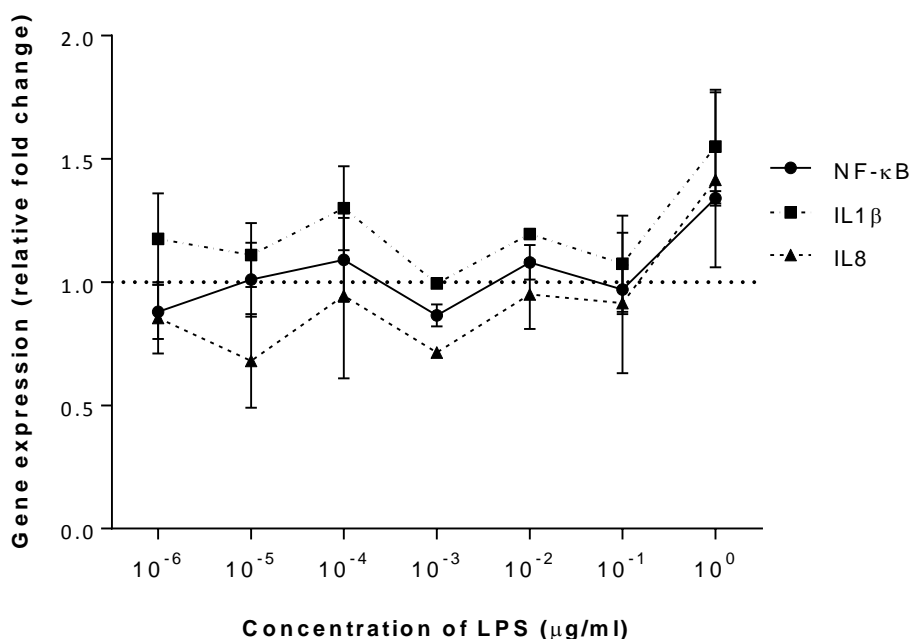
Figure 1. Response of transwell grown Caco-2 to a concentration range of LPS. Transwell grown Caco-2 cells were exposed to LPS ranging from 1 pg/ml to 1 µg/ml for 6 h. Gene expression of three marker genes (NF-κB, IL1β and IL8) were measured with qPCR and shown as fold change normalized to the geometric mean of GAPDH and RPLP0 and medium control (ΔΔct). The line chart shows mean values ± SEM, based on two independent experiments. Statistically significant differences between LPS and medium were calculated with one-way ANOVA by Graphpad Prism 6 and only found significant changes for IL-1β ($p < 0.05$) following 0.01 µg/ml LPS exposure.

used for the determination of polymer concentration. A refractive index increment per unit concentration increment (dn/dc) value of 0.146 ml/g was used for concentration determination. Data were collected with Omnisec 4.7 software (Viscotek), and all calculations on polymer peaks (concentration, average molecular weights, intrinsic viscosity) were carried out using Omnisec software.

Lipopolysaccharide (LPS) contamination was analysed by ToxinSensor™ Chromogenic LAL endotoxin Assay Kit (GenScript) and indicated as ng/mg NDP.

2.3 Cell culture and exposure

Caco-2 cells (American Type Culture Collection, Rockville, USA) were used between passage number 30 and 40 and grown on transwell inserts (Greiner Bio-one, Monroe, NC, USA) in 24-well plates. Cells were seeded with 3.375×10^4 cells in 150 µl per insert and grown for 21 days with 5% CO_2 at 37 °C. The medium DMEM (Gibco, Bleiswijk, The Netherlands) supplemented with 10% FBS (HyClone, Eindhoven, The Netherlands) was used to maintain Caco-2 cells and to replace medium of cells three times a week during differentiation on transwell inserts. One day before exposure to an NDP, we refreshed the apical and basolateral medium. The next day, cells were stimulated for 6 h in triplicate by replacing apical medium with medium containing an NDP or control treatment. Experiments were performed three times, including a medium control.

2.4 Cell Integrity and measurement of FD4 translocation

Trans-Epithelial Electrical Resistance (TEER) was measured using a MilliCell-ERS (Millipore) on transwell-grown Caco-2 before adding test samples. In all experiments, only Caco-2 monolayers with TEER values within the range of 280-400 $\Omega.cm^2$ were included in the experiment. TEER was measured directly, 1, 3 and 6 h after exposure of Caco-2 cells and depicted as percentages relative to medium stimulated cells at the same time point [34].

In order to measure permeability, 1 mg/ml FITC-dextran 4 kDa (FD4, Sigma, St. Louis, MO, USA) was added to the apical compartments with or without NDP [35]. After 8 h incubation, 100 µl medium was taken from the basolateral compartments for FD4 fluorescence intensity assay in multimode microplate readers (Infinite 200 PRO, Tecan, Männedorf, Switzerland) at 544 nm excitation and 590 nm emission wavelengths. FD4 concentration was calculated according to FD4 standard curve.

2.5 RNA extraction and cDNA synthesis

RNA was extracted and cDNA was prepared as described previously [36]. In brief, RNA extraction with 200 µL TRIzol (Invitrogen, Bleiswijk, The Netherlands) was followed by RNeasy (Qiagen, Venlo, The Netherlands) clean-up. RNA concentration and purity were checked using the Nanodrop spectrophotometer system (ND-1000 3.3; Nanodrop Technologies, Wilmington, DE, USA). The integrity of the ribosomal RNA was further checked using agarose (Eurogentec, Liège, Belgium) gel electrophoresis (1%). Only samples with a ratio (Abs 260/280 nm) between 1.8 and 2.1 were used for Reverse-Transcriptase quantitative Polymerase Chain Reaction (RT-qPCR) and microarray analysis experiments. cDNA was synthesised with iScript (Bio-Rad, Veenendaal, The Netherlands).
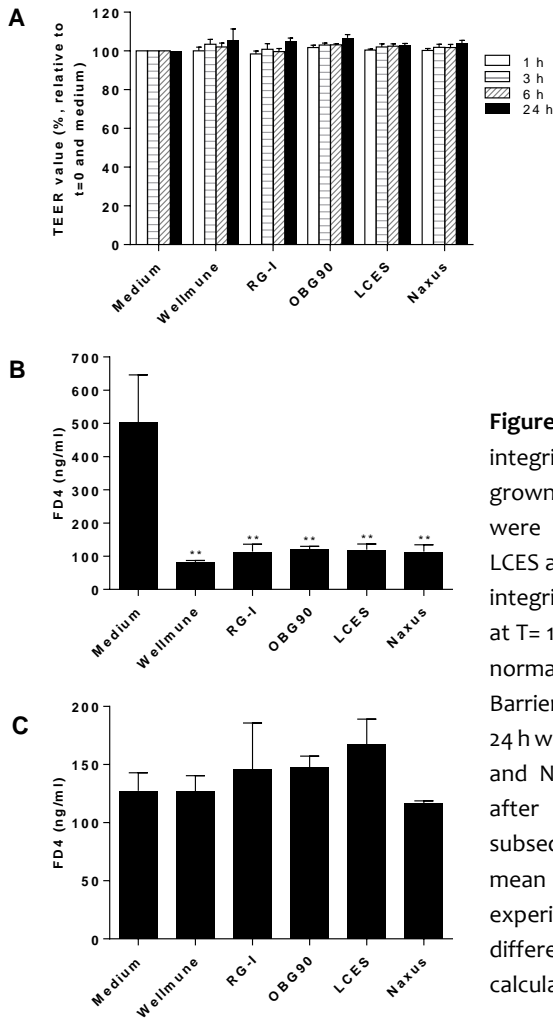
Figure 2. NDPs did not change monolayer integrity or barrier function of transwell grown Caco-2. Transwell grown Caco-2 cells were exposed to Wellmune, RG-I, OBG90, LCES and Naxus at 500 µg/ml. (A) Monolayer integrity was determined by measuring TEER at T= 1, 3, 6 and 24 and shown as percentage normalized to levels at T=0 and medium. (B) Barrier function was tested after exposure for 24 h with NDPs and subsequently 8 h with FD4 and NDPs. (C) Barrier function was tested after exposure for 24 h with NDPs and subsequently 8 h with FD4. Bar charts show mean values + SEM, n = 3 independent experiments. Statistically significant differences relative to medium were calculated with one-way ANOVA: **: $p<0.01$.

2.6 RT-qPCR

5 µL cDNA were mixed with 10 µl iQ™ SYBR Green supermix (Bio-Rad), together with forward and revers primers after which MQ was added to total 20 µl. Primer concentrations and sequences used for qPCR were chosen based on the sequences available in the GenBank database (www.ncbi.nlm.nih.gov), synthesized by Biolegio (Biolegio, Nijmegen, the Netherlands) and are listed in the supplementary Table 1. GAPDH, RPLP0, IL8, IL1 β and NF-κB were designed by Clone manager professional 9.
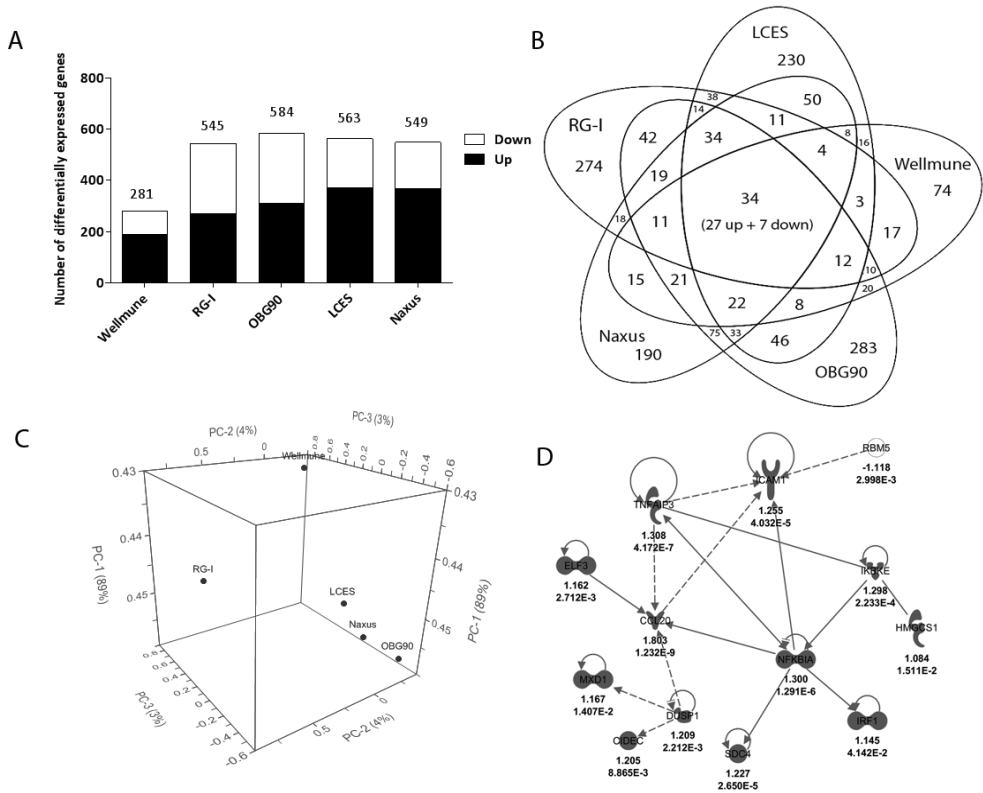
Figure 3. Transwell grown Caco-2 respond with different gene responses to exposure to various NDPs. Transwell grown Caco-2 cells were exposed to Wellmune, RG-I, OBG90, LCES and Naxus at 500 µg/ml and analysed for their gene expression profile using microarray. Genes that were assigned a UPC value >0.5 and a p-value <0.05 compared to medium control were included in these analysis. (A) Bar chart depicting the number of significantly up- and down-regulated genes for each NDP. (B) Venn diagram displaying NDP unique and shared differential gene expression responses. (C) PCA plot based on signal-log ratio compared to medium with numbers in parentheses indicating relative scaling of the principal variables. (D) IPA protein network based on the 34 genes in figure 3B excluded genes without demonstrated functional interaction with at least one other gene. Values represent fold change (upper number) and p-value (lower number) of differential gene expression following Naxus stimulation. Gene symbols in dark grey indicates a significant upregulation of expression and gene symbols in white indicates significant downregulation of expression. Data are expressed as mean values, n = 3 independent experiments.

CCL20 and CXCL10 were derived from the Harvard PrimerBank (http://pga.mgh.harvard.edu/primerbank/). The following thermal cycling conditions were used for amplifying the target sequences by C1000 thermal cycler (Bio-Rad): 90 s at 95 °C, followed by 40 cycles at 95 °C for 10 s, 58 °C for 10 s and 72 °C for 15 s, and a final elongation step at 72 °C for 2 min. qPCR were performed in duplicate, and all samples

were normalised to geometric means of GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) and RPLP0 (Ribosomal Protein, Large, P0) expression and medium ($\Delta\Delta$ct) using the qBase+ software (Biogazelle, Gent, Belgium). The melting curve and agarose gel analysis of amplified qPCR products were checked for amplification of a single product.

2.7 Microarray analysis

RNA quality was verified with the RNA 6000 Nano assay on an Agilent 2100 Bioanalyzer and was >8 (Agilent Technologies, Amstelveen, The Netherlands). Hybridization, washing and scanning of the Affymetrix Human Gene 1.1 ST array plate was performed according to standard protocols on an Affymetrix Gene Titan platform. Bioconductor was used to analyse the scans of the arrays [37]. Genes were considered to be expressed if the UPC algorithm valued expression with p>0.5 on at least one array [38]. Changes in gene expression were calculated as signal log ratio's or fold changes between NDP exposure and medium control. Genes which significant changed (p< 0.05 on at least one NDP, IBMT regularised paired *t*-test) were selected. The functional interpretation of gene signatures was executed using IPA 3.0 (Ingenuity, QIAGEN, Redwood, CA, USA). Microarray data were deposited in the Gene Expression Omnibus database.

2.8 Cholesterol uptake assay

The cholesterol uptake assay was performed using the Cholesterol Uptake Cell-Based Assay Kit (Cayman, Ann Arbor, MI, USA) according to the manufacturers' instructions. In brief, transwell grown Caco-2 were washed with medium (phenol red free DMEM without FBS) and exposed to medium (control) or NDPs at 500 μg/ml with or without NBD-cholesterol (20 μg/ml). After 24 h incubation, cells were trypsinized (Gibco), washed and resuspended in 100 μl of assay buffer. Fluorescence of the cells, apical and basolateral medium was analysed with excitation and emission at 485 nm and 535 nm, respectively. Fluorescent signals of NDP+NBD cholesterol exposed cells were subtracted with the signals of the NDP exposed cells to correct of auto-fluorescent signals. Subsequently the data were expressed as normalized to medium control.

2.9 ELISA analysis

Transwell grown Caco-2 were exposed to 500 μg/ml of NDPs for 24 h. Subsequently, Caco-2 spend medium, both apical and basolateral, was collected and chemokines (CCL20 (Sigma); CXCL10 (BioLegend, San Diego, CA, USA) measured by ELISA according to the manufacturer's instructions. The production of CCL20 and CXCL10 was calculated as total amount based on concentration measured and volumes in the transwell system (150 μl for apical and 700 μl for basolateral compartments).

2.10 Statistics

All parameters are presented as means ± SEM and differences between parameters were assessed for their significance with one-way ANOVA using Prism 6 (Graphpad, La Jolla, CA, USA). Differences with p<0.05 were considered significant.

3 Results

3.1 Biochemical analysis of NDP preparations

The five NDP preparations used in this study were characterized for composition, structure and endotoxin levels (Table 1). Wellmune Soluble, OBG90 and LCES were mainly composed of glucose, but have different backbone structure and side chain branches. OBG90 is a linear 1,3/1,4 linked β-D-glucan whereas LCES is a high MW, branched 1,3/1,6 linked β-D-glucan with a 1,3-linked backbone and 1,6-linked glucose side chains. Both preparations did not dissolve fully in regular culture medium. Wellmune Soluble is a low MW branched 1,3/1,6-linked β -D-glucan typical for yeast β-glucan. Naxus has a more complex composition with xylose, arabinose and galactose as major constituents with xylose and part of arabinose originating from arabinoxylans while galactose and part of the arabinose originate from arabinogalactan-proteins [39]. Naxus also contained some glucose originating from 1,3/1,4-linked β-D-glucan. The arabinoxylans, however, represents 62% of total polysaccharide in Naxus.

RG-I is composed of various neutral sugars (arabinose, galactose and rhamnose) and galacturonic acid. RG-I is primarily a mix of pectin backbone of rhamnogalacturonan I and homogalacturonan.

Besides NDP, the preparations contained proteins and other compounds of unknown origin which have not been examined in further detail. The levels of LPS contamination were determined as it is known that the presence of LPS can hamper certain *in vitro* analyses. The lowest contamination was found for Wellmune Soluble (<0.01 ng/mg) and the highest level of LPS contamination was found for LCES (~100 ng/mg). The NDP concentrations of the preparations ranged from 94.6% (*i.e.*, Wellmune Soluble) to 72.6% (*i.e.*, LCES). Sample concentrations for experiments were adjusted based on these total carbohydrate values to include equal amounts of NDPs.

3.2 Transwell grown Caco-2 cells are non-responsive to levels of endotoxin contaminations as found in NDP preparations

To investigate whether the observed levels of LPS contamination could lead to non-specific responses in Caco-2 cells, we exposed the Caco-2 cells for 6 h to a range of LPS

concentrations (Figure 1). Cell responses were determined by gene transcription analysis of known early response markers NF-κB, IL1β and IL8. Results indicate that gene expression of these markers are not significantly changed when cells are exposed to LPS concentrations of 1 μg/ml or below.

3.3 Selected experimental NDP concentration does not change transwell grown Caco-2 monolayer integrity

The main goal of the study is to investigate effects that NDP preparations can have on enterocytes mimicked by transwell 21-days-grown Caco-2 cells. First, the maximum NDP concentration was determined that did not induce unwanted side effects like apoptosis. Therefore, the cells were exposed to various concentrations of NDPs (ranging from 10 to 1000 μg/ml) for 6 h and we analysed cell viability and cell layer integrity. Cell viability was measured using the MTT assay and cell layer integrity was determined by measuring TEER. Both results indicated that Caco-2 cells were not significantly negatively (nor positively) affected by NDP concentrations up to 500 μg/ml. At an NDP concentration of 500 μg/ml all samples also contain LPS contamination levels below the threshold level (Table 1).

Time series experiments confirmed that 500 μg/ml NDP did not affect Caco-2 TEER values following 6 h of incubation. In addition, significant changes were also not observed after a shorter incubation period of 1 or 3 h, or a longer incubation period of 24 h (Figure 2A). FD4 is a marker for paracellular transport and was added together with the NDP and, following 8 h of exposure, the basolateral FD4 content was measured. Lowered FD4 levels in the basolateral compartment of NDP-exposed cells compared to control indicated that FD4 translocation was reduced by all NDPs (Figure 2B). However, when the Caco-2 cells were pre-incubated with NDP and subsequently exposed to FD4 in the absence of NDP, then no effect of the NDPs on FD4 translocation was observed (Figure 2C).

3.4 NDPs induce unique gene expression patterns in transwell grown Caco-2

To determine gene transcription responses of transwell grown Caco-2 following 6 h exposure to NDP we performed microarray analysis. NDP-mediated gene expression was compared to medium control and results showed that NDPs significantly affected expression of genes in Caco-2 cells. We observed the lowest number of significant differentially expressed genes for Wellmune Soluble (*i.e.* 281 genes) and the highest number for OBG90 (*i.e.* 584 genes) (Figure 3A). A minority of 34 genes were significant differentially expressed by all NDPs (Figure 3B). This overlap is relatively small as for instance OBG90 induces differential regulation of 283 genes that are not affected by any

Table 2. List of genes significantly affected in expression by all NDP preparations.

Genes	Wellmune	RG-I	OBG90	LCES	Naxus
ABI3BP	1.32	1.31	1.33	1.25	1.24
APOBEC3C	1.11	1.10	1.16	1.13	1.14
C17orf78	1.18	1.17	1.23	1.27	1.21
CCL20	1.21	1.18	1.35	1.56	1.80
CDC42EP2	1.13	1.14	1.12	1.11	1.13
CIDEC	1.19	1.19	1.22	1.15	1.20
CYP1A1	1.41	1.26	1.64	1.74	1.47
DUSP1	1.15	1.19	1.25	1.22	1.21
ELF3	1.13	1.14	1.14	1.14	1.16
GATAD2A	1.12	1.13	1.09	1.11	1.09
GNA13	1.13	1.14	1.11	1.12	1.17
HMGCS1	1.07	1.07	1.09	1.09	1.08
HNF4G	1.16	1.22	1.20	1.18	1.23
ICAM1	1.12	1.12	1.15	1.23	1.25
IKBKE	1.15	1.19	1.17	1.29	1.30
IRF1	1.18	1.21	1.16	1.25	1.14
MXD1	1.16	1.16	1.17	1.21	1.17
NFKBIA	1.15	1.11	1.20	1.22	1.30
OCEL1	1.13	1.10	1.11	1.16	1.12
PELO	1.13	1.17	1.13	1.10	1.10
SDC4	1.10	1.14	1.19	1.20	1.23
SLC20A2	1.11	1.19	1.17	1.11	1.15
SPRED2	1.08	1.09	1.08	1.17	1.07
STC1	1.31	1.26	1.27	1.39	1.26
TCN2	1.18	1.21	1.24	1.15	1.17
TNFAIP3	1.15	1.12	1.20	1.21	1.31
WBP2	1.10	1.14	1.13	1.19	1.14
B3GALNT2	-1.17	-1.15	-1.18	-1.22	-1.19
CLASP2	-1.12	-1.10	-1.19	-1.12	-1.12
FAM86EP	-1.17	-1.19	-1.22	-1.17	-1.15
PLXNC1	-1.16	-1.16	-1.14	-1.20	-1.19
RBM5	-1.08	-1.08	-1.09	-1.08	-1.12
SNORD52	-1.22	-1.41	-1.22	-1.33	-1.29
TAS2R13	-1.50	-1.57	-1.76	-2.03	-2.05

Values represent fold changes of gene expression, n=3 experiments. Statistically significant differences were calculated with IBMT regularised paired t-test: all genes have $p<0.05$.

other NDP. These strong differences between NDPs are reflected in the PCA plot showing a unique response of Caco-2 cells to each individual NDP (Figure 3C).

Interestingly, genes involved in immune-related processes are amongst the genes whose expression shows significant changes in response to all NDPs (Table 2), such as the 13 genes within the network centred around NFKBIA (Figure 3D). Furthermore, two genes related to G protein-coupled receptors (GPCRs) based signalling (GNA13 and TASR13) and two genes within the cholesterol pathway (HMGCS1 and APOBEC3C) showed significant changes for all NDPs.
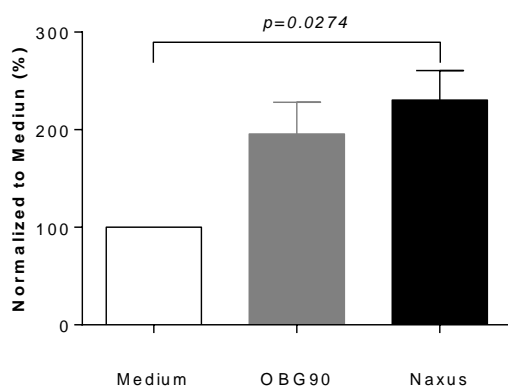
Figure 4. Naxus increases cholesterol uptake but not transport by Caco-2. Transwell grown Caco-2 cells were exposed to OBG90 and Naxus with or without fluorescent labelled cholesterol (20 µg/ml) for 24 h. Cholesterol uptake in cells was normalized to medium control. Bar chart shows mean values + SEM, n = 3 independent experiments. Statistically significant differences were calculated with one-way ANOVA: *: $p < 0.05$.

3.5 NDPs mediated minor change in expression of cholesterol pathway-related genes in transwell grown Caco-2

The NDP OBG90 is structurally related to the oat beta-glucan that has demonstrated cholesterol lowering effects *in vivo* [40]. Based on that knowledge and the observation that two genes related to the cholesterol pathway were identified within the overlap between all NDPs, we set out to detect other differentially regulated genes associated to the cholesterol pathway making use of the IPA software. Significant differentially expressed genes induced by the exposure to at least one NDP, compared to medium control, were listed in table 3. Results indicate that gene expression showed subtle, but significant changes and most differentially regulated genes were found to be upregulated. To analyse how the changes in gene expression induced by the NDPs relate to cholesterol uptake, Caco-2 cells were exposed to OBG90 and Naxus in the presence of fluorescently labelled cholesterol. Both OBG90 and Naxus increased the cholesterol uptake in the enterocytes and following Naxus stimulation we observed a significantly

increased fluorescent signal in the Caco-2 ($p<0.028$; Figure 4). No significant difference in fluorescent signal was found in the basolateral compartment indicating that the cholesterol was incorporated or utilised by the cells and not excreted, or at least not basolaterally (data not shown).

Table 3. Differential expression of cholesterol-metabolism-related genes.

Gene class	Genes	Wellmu	RG-I	OBG90	LCES	Naxus
Cholesterol biosynthesis	NSDHL	1.05	1.05	1.04	1.06*	1.08*
	DHCR24	1.06*	1.06*	1.03	1.04	1.06*
	EBP	1.05	1.12*	1.11*	1.10	1.13*
	FDFT1	1.07	1.09	1.11*	1.09	1.11*
	GGPS1	1.04	1.10	1.07	1.16*	1.08
	HMGCR	1.10*	1.08	1.07	1.09*	1.05
	HMGCS1	1.07*	1.07*	1.09*	1.09*	1.08*
	IDI1	1.06	1.07	1.13*	1.08	1.15*
	MSMO1	1.16*	1.09	1.14*	1.16*	1.17*
	MVD	1.07	1.13*	1.14*	1.13*	1.12*
	SC5DL	1.11	1.06	1.14*	1.06	1.16*
	SQLE	1.06	1.09*	1.05	1.09*	1.09*
Cholesterol influx	LDLR	1.08*	1.09*	1.07	1.12*	1.08*
	SCARB1	1.08	1.09*	1.06	1.06	1.05
HDL related	APOA1	1.05	1.04	1.07*	1.03	1.05
	APOD	1.17*	1.12*	1.18*	1.07	1.18*
Chylomicron related	MTTP	1.12*	1.14*	1.15*	1.11*	1.10
	APOBEC3	1.11*	1.10*	1.16*	1.13*	1.14*

Listed genes are part of the cholesterol biosynthesis pathway as described in IPA and shown significant different expression by at least one NDP exposure compared to medium control. Values represent fold changes of gene expression, n=3 experiments. Statistically significant differences were calculated with IBMT regularised paired t-test: *: $p<0.05$.

3.6 Most strongly differentially regulated genes in response to Naxus and LCES demonstrated a link to immunomodulation

In order to identify specific effects of NDPs towards transwell-grown Caco-2 cells, the top 10 up and down-regulated genes were studied (Table 4). Besides the genes that demonstrated significant differential expression by all NDPs (among others CYP1A1, CCL20 and ABI3BP; see Table 2), NDPs also show specific changes in gene expression, such as the group of five metallothioneins upregulated specifically by RG-I. From an immunological perspective, the CXCL10, CCL20, BIRC3, CASP1, PTGS2 and CYP1A1 genes

Table 4. Top 10 up- and down- regulated genes for each NDP.

Genes	Wellmune	Genes	RG-I	Genes	OBG90	Genes	LCES	Genes	Naxus
ARHGEF35	1.79	MT1X	1.50	CYP1A1	1.64	CYP1A1	1.74	CXCL10	1.83
MSL3P1	1.44	ARHGEF5	1.37	ARHGEF35	1.63	CCL20	1.56	CCL20	1.80
CYP1A1	1.41	MT1E	1.35	CHCHD7	1.45	ARHGEF35	1.52	BIRC3	1.59
ZNF487P	1.39	MT2A	1.33	HSPA1L	1.44	CAV1	1.46	CYP1A1	1.47
SNORD82	1.36	HSPA1L	1.32	RPE65	1.41	MIR215	1.41	ZNF701	1.44
ZNF701	1.34	ABI3BP	1.31	ORM2	1.37	MOGAT2	1.40	ALOX5AP	1.39
ABI3BP	1.32	MT1G	1.28	MSL3P1	1.37	STC1	1.39	SPARC	1.36
STC1	1.31	MT1H	1.28	ALOX5AP	1.36	BIRC3	1.39	MOSPD1	1.34
HSPA1L	1.31	GPR133	1.28	KMO	1.35	TAT	1.39	PTGS2	1.34
CST2	1.30	CYP1A1	1.26	CCL20	1.35	CXCL10	1.36	CASP1	1.33
SLC38A4	-1.22	CHI3L1	-1.41	LOC100131541	-1.64	SNORA75	-1.29	TAS2R20	-1.42
COX17	-1.22	TAS2R14	-1.41	TAS2R13	-1.76	TAS2R4	-1.31	METTL15	-1.43
CRIPAK	-1.23	SNORD52	-1.41	SNORA14B	-1.79	SNORD52	-1.34	TRBV5-1	-1.44
METTL6	-1.24	KRTAP5-7	-1.43	SNORA64	-1.84	TRNT1	-1.35	TAS2R19	-1.46
SNORD78	-1.25	TRIM34	-1.46	LOC100508226	-1.93	ACSM2B	-1.36	FLJ42393	-1.49
OSGEP	-1.28	FAM47E	-1.46	SNORA41	-1.96	TAS2R31	-1.36	UGT1A10	-1.63
TMEM99	-1.29	TAS2R13	-1.57	VTRNA1-1	-1.97	GOLGA8A	-1.39	LOC100131541	-1.77
C19orf40	-1.30	GOLGA8A	-1.57	SNORA14A	-1.97	GOLGA8B	-1.40	LOC100508226	-1.79
ACSM2B	-1.36	GOLGA8B	-1.64	SNORA52	-1.97	SNORD56B	-1.75	SNORD56B	-1.79
TAS2R13	-1.50	UGT1A10	-1.83	SCARNA4	-3.02	TAS2R13	-2.03	TAS2R13	-2.05

Values represent fold changes of gene expression, n = 3 experiments. Statistically significant differences were calculated with IBMT regularised paired *t*-test: All genes have p<0.05.

are of particular interest. These were all within the top 10 of genes which transcription was significant upregulated by Naxus and transcription of most of them was also significantly upregulated by LCES. These genes are involved in NF-κB signalling (BIRC3 and CASP1), code for chemokines (CXCL10, CCL20), or are involved in the regulation of inflammation processes via prostaglandins (PTGS2 and ALOX5AP).

3.7 NF-κB pathway in transwell-grown Caco-2 is most strongly activated by Naxus and LCES exposure

Next, we performed IPA analysis with all significant differentially regulated genes. All NDPs demonstrated to significantly alter gene expression of genes in the NF-κB downstream pathway (Figure 5 and Table 5). LCES (Figure 5D) and Naxus (Figure 5E) again showed the largest number of genes with altered transcription downstream of the NF-κB pathway. In addition, we analysed gene transcription of upstream NF-κB regulators which suggests CD14 and TLR2 might be involved in Naxus or RG-I and OBG90, respectively, mediated NF-κB pathway activation (Table 5). The gene transcription of other NF-κB upstream regulators following Naxus stimulation are presented in more detail in figure 5F. Furthermore, IPA predicted, indicated by z-scores above 2, that NDPs activate the NF-κB complex and REL-associated protein involved in NF-κB heterodimer formation, nuclear translocation and activation (Table 5). G-protein coupled receptors (GPCRs) are suggested to be involved in regulation of NF-κB pathway but are not integrated in the IPA pathways. Since a number of GPCRs were found to be in the top 10 of transcriptionally downregulated genes following Caco-2 exposure to NDPs, we generated an overview including all the GPCRs that revealed significant different expression following at least one NDP exposure (Table 6). This indicated that gene transcription of in particular GPCRs classified as taste receptors were downregulated by the NDPs.

3.8 Microarray data could partially be confirmed by qPCR and ELISA

As some of the most relevant changes in gene expression were connected to the NF-κB pathway and downstream effector genes, a validation of these effects was performed. First, transcriptional changes of two chemokine encoding genes, CXCL10 and CCL20, were analysed by qPCR. The qPCR data confirmed that the upregulation of gene expression of CXCL10 and CCL20 by Naxus were both significant (Figure 6A and B). Although both genes were also in the top 10 of LCES, we could only confirm significant increased expression of CCL20 by qPCR.

Subsequently, ELISAs were performed to analyse whether observed changes in gene expression translated to increased protein production and subsequently excreted
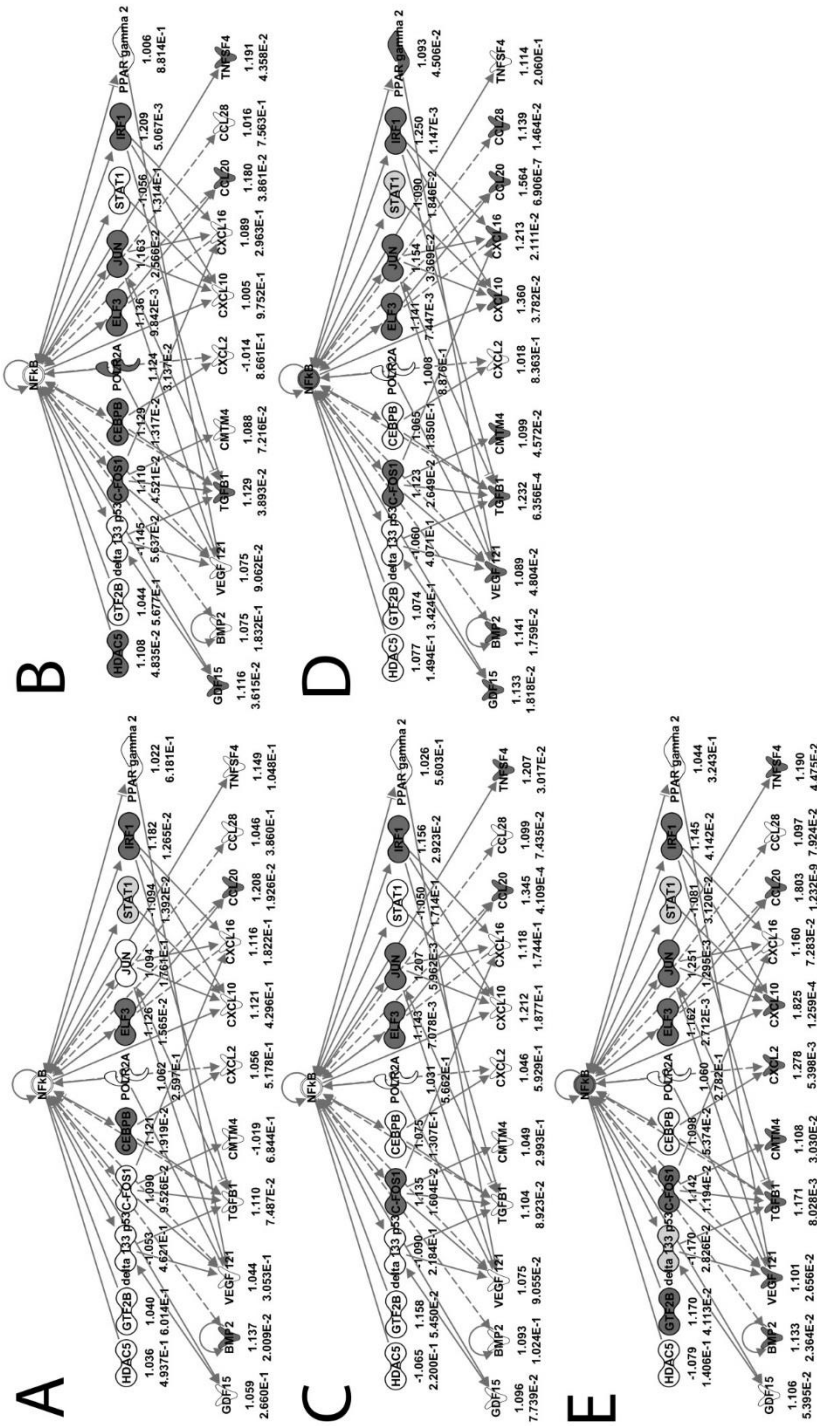
Figure 5. Transcription of genes linked to the NF-κB pathway in transwell grown Caco-2 cells were exposed to Wellmune, RG-I, OBG90, LCES and Naxus at 500 μg/ml and analysed for their gene expression profile using microarray. Genes that were assigned a UPC value >0.5 and a p-value <0.05 compared to medium (control) were included in these analysis. The schemes show gene expression responses in the pathway downstream of NF-κB when cells were treated with Wellmune (A), RG-I (B), OBG90 (C), LCES (D), and Naxus (E) and upstream of the NF-κB pathway when Caco-2 cells were treated with Naxus (F). The pathways were generated using IPA software and genes were removed of which transcription was not significantly affected by any of the NDPs. Values represent fold change (upper number) and p-value (lower number). Gene symbols in dark grey indicate significant upregulation of expression and gene symbols in light grey indicate significant downregulation of expression and gene symbols in white were not significantly affected.

Table 5. Differential expression of family members and inhibitor genes related to NF-κB complex following NDP exposure of Caco-2.

Genes	Wellmune	RG-I	OBG90	LCES	Naxus
Upstream regulators					
CD14	1.28*	1.17	1.21*	1.26*	1.30*
ICAM1	1.12*	1.12*	1.15*	1.23*	1.25*
TLR2	1.14	1.23*	1.27*	1.21	1.18
EGFR	1.08	1.07	1.09*	1.13*	1.06
ANXA1	1.15	-1.04	1.16	1.16	1.28*
CAV1	1.17	1.22*	1.21*	1.46*	1.17
BCAR1	1.08	1.07	1.09	1.10	1.06
GJA1	1.00	1.09	1.14*	-1.01	1.02
DLG1	-1.04	-1.07	-1.10*	-1.06	-1.06
Transcriptional change					
NFKB2	1.03	1.04	1.09	1.20*	1.20*
IKBKE	1.15*	1.19*	1.17*	1.29*	1.30*
IKBIP	1.11	1.24*	1.07	1.14	1.14
NFKBIA	1.15*	1.11*	1.20*	1.22*	1.30*
NFKBIB	1.10	1.04	1.25*	1.13	1.17*
NFKBIE	-1.01	1.08	1.11	1.10	1.13*
Predicated activation state					
EGFR	NA	1.05	1.01	1.87	2.77#
NFKB complex	2.61#	3.11#	2.44#	3.09#	3.30#
ReLA	2.22#	2.78#	2.61#	2.79#	2.97#

Genes were included when significantly affected in gene expression by one or more NDPs. Values represent fold changes of gene expression, n=3 experiments. Statistically significant differences were calculated with IBMT regularised paired t-test: *: $p<0.05$. According IPA predication: #: Z score >2, Activated. NA: Not available.

chemokines into the medium. To this end, we analysed CCL20 and CXCL10 protein in medium following exposure of transwell-grown Caco-2 to NDPs for 24 h. Results demonstrated that both LCES and Naxus induced a significant production of CXCL10 when compared to medium stimulated Caco-2 (Figure 6C). However, no significant changes in the medium levels of CCL20 could be observed (Figure 6D).

4 Discussion

Dietary non-digestible polysaccharides have been reported to have many biological effects, including cholesterol lowering and immunomodulatory effects. As these biological effects will partly be exerted through direct interaction with the IEC of the gut, we set out to study direct effects of NDPs towards enterocytes, making up approximately 90% of all IECs [19] .To this end, five NDP preparations were selected which originated from different sources (yeast, mushroom, oat grain, wheat grain and apple fruit), differed in compositional structure, molecular weight and solubility. The composition of the NDP preparations were in line with commonly reported compositions of such polysaccharide preparations in literature [39, 41-44], except for the MW of Wellmune Soluble which was smaller (~ 17kDa) than was previously reported for soluble yeast beta-glucan preparations (*i.e.* ~150kDa) [15]. This is important in comparing results from this research with previous studies as it has been demonstrated that bioactivity of polysaccharides can depend on the structure. For instance, lowering the molecular weight can reduce or abolish bioactivity [45, 46].

IEC models like Caco-2 are a promising strategy in the analysis of bioactivity of NDPs as Caco-2 cells can be grown routinely, display many *in vivo* features of enterocytes and have been shown to respond to different interventions including food compounds. Caco-2 cells are used in many labs, resulting in a collection of microarray data that can be systematically exploited to support dedicated enterocyte analysis and the generation of new hypothesis on the relation between food and intestinal function [47]. For research towards the effects of food compounds, such as NPDs, Caco-2 cells have another advantage as they are reasonably tolerant to LPS upon differentiation and polarisation [48]. The sensitivity of Caco-2 cells to LPS, as determined here by studying the threshold for induced expression of major response genes, has been set around 0.1 µg/ml. Compared to immune cells such as monocytes and macrophages this makes the Caco-2 cells approximately 1×10^7 times less sensitive [49]. LPS concentrations present in the NDPs used in our exposure experiments most likely do not affect the Caco-2 gene responses directly, although we cannot exclude synergistic effects between LPS and NDPs.

To fully characterize gene transcription responses of Caco-2 to NDPs we performed microarray analysis which provides a broad overview of transcriptional responses. IPA-mediated analysis of all significant differentially regulated genes demonstrated a potential role for NF-κB signalling in recognition of NDPs by Caco-2. Our analysis indicated that NDP stimulation of Caco-2 cells significantly upregulated transcription of NF-κB-complex-related genes and upstream regulators and IPA predicted RelA as
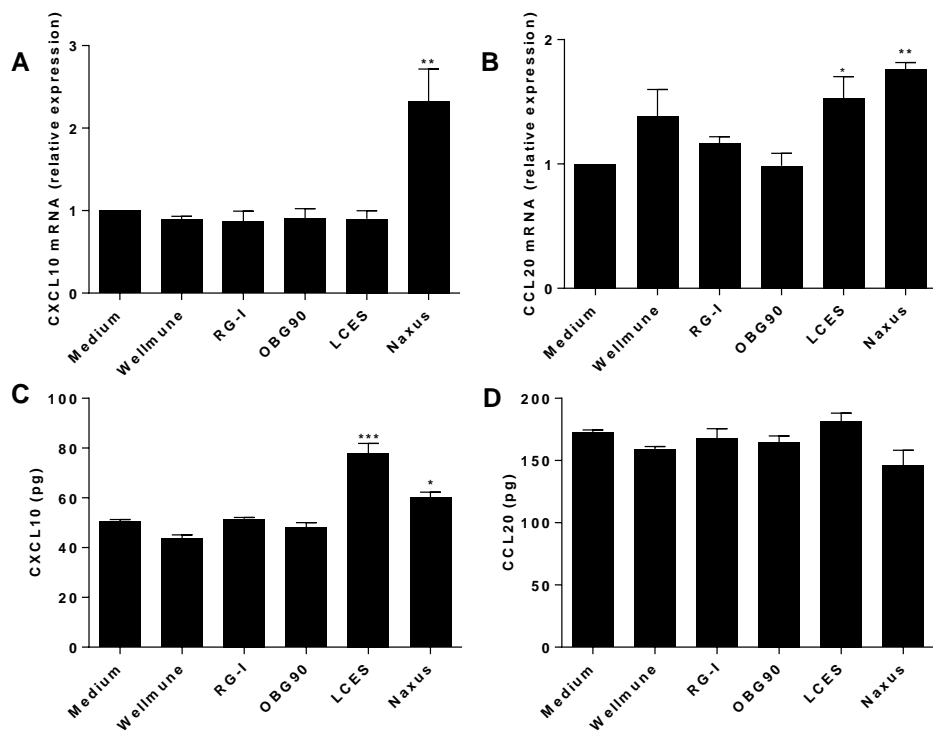
Figure 6. Q-PCR and ELISA validate CXCL10 expression and protein production following Naxus and LCES stimulation of Caco-2. Transwell grown Caco-2 cells were exposed to Wellmune, RG-I, OBG90, LCES and Naxus at 500 μg/ml for 6 h and analysed for gene expression by qPCR (A and B) or exposed for 24 h and analysed for protein level by ELISA (C and D). Bar charts represent (A) CXCL10 and (B) CCL20 gene expression are shown as fold change normalized to geometric mean of GAPDH and RPLP0 and medium control ($\Delta\Delta$ct) and (C) CXCL10 and (D) CCL20 protein totals in the medium 24 h after exposure. Protein analysis was performed on a mixture of apical and basolateral medium and corrected for combined medium volumes to yield a total protein amount. Bar chart shows mean values + SEM, n = 3 independent experiments. Statistically significant differences were calculated with one-way ANOVA: *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$.

activated upstream regulator. This is in line with previous findings for enterocytes [50, 51] and other immune cells [52] including *in vivo* and *in vitro* studies [53-55].

We also observed enhanced transcription of TLR2 and CD14, which are known upstream activators of NF-κB [50]. This supports previous studies that identified TLR2 as receptor for other NDPs [56, 57]. Besides, we also observed upregulation of CYP1A1, a key enzyme in elimination and detoxification of compounds in different cell types, which has been shown to be under control of TLR2 activation [58]. This TLR2 receptor and co-receptor

CD14 have been found to work together in pathogen recognition and innate defence response against bacterial pathogens like *Yersinia spp, Listeria* and *Rickettsia akari* [59] and are important for proper immune function [60]. This would be in line with the hypothesis that some NPSs can activate the innate defence responses similar as pathogens.

The NF-κB complex controls, amongst a number of functions, the production of cytokines and chemokines such as CXCL10 and CCL20. Gene expression of these signalling molecules was upregulated by Naxus and LCES.

Table 6. Differential expression of GPCRs following NDP exposure of Caco-2

Genes	Wellmune	RG-I	OBG90	LCES	Naxus
GPR108	1.07	1.06	1.08	1.08	1.10*
GPR114	1.13	1.15*	1.09	1.09	1.09
GPR133	1.18	1.28*	1.21*	1.13	1.14
GPR160	1.15*	1.05	1.12	1.08	1.09
TAS2R13	-1.50*	-1.57*	-1.76*	-2.03*	-2.05*
TAS2R14	-1.25	-1.41*	-1.38*	-1.20	-1.39*
TAS2R19	-1.31	-1.32	-1.38*	-1.33	-1.46*
TAS2R20	-1.15	-1.27	-1.30	-1.27	-1.42
TAS2R3	-1.15	-1.35*	-1.42*	-1.30	-1.27
TAS2R31	-1.14	-1.27	-1.26	-1.36*	-1.40*
TAS2R4	-1.21	-1.27	-1.41*	-1.30*	-1.42*
TAS2R50	-1.15	-1.27	-1.22	-1.24	-1.38*

Genes were included when significantly affected in gene expression by one or more NDPs. Values represent fold changes of gene expression, n=3 experiments. Statistically significant differences were calculated with IBMT regularised paired *t*-test: *: $p<0.05$.

Further analysis demonstrated that CXCL10 protein secretion was also increased following Naxus and LCES stimulation of Caco-2, in contrast to CCL20. CXCL10 production by Caco-2 cells was previously reported in response to stimulation with various mixtures of pro-inflammatory cytokines [61]. CXCL10 was demonstrated to control intestinal epithelial cell movement together with IL-13 [62] and, together with CXCR3, held responsible for recruiting CXCR3-positive immune cells in inflammatory bowel disease [63]. It remains to be proven whether observed significant changes in gene transcription of, for instance, ICAM-1 also contributes to attraction of immune cells as these have shown to be involved in adhesion of activated T cells [64]. This potential stimulation of immune cell migration by intestinal enterocytes following exposure to NDPs is

supported by previous results for 1,3/1,6-β-glucan from baker's yeast that has been shown to increase the number of intraepithelial lymphocytes in the intestine in C57BL/6 mice [65].

NDPs are known for their capability to bind compounds like proteins, cholesterol, bile acids, fatty acids, and phytochemicals [66-68]. Interestingly, we found that NDPs reduced FD4 translocation without changing monolayer integrity, but only when NDPs and FD4 were present in the medium simultaneously. Pre-incubation with NDPs had no effect on FD4 translocation suggesting that exposure to NDPs did not change active paracellular transport of Caco-2 but that a direct physical interaction between NDPs and FD4 is preventing translocation. In addition, NDPs increased gene transcription of genes involved in the cholesterol biosynthesis pathway and uptake of cholesterol from medium. Those results indicate that NDPs induce cellular cholesterol levels potentially leading to changed characteristics of cell membranes [69]. The induction of cholesterol related genes was found for all NDPs, but most strongly for LCES, Naxus and OBG90. Oral administration of oat β-glucan was shown to support maintenance of normal [70] and reduction of blood cholesterol levels [71] by a proposed direct binding of bile acids in the lumen and thereby increased faecal excretion [72]. Arabinoxylans from wheat bran also have been shown to reduce total plasma cholesterol and LDL cholesterol concentrations in hamsters [73]. Our results suggest that a cellular mechanism of intestinal cells might contribute to this cholesterol lowering effects of NDPs *in vivo*.

In this study, we have attempted to characterize NDPs through their stimulation of enterocytes. We performed gene expression profiling in combination with dedicated functional assays such as analysis of barrier integrity, translocation, cholesterol regulation and chemokine production. The results demonstrated strong differences in Caco-2 responses to the various NDPs, indicating that these analyses discriminate between functional activities of the NDPs. The next step in research to characterize the bioactivity of these NDPs should be human intervention studies to couple our *in vitro* data to *in vivo*. Cholesterol lowering effects, which are relatively easy to study and for which trial design and biomarkers are validated, can be expected for some of these NDPs. Besides, the immune modulatory effects could be studied by vaccination efficacy studies and blood cellular parameters.

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Supplementary Table 1. Primer details

Genes	Forward (5'-3')	Reverse (5'-3')	Accession
GAPDH	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG	NM_002046
RPLP0	GCAATGTTGCCAGTGTCTG	GCCTTGACCTTTTCAGCAA	NM_001002.3
IL8	CTGATTTCTGCAGCTCTGTG	GGGTGGAAAGGTTTGGAGTATG	NM_000584.2
IL1β	GTGGCAATGAGGATGACTTGTTC	TAGTGGTGGTCGGAGATTCGTA	NM_000576.2
NFκB	TGAGTCCTGCTCCTTCCA	GCTTCGGTGTAGCCCATT	NM_003998.2
CCL20	TGCTGTACCAAGAGTTTGCTC	CGCACACAGACAACTTTTTCTTT	NM_004591
CXCL10	GTGGCATTCAAGGAGTACCTC	TGATGGCCTTCGATTCTGGATT	NM_001565

ª Gene accession number refer to the sequence on which the primer design was based.

Chapter 3

Macrophages treated with non-digestible polysaccharides reveal a transcriptionally unique phenotype

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Abstract

Dietary non-digestible polysaccharides (NDPs) might promote intestinal health via immuno-modulation. Immunomodulatory effects of NDP are most likely brought about by antigen processing cells such as macrophages that populate the intestine, although the mechanisms are still poorly understood. We validated the in vitro model of M1 and M2 macrophages to mimic the intestinal inflammatory and tolerant macrophages using literature and microarray-derived gene markers. All these markers were used to characterise the macrophage phenotype following NDP stimulation. This identified an alternative subset, termed M_{NDP}, which commonly modulated a set of 126 genes, involved in migration, metabolic processes, cell cycle, and inflammatory immune function. This gene-based analysis for macrophage subsets provides an additional tool to characterise NDP bioactivity for their in vivo potential.

Keywords: Arabinoxylan; β-Glucans; Intestinal immunity; Macrophage polarisation; Gene expression markers

Abbreviations: CLRs, C-type lectin receptors; **GO-BP,** gene ontology – biological process; **IECs,** intestinal epithelial cells; **LCES,** lentinan containing extracted from shiitake; **M_{NDP},** NDP specific macrophage subset; **NDP,** non-digestible polysaccharide; **PCA,** principal component analysis; **PRRs,** pattern recognition receptors; **TLRs,** toll‑like receptors;

Graphical abstract:
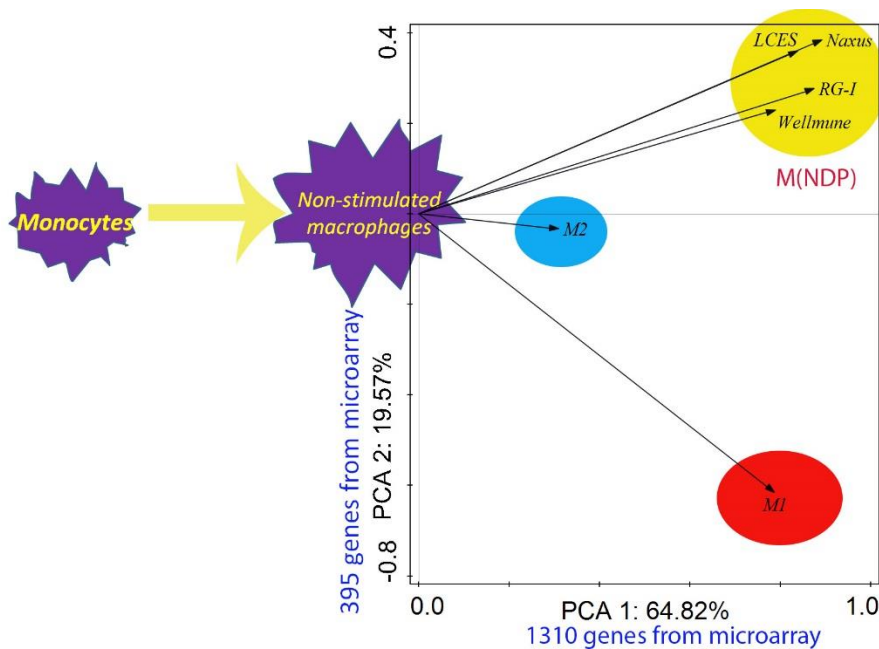
1 Introduction

Dietary non-digestible polysaccharides (NDPs), amongst which glucans [1], pectin [2], arabinoxylan [3], inulin [4], and resistant starch [5] are described to possess immune modulatory activity. NDPs have been shown to trigger innate immune responses by activating antigen processing cells of the intestinal immune system [6] and to increase the regulatory T cell population in the intestine and tip the balance towards an anti-inflammatory phenotype [7]. The mechanism behind this is of great interest and only partially understood. Also, NDPs have demonstrated to directly interact with the microbiota and gut epithelium. Fermentation of NDPs by intestinal microbiota results in changes of microbiota composition and short chain fatty acid production in both the cecum [8] and the colon [9]. We and others found that NDPs were also able to directly interact with intestinal epithelial cells. NDPs have been demonstrated to increase cytokine and chemokine gene expression and protein production in Caco-2 cells [10], illustrating that NDPs might modulate immune responses via the intestinal epithelial cells.

Since a large amount of macrophages are to be found in the intestine [11], these cells likely are important in maintaining homeostasis. Within these intestinal macrophage population tolerant and inflammatory subsets have been described [12]. Tolerant macrophages are the main subset found in homeostasis. They have a low turnover and are located underneath the submucosal region in close proximity to CD4 T cell populations [13] and are sustained by stromal cues [14]. These cells lack expression of CD14, TLR2, CCR2 and MHC class II and production of TNFα [15], but demonstrate phagocytic and bactericidal activity [14], produce IL-10 and balance T cell differentiation away from Th17 cells towards a regulatory T cell phenotype [13]. Inflammatory macrophages appear to be absent under homeostatic conditions but are rapidly recruited in large numbers upon infection following CCR2-dependent monocyte migration and differentiation [16]. These macrophages have a high rate of turnover and express various pattern recognition receptors and produce TNFα and multiple other pro-inflammatory cytokines [17, 18]. Both subsets were found to be able to directly sample the luminal content by extending protrusions between epithelial cells in a CX3CR1-dependent manner [19, 20].

We are interested in these direct interactions of NDP with intestinal macrophages and used *in vitro* cultures and conditions to generate non-polarised macrophages (*i.e.* M0), inflammatory or classically activated macrophages (*i.e.* M1), or tolerant or alternatively activated macrophages (*i.e.* M2). Besides markers that have been described in the literature additional markers were selected and subsequently used to characterise the macrophage subset after exposure to four different NDPs. This revealed that NDPs

induce a distinct phenotype characterised by putative epigenetic modification resulting in differential transcription of genes related to migration, metabolic processes, cell cycle progression and inflammatory responses and which combined may prove a practical resource for studying the effects of NDPs *in vitro* and potentially *in vivo*.

2 Materials and Methods

2.1 NDP characterisation

We used four NDPs being: Wellmune Soluble (referred to as 'Wellmune' in table and figures), RG-I, LCES and Naxus. Wellmune Soluble is a 94% pure preparation of water soluble β-1,3/1,6-glucan of approximately 17 kDa isolated from the yeast *Saccharomyces cerevisiae* and provided by BioThera (Eagan, MN, USA). RG-I is a 80% pure preparation of rhamno-galacturonan-I with branced L-arabinan, type II arabinogalactan and probably linear L-arabinan side chains with a molecular weight (MW) of approximately 95 and 14 kDa isolated from apple and provided by INRA (Paris, France). The isolation procedure including pectin methyl esterase (30 U/L, Rapidase SPE, DSM) and polygalacturonase (20 UI/L, PG, Megazyme, IL, USA) treatment at 4°C for 12 h after which the extract was concentrated by cross flow ultrafiltration using a ceramic membrane (0.5 m², diameter 25 mm, cut-off 8 kDa). Next, a diafiltration step was applied (dilution factor 1000), and the sample was centrifuged (2800 x g, 30 min, 4 °C) and lyophilized. LCES (lentinan containing extract from shiitake) is a 73% pure preparation of β-1,3/1,6-glucan, with a minor amount of α-1,4/1,6 glucan and mannose and galactose units, of approximately 21000 kDa isolated from shiitake mushrooms. For isolation of LCES the mushrooms were minced and boiled for 8 h in water, filtered over cheesecloth, and LCES was precipitated with ethanol and finally lyophilized. Naxus is 77% pure preparation of arabinoxylans with additional arabinogalactan peptides together with some 1,3/1,4-linked β- glucans and β(1,4)-mannans with an average MW of ~95 kDa isolated from wheat and provided by BioActor (Maastricht, The Netherlands).

To remove LPS contamination, NDPs were treated as previously described [21]. In brief, RG-I was treated with alkaline-ethanol at 4 °C for 5 h, while LCES and Naxus were treated with alkaline-ethanol at 56 °C for 5 h. NDPs were lyophilized following neutralization with HCl. For Wellmune Soluble, low in LPS contamination, no LPS removal treatment was applied. For all NDPs, LPS levels were below those that would result in immune stimulations [21].

2.2 Primary macrophage differentiation and polarization

Human monocytes were obtained from buffy coats from healthy donors (Sanquin, Nijmegen, The Netherlands) using the quadroMACS system and CD14 microbeads according to the manufacturer's protocol (Miltenyi Biotec, Leiden, The Netherlands). A written informed consent was obtained before sample collection. Monocytes were differentiated into macrophages following 7 days culturing in RPMI 1640 - Glutamax medium (Gibco, Bleiswijk, The Netherlands) supplemented with 10% fetal bovine serum (FBS, Hyclone, Eindhoven, The Netherlands), 1% MEM non-essential amino acids (Gibco), 1% Na-pyruvate (Lonza, Breda, The Netherlands), 1% Pen/strep (Sigma, St. Louis, MO, USA) and 50 ng/ml MCSF (R&D systems, Minneapolis, MN, USA) at 1×10^6 cells / 2 ml / well in a 24-well plate. Half of the medium was replaced after 3 and 5 days of culture with medium containing 100 ng/ml MCSF. On day 7, macrophages were polarized by replacing all the culture medium with medium containing no additions (M0), 20 ng/ml TNF-α (R&D systems) and 20 ng/ml IFN-γ (R&D systems) (M1), 20 ng/ml IL-4 (R&D systems) (M2) or 500 µg/ml NDPs without MCSF and incubated for 18 h.

2.3 RNA extraction and cDNA synthesis

RNA was extracted by lysing cells with 200 µL TRIzol (Invitrogen, Bleiswijk, The Netherlands) for each well in the 24-well plates. This was followed by RNeasy (Qiagen, Venlo, The Netherlands) clean-up according to manufacturer's protocol. The integrity of the ribosomal RNA was analysed using agarose (Eurogentec, Liège, Belgium) gel electrophoresis (1%). RNA concentration and purity were checked using the Nanodrop spectrophotometer system (Nanodrop Technologies, Wilmington, DE, USA) and only samples with a ratio (Abs 260/280 nm) between 1.8 and 2.1 were used for qPCR and microarray analysis. Subsequently, cDNA was synthesised with iScript (Bio-Rad, Veenendaal, The Netherlands) according to manufacturer's protocol.

2.4 qPCR

qPCR was performed as described elsewhere [22]. In brief, 5 µl cDNA was mixed with 10 µl iQ™ SYBR Green supermix (Bio-Rad), forward and reverse primers, and milliQ to a total volume of 20 µl. Primers were derived from the Harvard Primerbank (http://pga.mgh.harvard.edu/primerbank/) or designed using Clone Manager Professional 9 and synthesized by Biolegio (Nijmegen, The Netherlands). Primers and their final used concentrations are listed in supplementary Table 1. The following thermal cycling conditions were used for amplifying the target sequences: 90 s at 95 °C, followed by 40 cycles at 95 °C for 10 s, 58 °C for 10 s and 72 °C for 15 s, and a final elongation step

at 72 °C for 2 min and performed on the CFX96 Touch Real-Time PCR Detection System (Bio-Rad). qPCR was performed in technical duplicate, and all samples were normalised to geometric means of PUM1 and RPLP0 expression and medium-stimulated macrophages using the qBase$^+$ software (Biogazelle, Gent, Belgium). To allow comparison in terms of fold change, transcript levels below detection thresholds in M0 were set at 40 cycles.

Table 1. Most differentially expressed genes in M1 and M2 macrophages as determined by microarray

Subsets	Genes	Mean	Description
	IDO1	122.00	indoleamine 2,3-dioxygenase 1
	IRG1	121.95	immunoresponsive 1 homolog (mouse)
	ANKRD22	105.57	ankyrin repeat domain 22
	GBP5	91.66	guanylate binding protein 5
	GBP4	89.87	guanylate binding protein 4
	RARRES3	59.52	retinoic acid receptor responder (tazarotene
	IFITM1	50.21	interferon induced transmembrane protein 1
	GCH1	48.16	GTP cyclohydrolase 1
	HAPLN3	46.59	hyaluronan and proteoglycan link protein 3
	VCAM1	43.32	vascular cell adhesion molecule 1
	CLEC6A	42.85	C-type lectin domain family 6, member A
	UBD	40.35	ubiquitin D
M1	SCIN	39.03	scinderin
	RSAD2	36.36	radical S-adenosyl methionine domain containing 2
	IDO2	35.93	indoleamine 2,3-dioxygenase 2
	PLA1A	35.15	phospholipase A1 member A
	LAMP3	34.38	lysosomal-associated membrane protein 3
	IL31RA	32.74	interleukin 31 receptor A
	IFI27	32.71	interferon, alpha-inducible protein 27
	CXCL11	32.48	chemokine (C-X-C motif) ligand 11
	FAM26F	32.20	family with sequence similarity 26, member F
	CCL15	31.92	chemokine (C-C motif) ligand 15
	CLEC4E	30.64	C-type lectin domain family 4, member E
	C1S	29.65	complement component 1, s subcomponent
	ETV7	27.78	ets variant 7

	Gene	Fold	Description
	IL17RB	59.72	interleukin 17 receptor B
	ALOX15	42.4	arachidonate 15-lipoxygenase
	CD180	31.9	CD180 molecule
	ST8SIA6	30.81	ST8 alpha-N-acetyl-neuraminide alpha-2,8-
	CHDH	24.61	choline dehydrogenase
	GPR34	24.54	G protein-coupled receptor 34
	CCL13	20.16	chemokine (C-C motif) ligand 13
	FCER2	19.13	Fc fragment of IgE, low affinity II, receptor for
	MAOA	18.49	monoamine oxidase A
	CCL26	18.08	chemokine (C-C motif) ligand 26
	PTGFRN	18.01	prostaglandin F2 receptor inhibitor
	TSPAN15	17.8	tetraspanin 15
M2	THBD	17.65	thrombomodulin
	PPM1L	17.26	protein phosphatase, Mg2+/Mn2+ dependent, 1L
	TMEM37	17.02	transmembrane protein 37
	CD200R1	15.65	CD200 receptor 1
	DNASE1L3	15.59	deoxyribonuclease I-like 3
	KLHL13	15.1	kelch-like family member 13
	FAXDC2	14.69	fatty acid hydroxylase domain containing 2
	CTNNAL1	14.38	catenin (cadherin-associated protein), alpha-like 1
	TMEM71	14.35	transmembrane protein 71
	GNG2	13.81	guanine nucleotide binding protein (G protein),
	METTL7A	13.63	methyltransferase like 7A
	GSDMA	13.22	gasdermin A
	IL1R1	13.1	interleukin 1 receptor, type I

Values represent fold changes of gene expression of M1 over M2, or M2 over M1, n=3 different donors.

2.5 Microarray analysis

Microarray analysis was performed as described previously [23]. Briefly, RNA quality was verified with the RNA 6000 Nano assay on an Agilent 2100 Bioanalyzer (Agilent Technologies, Amstelveen, The Netherlands). Hybridization, washing and scanning of the Affymetrix Human Gene 1.1 ST array plate was performed on an Affymetrix Gene Titan platform according to standard protocols. Scans of arrays were analysed using package from Bioconductor project. Raw signal intensities were obtained by Robust Multi-array Average method. Probe sets were defined using remapped chip definition file (CDF) based on Entrez gene database. Individual Genes were considered to be

expressed if UPC algorithm values are above 0.5 on at least one array [24]. Genes were selected that meet the cut-off of absolute fold change > 1.8 and false discovery rate corrected p<0.05 (IBMT regularised paired t-test) on at least one treatment. The functional interpretation of gene signatures was executed using IPA 3.0 (Ingenuity, QIAGEN, Redwood, CA, USA). Microarray data were deposited in the Gene Expression Omnibus (GEO) database.

2.6 Statistics

All parameters are presented as means ± SEM and differences between parameters were assessed for their significance with one-way ANOVA using Prism 6 (Graphpad, La Jolla, CA, USA). Differences with p<0.05 were considered significant. To relate changes in groups and genes, principal component analysis [25] was used as implemented in the CANOCO 5 software package [26].
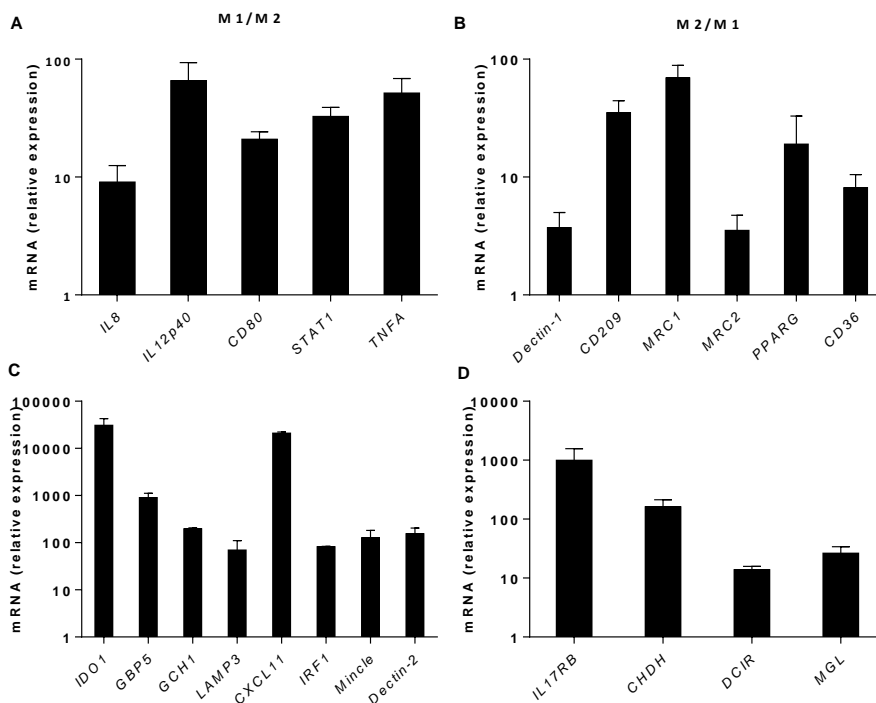
Figure 1. Literature marker list substantiates macrophage polarization and is expanded using microarray. Primary monocytes were differentiated for 7 days to macrophages and were subsequently polarised to M1 or M2. Markers identified in literature for M1 (A) and M2 (B) and by

microarray for M1 (C) and M2 (D) were tested for gene expression in polarised macrophages by qPCR. Results are shown as fold change between M1 and M2 macrophages with bars representing mean values ± SEM, n=3 or 6 different donors.

3 Results

3.1 Polarised primary macrophages can be characterized by specific gene transcription markers

Inflammatory and tolerant macrophages were generated according to established protocols [27]. Stimulation with TNFα and IFNγ yielded inflammatory macrophages (M1) and stimulation with IL-4 yielded tolerant macrophages (M2). To verify polarization a qPCR-based approach was used to analyse the gene expression of reported M1 markers (*i.e.* IL8, IL12p40, CD80, TNFA, and STAT1) and M2 markers (*i.e.* Dectin-1, CD209, MRC1, MRC2, CD36 and PPARG). Fold change expression ratios of M1 markers and M2 markers indicated that the polarisation protocol applied indeed yielded M1 and M2 macrophages (Figure 1A and 1B).

Considering the lack of standards in macrophage culture and polarization, we decided to further characterize our M1 and M2 macrophages by microarray analysis. Interestingly, when ranking the differential expressed genes, some genes revealed to be more discriminative than most genes reported in literature. The top 25 most differentially expressed genes between M1 and M2 macrophages are listed in Table 1. From this table, we selected genes to serve as M1 and M2 markers based on their expression level or potential function in macrophage reactivity towards NDPs (supplementary Table 2). qPCR analysis to validate these genes, similar to the markers reported in literature, verified the high expression levels and discrimination between M1 and M2 macrophages (Figure 1C and 1D). In fact, differential fold changes were between 1.1 and 657 times higher when compared to microarray analysis.

3.2 Based on M1 and M2 markers, NDPs induce an alternative macrophage phenotype

We used the newly identified gene marker set as means to identify macrophage responses following exposure to NDPs in comparison to M1 or M2 macrophage polarisation. We treated macrophages with a yeast- (Wellmune Soluble), a shiitake-derived (LCES) β-glucan, an apple rhamnogalacturonan I-derived from pectin (RG-I) and a wheat-derived arabinoxylan (Naxus). Gene transcription responses for M1 and M2 polarisation were similar as before, whereas the NDPs demonstrated a variety of changes to the transcriptional response (Figure 2). We did not observe any significant alteration in gene transcription following macrophage exposure to Wellmune Soluble.
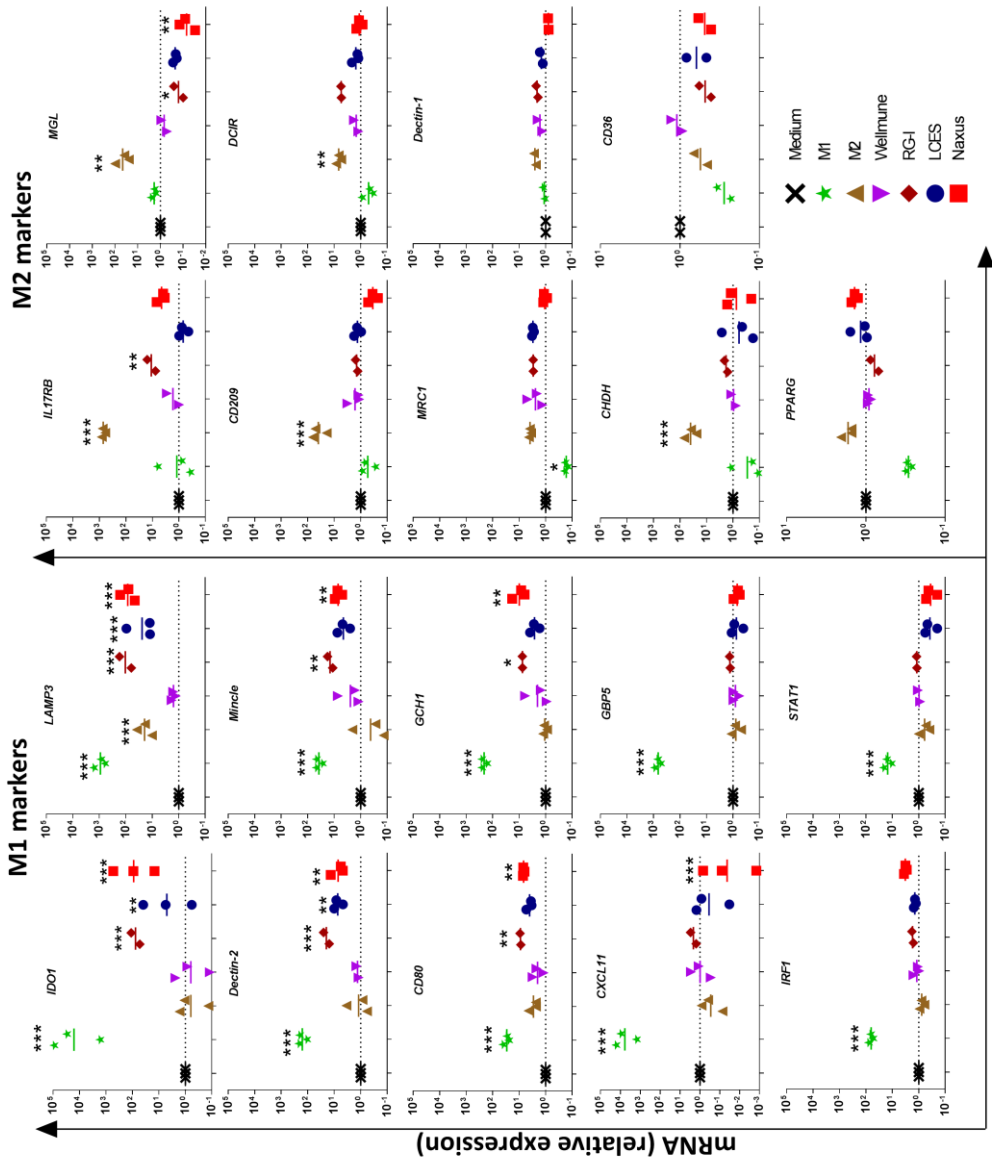
Figure 2. NDPs induce a M1 and M2 independent macrophage phenotype. Primary monocytes were differentiated for 7 days to macrophages and were subsequently polarised to M1 or M2 or exposed to Wellmune, RG-I, LCES, or Naxus. Macrophages were analysed for gene expression of M1 and M2 markers by qPCR and results are shown in mean values as fold change compared to non-treated macrophages, n=2 or 3 different donors. Statistically significant differences were analysed by one-way ANOVA: * p<0.05, ** p<0.01, *** p<0.001.
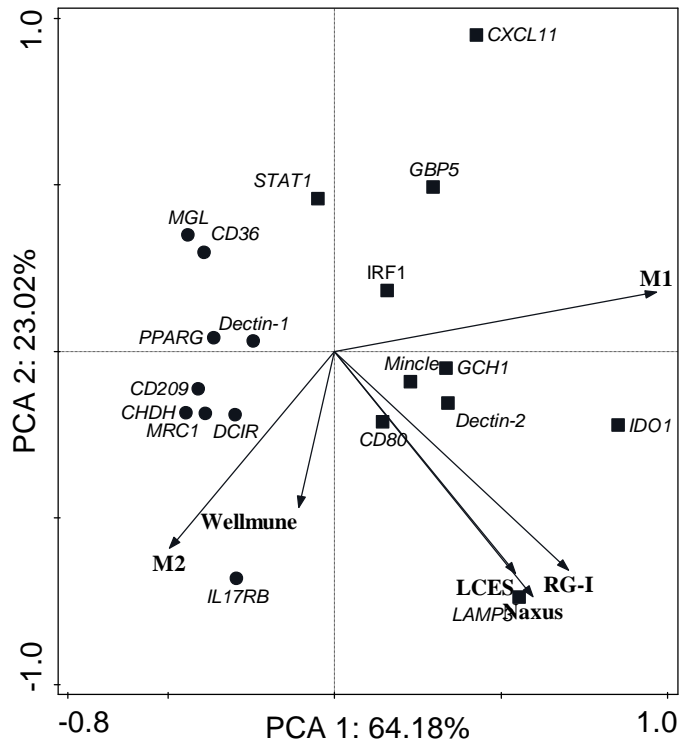
Figure 3. NDPs induce a transcriptionally unique macrophage subset. Primary monocytes were differentiated for 7 days to macrophages and were subsequently polarised to M1 or M2 or exposed to Wellmune, RG-I, LCES, or Naxus. Gene expression of M1 (■) and M2 (●) markers as analysed by qPCR and shown in Figure 2 were log-2 manipulated and shown in a PCA plot with relative scaling of the principal variables indicated, n=3 different donors.

LCES only significantly enhanced expression of three M1 markers. In contrast, RG-I and Naxus comparably enhanced transcription of most of the M1 markers and at the same time significantly downregulated expression of the M2 marker gene MGL. Of interest are also the gene markers of which expression is not altered by the NDPs. The M1 specific markers GBP5, IRF1, and STAT1 were not significantly increased in expression indicating NDPs do not induce a full M1 phenotype. PCA analysis clearly indicates that NDP exposure induced a macrophage phenotype that differs from M1 and M2 macrophages (Figure 3). Naxus, RG-I, and LCES appeared to induce a comparable specific macrophage subset which we coined M$_{NDP}$. The PCA analysis also suggested that IDO1 and LAMP3, in addition to being M1 markers, might also function as M$_{NDP}$ markers.

## 3.3 NDPs affect expression of a specific subset of genes in macrophages

Following our finding that Naxus, RG-I, and LCES induced a transcriptional unique NDP-macrophage subset, we aimed to identify M_{NDP} specific markers. To this end, we performed microarray analysis of the NDP-treated macrophage transcriptome. When analysing the metadata of these microarrays, we observed the most significantly altered gene transcription compared to medium following Naxus treatment (945 genes) and the least following Wellmune Soluble treatment (4 genes; Figure 4A). Furthermore, using PCA the NDP-treated macrophages could clearly be distinguished from M1 and M2 macrophages (Figure 4B). As could be expected, Wellmune Soluble appeared to be most similar to medium-treated macrophages while Naxus demonstrated the strongest changes over especially PC-1. The NDPs that strongly affected macrophage gene transcription (*i.e.* RG-I, LCES, and Naxus) demonstrated similar effects for 126 genes (Figure 4C), of which expression was upregulated for 59 genes and downregulated for 67 genes (Figure 5). These genes therefore appear most related to the M_{NDP} macrophage subset.

We investigated this list of 126 genes for functional relationships using gene ontology – biological process (GO-BP) descriptions (Figure 5). GO-BP terms describing migration were observed for 6 transcriptionally downregulated genes and 12 upregulated genes and included a number of C-C motif chemokine ligands. We also found a subset of genes involved in cell cycle. Expression was downregulated for 17 genes and upregulated for 9 genes, which in fact negatively influenced cell cycle. Within the group of 17 downregulated genes, 3 were part of the replication-dependent HIST1-family, of which another 4 were downregulated but lack a GO-BP description. GO-BP terms describing metabolic processes were observed for 9 transcriptionally downregulated and 10 transcriptionally upregulated genes. Not to our surprise, within the cluster of inflammatory immune function, genes of which expression was upregulated included IDO1 and LAMP3. These genes were clustered together based on GO-BP terms demonstrating inflammatory responses and were already linked to the M_{NDP} phenotype in the PCA plot (Figure 3).

3.4 NDPs demonstrate varying transcriptional profiles of CLRs

Many C-type lectin receptors (CLRs) have been described to be important in NDP recognition. We observed 2 CLRs, CLEC5A and CLEC6A, to be similar transcriptionally regulated following macrophage exposure to RG-I, LCES or Naxus (Figure 5). Further analysis revealed that each macrophage subset affects transcription of a number of CLRs. Strikingly, M1 polarisation strongly affected transcription of CLEC4E (Mincle) and CLEC6A (Dectin-2) and M2 polarisation strongly affected transcription of CLEC4A,
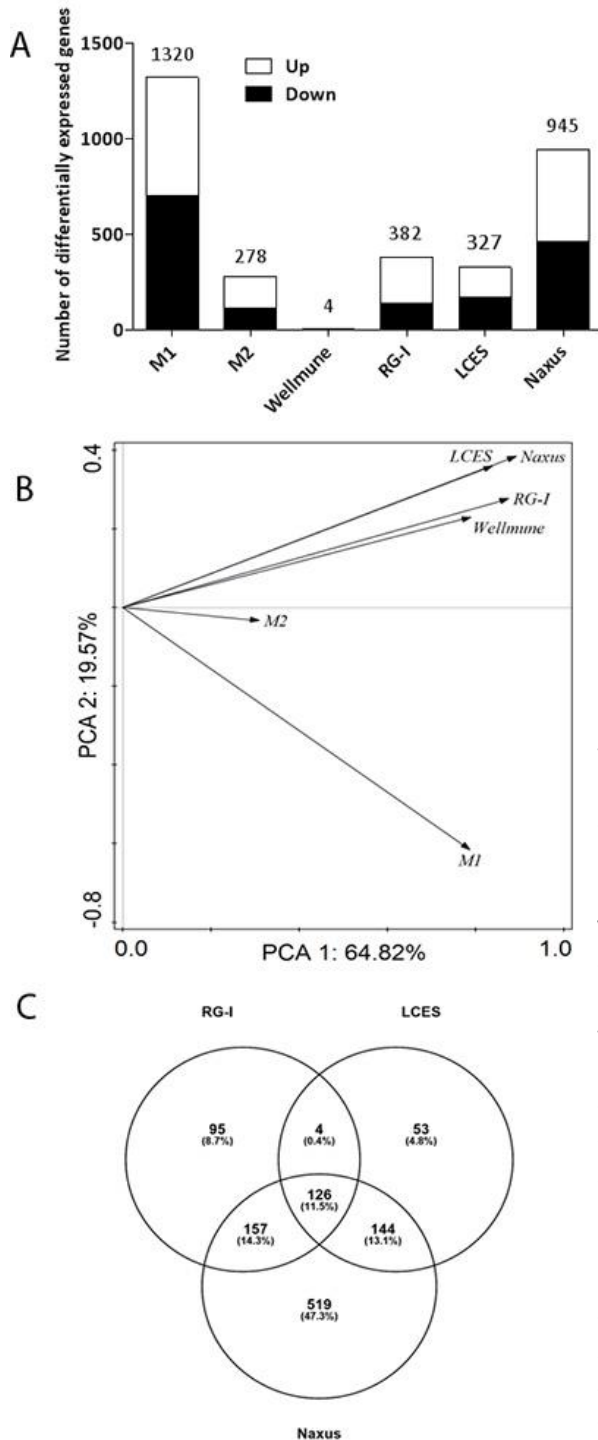
Figure 4. NDPs vary in their genome-wide differential gene expression. Primary monocytes were differentiated for 7 days to macrophages and were subsequently polarised to M1 or M2 or exposed to Wellmune, RG-I, LCES, or Naxus. Gene expression was analysed using microarray and compared to non-treated macrophages. The number of up- and down-regulated genes for each treatment is depicted in a bar chart (A). Relative gene expression is shown as PCA plot, based on a signal-log ratio, with relative scaling of the principal variables indicated (B). Number of NDP unique and shared differentially regulated genes is shown in a Venn diagram (C). Data are expressed as mean values, n=3 different donors.

Table 2. C-type lectin expression profiles as determined by microarray

Genes	Aliases	M1	M2	Wellmune	RG-I	LCES	Naxus
CLEC6A	Dectin-2	24.53***	1.04	4.28	8.59*	5.71*	5.34*
CLEC4E	Mincle	12.52***	-2.99	3.77	8.99**	3.94	6.36**
CLEC4D	Dectin-3/MCL	6.74**	-1.08	2.45	5.77*	1.99	4.86**
CLEC2C	CD69	2.32***	-1.1	-1.08	1.01	1.00	-1.05
CLEC4J	FCER2/CD23	1.21	23.73***	1.82	1.58	2.52	2.60
CLEC10A	MGL	1.04	19.81***	2.01	-1.11	-1.39	-1.93
CLEC4L	DC-SIGN/CD209	-2.40*	13.08***	1.93	1.59	-1.17	-1.55
CLEC4A	DCIR	-3.11**	4.28***	2.12	3.16**	1.22	1.47
CLEC13D	MRC1	-4.69***	2.73*	2.12	1.67	1.68	1.10
CLEC8A	LOX-1/OLR1	-4.60*	-1.61	1.89	3.11	6.99**	9.88***
CLEC13A	DCL-1/CD302	-3.35***	1.01	-1.52	-2.60*	-2.84**	-4.92***
CLEC13E	MRC2	-1.95**	-1.19	-1.61	-2.14*	-1.63	-2.56***
CLEC7A	Dectin-1	-1.44	1.87**	1.34	1.32*	-1.03	-1.65*
CLEC5A	MDL1	-1.50	-1.96	3.39	3.33**	3.52**	2.06*
CLEC4G	LSECtin	1.05	2.40**	1.21	1.17	1.05	-1.03
CLEC1A	-	-1.10	2.60**	1.71	2.07*	1.26	1.13

Values represent fold changes of gene expression, n=3 experiments. Statistically significant differences were calculated with IBMT regularised paired *t*-test: * FDR p<0.05, ** FDR p<0.01; *** FDR p<0.001.

CLEC4L, CLEC10A (MGL), and CLEC13D (Figure 1C and D). In total, we found significant transcriptional upregulation of 4 CLRs in M1 and 8 CLRs in M2 macrophages, and downregulation of 6 CLRs in M1 and none in M2 macrophages (Table 2).

There are clear differences in CLR gene expression profiles between M1, M2 and NDP-treated macrophages. Gene expression of CLEC5A and CLEC8A (LOX-1) was found

Cell migration			
Gene	RG-I	LCES	Naxus
CCL1	4.6	5.8	7.9
CCL24	4.2	6.9	7.2
CXCL8	5.1	4.1	5.7
IL1B	4.3	3.4	5.0
SLC7A11	4.5	3.9	4.5
CCL4	2.1	3.3	3.6
SERPINE1	2.3	2.6	3.3
CCL3L3	1.4	2.0	2.8
PDPN	3.1	2.0	2.7
CCL3	1.4	2.0	2.7
ICAM1	1.7	1.2	2.1
MMP14	1.6	1.1	1.7
ITGA4	-1.0	-1.0	-1.8
PRR5L	-1.7	-1.2	-2.0
SORL1	-2.5	-1.8	-2.3
HGF	-2.5	-2.3	-2.4
IGF1	-2.1	-2.3	-2.7
ADORA3	-2.8	-3.3	-4.1

Inflammatory immune function			
Gene	RG-I	LCES	Naxus
IDO1	4.0	2.0	5.8
IL3RA	4.5	4.3	5.8
TNIP3	5.4	2.4	5.5
TNFAIP6	3.7	1.9	5.3
LAMP3	3.6	1.5	4.0
TRAF1	2.4	2.7	4.0
BIRC3	1.7	1.6	3.3
IRAK2	1.3	1.7	3.0
CD274	2.0	1.3	2.6
CLEC6A	3.1	2.5	2.4
TNFAIP3	1.3	1.6	2.4
TNIP1	1.8	1.3	2.4
CD40	1.2	1.1	1.6
KCNN4	0.9	1.3	1.3
CLEC5A	1.7	1.8	1.0
PIK3IP1	-1.2	-1.1	-1.0
APOBEC3B	-1.0	-1.1	-1.3
CD28	-1.4	-1.4	-1.7
SIGLEC16	-1.4	-2.0	-1.8
CD200R1	-1.4	-1.0	-1.9
TLR5	-1.3	-1.3	-2.1
DEPTOR	-1.6	-1.8	-2.1
CD302	-1.4	-1.5	-2.3
FFAR4	-1.8	-1.0	-2.3
MEF2C	-1.7	-1.6	-2.3
LILRB5	-1.4	-2.3	-2.7
GAPT	-3.1	-3.6	-3.3
TLR7	-3.0	-3.0	-3.5

Cell cycle			
Gene	RG-I	LCES	Naxus
INHBA	3.4	6.0	7.5
IL7R	5.4	4.2	5.2
MMP12	3.9	4.3	4.7
SLAMF1	3.2	2.2	4.9
SOCS3	2.8	2.3	4.0
KDM6B	0.9	1.5	2.2
LRP12	1.6	1.2	2.1
DCUN1D3	1.4	0.9	1.4
VDR	1.2	0.9	1.1
HIST1H2BF	-1.1	-1.3	-1.0
HIST1H2AI	-0.9	-1.0	-1.3
HIST1H3B	-1.9	-1.3	-2.0
HIST1H2BM	-2.0	-1.6	-2.4
HIST1H3G	-2.2	-1.5	-2.6
HIST1H3F	-1.5	-1.3	-2.0
HIST1H1B	-1.1	-1.1	-1.6
ANLN	-1.3	-1.0	-1.5
MYBL2	-1.1	-0.9	-1.5
PLK1	-1.3	-1.0	-1.3
APPL2	-0.9	-1.2	-1.2
FANCI	-1.0	-1.0	-1.2
PRLR	-1.3	-1.3	-1.3
REPS2	-1.3	-1.1	-1.4
ESCO2	-1.0	-1.1	-1.9
TOP2A	-1.4	-1.1	-2.0
RRM2	-1.8	-1.0	-2.0

Metabolic process			
Gene	RG-I	LCES	Naxus
HS3ST3B1	2.9	1.7	4.1
G0S2	3.3	1.9	2.9
SH3PXD2B	2.9	1.8	2.7
ABCG1	1.2	1.2	2.6
CYP27B1	1.7	1.6	2.5
STK17A	1.4	0.9	1.8
MAOA	2.0	1.0	1.6
SOD2	1.7	0.9	1.6
ST3GAL2	0.9	1.0	1.5
ABCA1	1.0	0.9	1.2
DPEP2	-1.3	-1.1	-1.2
AMDHD1	-1.1	-0.9	-1.2
CD101	-1.6	-1.1	-1.4
CMBL	-1.1	-1.5	-1.9
GATM	-1.5	-1.4	-2.1
GCNT1	-1.4	-1.6	-2.6
SLC46A1	-1.8	-2.1	-2.8
GAL3ST4	-1.9	-3.2	-3.3
SEPP1	-3.0	-3.7	-4.1

Other functions			
Gene	RG-I	LCES	Naxus
HIVEP2	1.1	1.6	3.2
MSC	2.0	1.8	2.4
C17orf96	2.0	1.8	2.2
MB21D2	1.3	1.0	2.1
ARRDC4	1.9	1.3	2.1
FAM20A	2.0	1.0	1.9
MOB3B	1.1	1.2	1.8
C15orf48	1.2	1.0	1.7
SRXN1	1.9	1.2	1.6
STAC	2.2	1.6	1.5
ZSWIM4	1.3	0.9	1.3
SLC41A2	1.4	1.2	1.8
RHBDF2	0.9	0.9	1.3
ZNF836	-1.1	-1.0	-1.1
SRGAP3	-1.3	-1.3	-1.1
ZNF33B	-0.9	-1.1	-1.2
LPAR5	-1.1	-1.1	-1.2
GPBAR1	-1.1	-1.1	-1.3
RHOBTB1	-1.3	-1.1	-1.3
MAML3	-1.0	-1.1	-1.3
ASRGL1	-1.0	-0.9	-1.4
FXYD6	-1.3	-1.2	-1.5
DBP	-1.2	-1.2	-1.7
FAXDC2	-2.0	-1.7	-1.7
PTGFRN	-1.4	-0.9	-1.7
RAD51AP1	-1.2	-1.0	-1.9
SLC46A3	-0.9	-1.3	-1.9
CRYBB1	-1.3	-1.6	-2.1
RGS18	-1.6	-1.5	-2.2
GPR155	-1.3	-1.5	-2.3
SLC40A1	-2.9	-3.2	-2.8
MRO	-2.2	-2.5	-2.9
P2RY8	-2.5	-2.6	-3.1
TMEM37	-2.1	-3.5	-4.3
MS4A6A	-2.1	-4.0	-4.6

Figure 5. Heatmaps of genes differentially expressed in M$_{NDP}$. Primary monocytes were differentiated for 7 days to macrophages and were subsequently exposed to RG-I, LCES, or Naxus. Gene expression was analysed using microarray and compared to non-treated macrophages. Genes were selected based on significant differential expression for all three NDPs and grouped based on GO-BP terms in genes related to cell migration, cell cycle, inflammatory immune function, metabolic process, and other functions. Data are expressed as mean values of log 2 transformed fold change (red to blue scale) with n=3 different donors.

decreased and CLEC10A increased in both M1 and M2 macrophages, albeit not all significant, but oppositely regulated in M$_{NDP}$. Furthermore, gene expression of CLEC4L and CLEC13D were decreased in M1 and increased in M2 but not affected by NDPs. NDPs also affected transcription of some CLRs similarly to M1 or M2 macrophages. Differential CLR gene expression as in M1 macrophages was observed in LCES-treated macrophages in case of 2 CLRs and in Naxus-treated and RG-I-treated macrophages in case of 5 CLRs, which include CLEC6A. However, only RG-I also significantly affected gene expression of CLRs (CLEC1A, CLEC4A and CLEC7A) similar as in M2 macrophages. The CLR transcriptional profile therefore appears representative for the alternative M$_{NDP}$ subset when compared to M1 and M2.

### 3.5 Naxus and RG-I differ in the kinetics of induction of IDO1 and LAMP3 gene expression

The 18 h exposure time point used in the previous analyses was based on pilot experiments when setting up the polarisation towards M1 and M2 macrophages characterised by the markers from literature. In order to better understand the expression kinetics related to MNDP, we analysed gene transcription levels of IDO1 and LAMP3, being markers for M1 (Figure 1) and MNDP (Figure 6). Analysis of a time series of M1 polarisation or macrophage exposure to RG-I or Naxus demonstrated that IDO1 and LAMP3 expression was already significant upregulated in M1 polarised and RG-I-treated macrophages after 4 h, with an expression that peaked at 18 h for M1 and 8 h for RG-I.

Differential IDO1 and LAMP3 gene expression following Naxus treatment reached significance only after 8 h and also peaked at 18 h after which the expression remained stable until the 48 h time point. In contrast, IDO1 expression in response to RG-I declined after peaking at 8 h.

4 Discussion

Macrophages that can sample antigens in the lumen can, in particular, be expected to have a central role in responding to luminal stimuli and maintaining homeostasis in the intestine [20, 28]. Non-digestible polysaccharides are not or to a limited extent degraded in the upper gastric tract and can therefore act as luminal stimuli to these sampling macrophages. Indeed, macrophages express receptors such as dectin-1 to respond to treatment with β-glucan preparations with cytokine release [29].

To investigate the effects of NDPs on macrophages in more detail, we used a monocyte-derived macrophage culture system as a follow-up to previous work on THP-1 macrophages [30]. Upon differentiation, the macrophages were treated with NDPs or polarised towards inflammatory or tolerant subsets. The latter two subsets can be
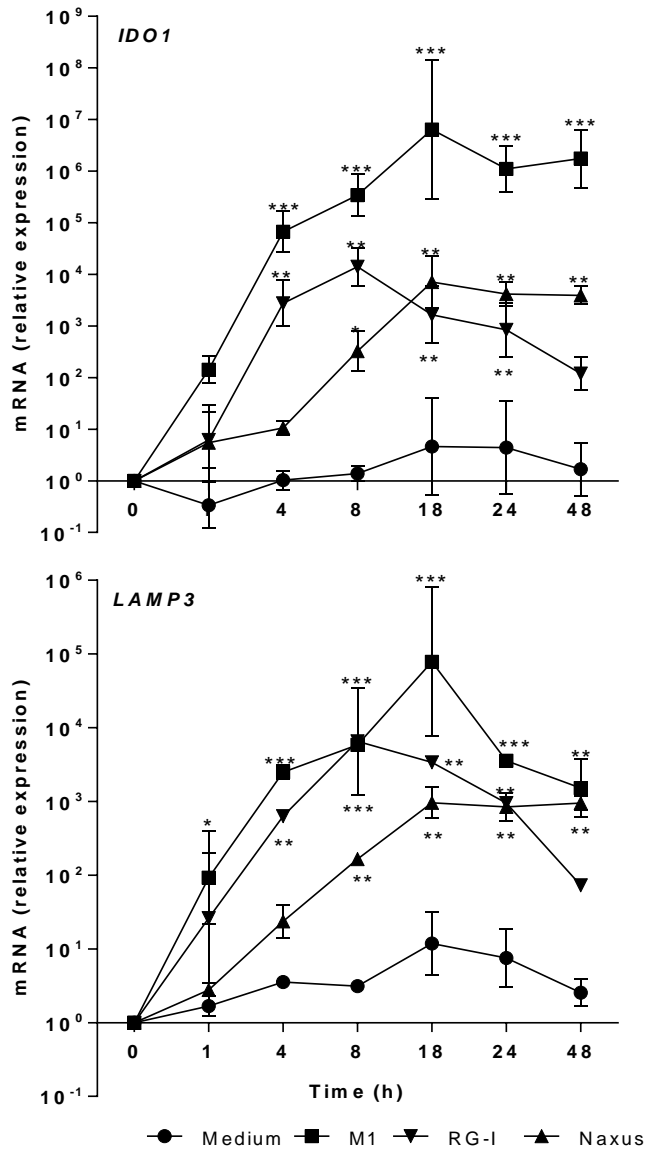
Figure 6. Naxus and RG-I demonstrate an opposite gene expression kinetics for IDO1 and LAMP3. Primary monocytes were differentiated for 7 days to macrophages and were subsequently polarised to M1 or exposed to RG-I or Naxus. IDO1 and LAMP3 gene expression was analysed by qPCR at 1 h, 4 h, 8 h, 18 h, 24 h, 48 h and results shown as fold change compared to non-treated macrophages by mean values ± SEM, n=2 different donors. Statistically significant differences were analysed by one-way ANOVA: * p<0.05, ** P<0.01, ***

considered extremes to be found in the intestine depending on whether there is a homeostatic or inflammatory environment [12]. Polarisation of macrophages to a tolerant (M2) or inflammatory (M1) phenotype can be determined by gene markers reported in the literature [31-33], which were confirmed in our study. For IL12p40 it was shown that this gene was also specifically upregulated in inflammatory macrophages in the intestine of mice as it was in M1 macrophages in our system [19]. Similarly, MRC1 was upregulated in tolerant M2 type macrophages. Our microarray analysis, supported by qPCR validation, revealed gene markers that are even more strongly differentiating between M1 and M2 macrophages in our culture system. Among those genes are IDO1, LAMP3, CXCL11, Dectin-2, and Mincle that can be used as M1 markers and IL17RB, CD209, and MRC1 to identify M2 macrophages. This resulted in a final marker gene list as shown in Figure 2 which can be used for improved characterisation of inflammatory and tolerant macrophages *in vitro* and potentially also *in vivo*. This list mainly contains markers related to microbial carbohydrate recognition (M1 or M2), inflammatory responses (M1), immunoregulation and energy supply (all M2; supplementary Table 2). For both M1 and M2 macrophages we observed a strong transcriptional regulation of CLRs, which are receptors specific for carbohydrate recognition [34] enabling recognition of microbial structures [35].

When using the marker gene set to characterise macrophage phenotypes following NDP exposure, we observed a transcriptionally unique macrophage subset that we termed M_{NDP}. Remarkably, PCA analysis based on all differential expressed genes demonstrated that NDPs induced a similar macrophage subset but with different intensity, although only limited effects on gene expression were observed for Wellmune Soluble. Wellmune Soluble was described to interact with the CR3 receptor to prime this for binding to antibodies [36]. In contrast, Wellmune Soluble does not cluster cell surface receptors and fails in initiating intracellular signalling [29]. Therefore, it is likely that Wellmune Soluble does not induce gene transcription in our model. The other NDPs induced macrophage gene transcription to various extents, with Naxus being the most potent. This likely occurred via interactions with carbohydrate binding receptors [35]. For example, it has been shown that dectin-1 binds β-glucans, the major polysaccharide in LCES, and arabinoxylan, the major constituent of Naxus [37, 38]. Furthermore, Mincle was demonstrated to recognize α-mannose, which can be found in the Naxus and LCES preparations, and both Dectin-2 and Dectin-3 were shown to bind α-mannans [39-41], which are also present in Naxus. As a result of NDP recognition, macrophages mainly increased expression of CLRs, which might constitute a positive feedback mechanism to enhance vigilance towards microbes or fungi.

Gene markers that related to the M_{NDP} phenotype are LAMP3 and IDO1. LAMP3 was reported to associate with dendritic cell differentiation and maturation by performing a key role in processing of exogenous antigens [42]. IDO1 is an enzyme that catalyses the first and rate-limiting step in tryptophan catabolism to N-formyl-kynurenine [43], and thereby modulates innate and adaptive immune responses [44] and potentially represents an important feedback mechanism for macrophage activity. Both genes therefore have an important role in macrophage functionality which prompted us to analyse the expression pattern in more depth following NDP exposure that showed a strong upregulated LAMP3 and IDO1 gene expression (*i.e.* Naxus and RG-I). M1 macrophages and macrophages treated with RG-I or Naxus all demonstrated a different kinetic profile of gene transcription of both IDO1 and LAMP3. A striking difference between M1 and RG-I on the one hand and Naxus-treated macrophages on the other hand is the rapid increase versus the lag time in transcription, respectively. IDO1 transcription has been described to be regulated by STAT1, which can directly bind to the gene promotor, or indirectly regulated by STAT3, via activation of the non-canonical NFκB pathway which could lead to alternative kinetics in IDO1 gene transcription [45, 46]. Activity of transcription factors can be inferred from gene transcription profiles using IPA Upstream Regulator Analysis resulting in Z-scores. In concordance with observed IDO1 transcription and anticipated kinetic difference in STAT transcriptional activity, STAT1 was predicted to be active in M1 with a Z-score above 2 (*i.e.* 3.4). For RG-I-treated macrophages, demonstrating a minor lag time compared to M1 macrophages, STAT1 but also STAT3 were predicted to be active with a Z-score of 2.0 and 2.2, respectively. Finally, for Naxus-treated macrophages STAT1 was not predicted to be active (Z-score of 0.1), but STAT3 activation was predicted with a Z-score of 1.6 suggesting a mainly STAT3 driven IDO1 transcription. Differences in carbohydrate recognition receptor ligation might result in variable STAT1 and STAT3 activation which likely can result in alternative kinetics of IDO1 gene transcription. So, although these genes represent suitable M_{NDP} markers between 8 and 48 h of treatment, their expression kinetics might enable further discrimination between NDP bioactivity.

A continuous concern for *in vitro* immune analysis of for instance NDPs, is lipopolysaccharide [47] contamination. LPS is often found in food-derived preparations and can be a cause of false positive results [48]. To be able to identify LPS-mediated macrophage responses we opted to induce M1 polarisation with TNFα and IFNγ instead of the frequently used combination of LPS and IFNγ. LPS also demonstrated the potency to induce IDO1 and LAMP3 expression in macrophages (supplementary Figure 1A and B). CXCL11, which serves in our panel as M1 marker, has also been shown to be a strong response marker for LPS recognition by primary monocytes [49]. Indeed, CXCL11

expression is significantly enhanced in our macrophages when exposing these to as low as 100 fg/ml of LPS (supplementary Figure 1C). In a previous study we demonstrated that LPS was efficiently removed from NDPs using a NaOH-based treatment [21]. Exposing macrophages to treated NDPs confirms the absence of LPS in our samples as no significant differential CXCL11 transcription was observed (supplementary Figure 1D).

Extended analysis of macrophage transcriptional responses to NDP treatment revealed the downregulation of HIST1 family genes. These replication-dependent histones are actively transcribed during mitosis and reach a peak during the S-phase of the cell cycle [50, 51]. Not only via alternative HIST1 gene regulation, but also by expression of lysine-specific demethylase 6B (KDM6B/Jmjd3; Figure 5), that can demethylate both trimethylated and dimethylated H3K27 histones [52], NDP appear to affect macrophage phenotype via epigenetic regulation. This demethylation increases transcription of inflammatory genes via activation of various signalling pathways, including NFκB, STAT, SMAD, and T-bet transcription factors [53]. This is in line with *candida*-derived NDP, from the sub-group of glucans which includes lentinan, which demonstrated to use epigenetic regulation to induce so-called 'trained immunity' [54]. Epigenetic changes upon β-glucan-induced trained immunity results in transcriptional profiles most similar to classical macrophages [55]. In contrast, putatively induced HIST1-dependent proliferation linked to the M2 phenotype.

Taken together, we describe a qPCR marker set to characterise inflammatory (M1) or tolerant (M2) phenotypes and identified a distinct macrophage subset following exposure to NDPs. It will be of interest to analyse whether this transcriptionally unique M_{NDP} subset can also be distinguished *in vivo* and how this depends on the NDPs in our diet or other luminal triggers such as microbiota composition or metabolites. The unique and overlapping set of gene markers to identify M1, M2, and M_{NDP} can likely contribute to those analyses.

Y.T., C.G., and J.J.M. conceived and designed the experiments. Y.T. performed the experiments. Y.T., C.G., and J.J.M. analysed the data. Y.T., C.G., J.J.M., and H.J.W. wrote the manuscript.

The authors have declared no conflicts of interest.

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Supplementary Table 1. Primer details

| Genes	Forward (5'-3')	Reverse (5'-3')
PUM1	TGAGGTGTGCACCATGAAC	CAGAATGTGCTTGCCATAGG
RPLP0	GCAATGTTGCCAGTGTCTG	GCCTTGACCTTTTCAGCAA
IL8	CTGATTTCTGCAGCTCTGTG	GGGTGGAAAGGTTTGGAGTATG
IL12p40	CTCTGGCAAAACCCTGACC	GCTTAGAACCTCGCCTCCTT
CD80	ACCATCCAAGTGTCCATACC	CACGTGGATAACACCTGAAC
STAT1	CTGGCACCAGAACGAATG	GCTGACGTTGGAGATCAC
TNFA	GGCGTGGAGCTGAGAGATAA	GATGGCAGAGAGGAGGTTGA
CD209	AGGAGCAGAACTTCCTAC	GCTCTCCTCTGTTCCAATAC
MRC1	CAGCGCTTGTGATCTTCATT	TACCCCTGCTCCTGGTTTTT
MRC2	GGAAACTCCCACGGAAAG	GTCTCGCAGTCGTTACTC
PPARG	TCTAAAGAGCCTGCGAAAGC	GCGGTCTCCACTGAGAATAATG
CD36	GCCAAGGAAAATGTAACCCAGG	GCCTCTGTTCCAACTGATAGTGA
IDO1	GCCAGCTTCGAGAAAGAGTTG	ATCCCAGAACTAGACGTGCAA
GBP5	ACATTAGTTCTGCTTGACACCG	GCTGCTCAGTAAGAGTGCCAG
GCH1	ACGAGCTGAACCTCCCTAAC	GAACCAAGTGATGCTCACACA
LAMP3	ACTACCCCAGCGACTACAAAA	CTAGGGCCGACTGTAACTTCA
CXCL11	GACGCTGTCTTTGCATAGGC	GGATTTAGGCATCGTTGTCCTTT
IRF1	GCAGCTACACAGTTCCAGG	GTCCTCAGGTAATTTCCCTTCCT
Mincle	CTGAAACACAATGCACAGAGAGA	AAAGATGCGAAATGTCACAACAC
Dectin-1	AACCACAGCTACCCAAGAAAC	GGGCACACTACACAGTTGGTC
Dectin-2	GCTTTCAGACCCACAAGGTAAT	GCAGAATGATTGGGCTCACCTA
IL17RB	GGCTGCCTAGACCACATAATG	GCTGTGTTGGATAAGAGCCAT
CHDH	TATCACCCTCCATTCAGCACA	GAACCCACCTGTTTCCAGATG
DCIR	GACTGTGCTAGAATGGAGGCT	GTCGCTGACCTTCTGGATCTG
MGL	AGCAACTTCACCTCAAACACTG	AGATGCTATCGTTTCTTCCAAGC

Supplementary Table 2: Function of gene markers for M1 and M2

Subset	General function[a]	Gene	Microarray[b]	qPCR[b]	Notes
M1	Carbohydrate recognition	Dectin-2	23	154	Binds high-mannose carbohydrates
		Mincle	16	128	Binds damaged cells, fungi and mycobacteria
	Interferon related transcription factor	STAT1	5	27	Mediates cellular responses to interferons
		IRF1	23	82	Activator of interferon-α and-β transcription
	Functional inflammatory responses to pathogens	CD80	10	11	Provides co-stimulatory signals essential for T
		LAMP3	81	70	Involved in antigen processing
		GBP5	74	904	Activator of NLRP3 inflammasome
		GCH1	32	196	Positively regulates nitric oxide synthesis
		IDO1	799	30780	Catalyzes rate-limiting step in tryptophan
		CXCL11	32	21017	Induces chemotactic responses in activated T
M2	Energy supply	CD36	1	8	Binds long-chain fatty acids and facilitates
		PPARG	2	6	Controls the peroxisomal beta-oxidation pathway of
		CHDH	25	162	Mitochondrial oxidoreductase activity
	Carbohydrate recognition	MRC1	7	20	Mediates the endocytosis of
		Dectin-1	3	4	Specifically binds β-1,3-linked and β-1,6-linked
		CD209	15	70	Mediates the endocytosis of
		DCIR	7	14	Putative role in regulating immune
		MGL	19	26	Binds to glycoproteins
	Intestinal immunoregulatory activity	IL17RB	12	995	Receptor that can activate NF-κB and induce IL-8 production

[a] Function was extracted from description in www.genecards.org. [b] Fold-change of M1/M2 (for M1 cells) or M2/M1 (for M2 cells).

Supplementary Figure 1
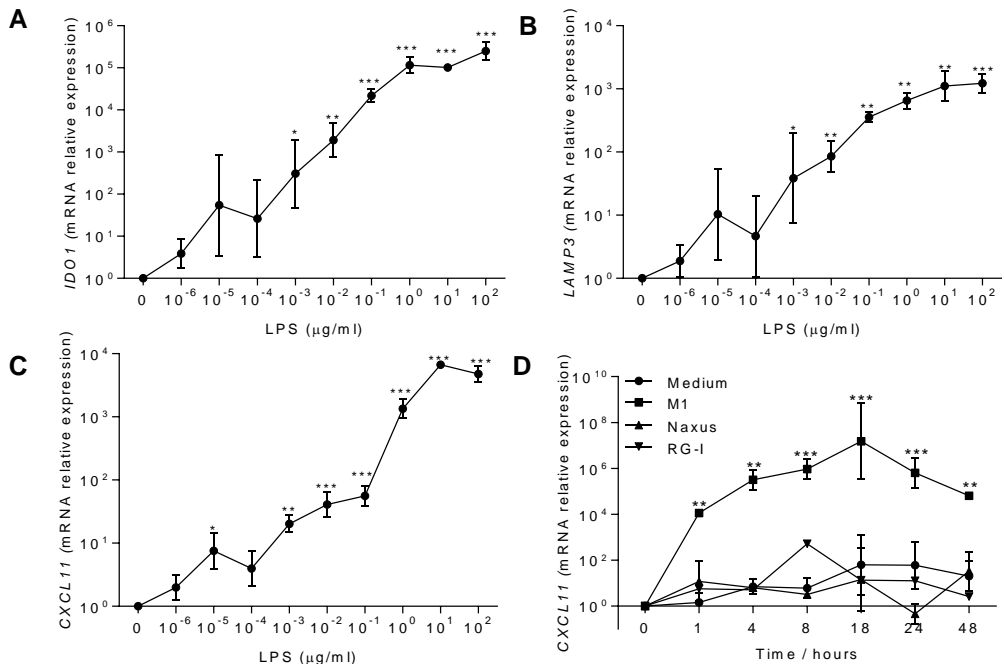
Supplementary Figure 1. LPS induces IDO1, LAMP3 and CXCL11 expression in macrophages, of which the latter is not observed in RG-I- and Naxus-stimulated macrophages. Primary monocytes were differentiated for 7 days to macrophages which were subsequently stimulated with various concentrations of LPS or NDPs. Gene expression of (A) IDO1, (B) LAMP3, and (C) CXCL11 was analysed by qPCR following M0 stimulation with various concentrations of LPS, n=3 different donors. (D) CXCL11 gene expression was analysed by qPCR in more details following 1, 4, 8, 18, 24, and 48 h of M1 polarisation or RG-I or Naxus stimulation. Results are shown as fold change compared to non-stimulated macrophages by mean values ± SEM. Statistically significant differences were analysed by one-way ANOVA: * p<0.05, ** P<0.01, *** p<0.001.

Chapter 4

Human macrophages stimulated with non-digestible polysaccharides are in function and chemokine production different from inflammatory and tolerant macrophages

Yongfu Tang, Coen Govers, Ellen H. Stolte, Harry J. Wichers, Jurriaan J. Mes

Chapter 4

Abstract

Scope: The immunomodulatory properties of non-digestible polysaccharides (NDPs) have been recognized in *in vitro* and *in vivo* studies. Macrophages represent an important cell type for mediating immune signaling following NDP consumption. In a previous study we analyzed macrophage gene transcription following NDP treatment with four different NDPs which revealed a transcriptionally unique phenotype when compared to M1 or M2 macrophages. The aim of this study was to functionally characterize M_{NDP} in comparison to M1 and M2 macrophages.

Methods and results: We assessed M1 and M2 and NDP-treated macrophages for IDO activity, characteristic of M1 macrophages, and antigen uptake and processing capacity, characteristic of M2 macrophages, and for phagocytotic capacity. Surprisingly, NDP-treated macrophages showed no IDO activity and inhibited antigen uptake and processing. Their phagocytotic capacity was reduced compared to M1 or M2 macrophages. Furthermore, microarray analysis revealed an alternative expression pattern for NDP-treated macrophages which was confirmed by protein measurements. The signature mix of CCL1, CCL5, CCL20, CCL24, CXCL8, and IL1β secreted by M_{NDP}, and in particular when macrophages were treated with Naxus, was shown to induce migration of monocytes.

Conclusion: We have shown that NDP-treatments results in a macrophage phenotype that is transcriptionally and functionally different from M1 and M2 macrophages. This M_{NDP} might represent an intermediate phenotype that contributes to the maintenance of intestinal homeostasis.

Keywords: Arabinoxylan; Cytokines; β-Glucans; Migration; Phagocytosis; Wheat

Abbreviations: LCES, lentinan containing extracted from shiitake; **MFI**, Mean fluorescence intensity; **M$_{NDP}$,** NDP specific macrophage subset; **NDP**, non-digestible polysaccharide;

1 Introduction

Non-digestible polysaccharides (NDPs) are known for their immune-modulating properties [1, 2]. These properties were not only demonstrated *in vitro* but also reported in clinical studies. NDP intake by obese people resulted in a reduction of pro-inflammatory cytokines (i.e. IL6 and TNFα) and an increase in the anti-inflammatory IL10 levels in peripheral blood [3]. These changes in cytokine balance were accompanied by a reduced waist circumference and blood pressure which are considered risk factors. Furthermore, NDPs demonstrated to increase NK cell activity and peripheral blood levels of myeloid dendritic cells and T_H1 related cytokines in multiple myeloma patients, however without reported beneficial effects on disease progress [4]. Other examples in which NDPs demonstrated effects in clinical trials include an increase in NK cell activity linked to a reduction in metastasis in lung and breast cancer patients [5]; an increase in blood leukocytes, neutrophils, IgG and IgM accompanied by a delayed progression of non-small cell lung cancer [6]; and transiently increased blood levels of IgG and IgM and NK cell counts linked to a reduction in flu and flu-like symptoms and respiratory tract infections in children [7].

To benefit health through immunomodulation, NDPs need to be recognized and/or sampled to induce cellular responses. NDPs can come into contact with intestinal immune cells located in the Peyer's patches by transcytosis across the intestinal epithelium by microfold cells [8]. Peyer's patches consist of a wide array of immune cells including T cells, B cells, dendritic cells and macrophages. Alternatively, NDPs have been shown to directly interact with the intestinal immune system [9]. Especially CX3CR1-positive antigen presenting cells can directly sample the lumen and were shown to consist for 75-80% of macrophages [10]. These CX3CR1-macrophages were demonstrated to be of an immune suppressive subset (often referred to as M2) [11]. In case of intestinal inflammation the influx of an inflammatory macrophage subset can be observed (often referred to as M1) [12, 13]. Both macrophages subsets have an important role in maintaining homeostasis [14]. β-Glucans can potentially influence the polarisation of macrophages through interaction with expressed pattern recognition receptors (PRRs) such as dectin-1. This was the first receptor proven to bind β-glucans to initiate functional responses in macrophages [15]. More and more PRRs are being identified through which NDPs can modulate immune responses including dectin-2 [16], mannose receptor [17], complement receptor 3 [18] and scavenger receptors [19]. We previous showed that NDPs increased gene expression of dectin-2, dectin-3, Mincle, and LOX1, and decreased CD302, MRC2, and MDL1 on [20], which might suggest a positive-feedback signal.

In a previous study we demonstrated that NDPs from different sources induced a transcriptionally unique macrophage subset, termed M_{NDP}, compared to inflammatory and tolerant macrophages [20]. Here, we analysed the functionality of M_{NDP} in comparison to M1 and M2 macrophages to gain insight into the potential implications of interactions between NDP and macrophages in the intestine.

2 Materials and Methods

2.1 NDP characteristics

Macrophages were treated with Wellmune Soluble, RG-I, LCES, or Naxus as described previously [20]. In brief, Wellmune Soluble (referred as Wellmune in tables and figures) is a water soluble β-1,3/1,6-glucan from the yeast *Saccharomyces cerevisiae* provided by BioThera (Eagan, MN, USA). RG-I is a concentrate of rhamnogalacturonan I from apple juice provided by INRA (Paris, France). LCES (lentinan containing extract from shiitake) is a β-1,3/1,6-glucan extract from shiitake. Naxus is a polysaccharide from wheat, rich in arabinoxylan, provided by BioActor (Maastricht, The Netherlands). Before exposure to macrophages, RG-I was treated with alkaline-ethanol at 4 °C for 5 h, and LCES and Naxus were treated with alkaline-ethanol at 56 °C for 5 h, followed by neutralization with HCl and freeze-drying to inactivate lipopolysaccharide (LPS) [21]. Resulting functional LPS contaminations of all NDPs samples was demonstrated to be below 0.01 EU/mg [21].

2.2 Primary macrophage differentiation and polarization

Primary macrophages were differentiated as previously described [20]. In brief, human monocytes were obtained from buffy coats from healthy donors (Sanquin, Nijmegen, The Netherlands) using the QuadroMACS system and CD14 microbeads according to manufacturer's protocol (Miltenyi Biotec, Leiden, The Netherlands). A written informed consent was obtained before sample collection. Monocytes were differentiated into macrophages following 7 days culturing in presence of 50 ng/ml MCSF (R&D systems, Minneapolis, USA). MCSF was replenished after 3 and 5 days of culture. On day 7 macrophages were polarized by addition of medium containing 20 ng/ml TNFα and 20 ng/ml IFNγ (for M1), 20 ng/ml IL4 (for M2) or 500 μg/ml NDPs (for M_{NDP} generation) for 18 h (unless stated otherwise).

2.3 RNA extraction and microarray analysis

RNA extraction and microarray analysis was performed as described previously [20]. Briefly, RNA was extracted with TRIzol (Invitrogen, Bleiswijk, The Netherlands) and RNeasy kits (Qiagen, Venlo, The Netherlands). RNA quality was verified with the RNA

6000 Nano assay on the Agilent 2100 Bioanalyzer (Agilent Technologies, Amstelveen, The Netherlands) and microarray analysis was performed using Affymetrix Human Gene 1.1 ST array plates and an Affymetrix Geen Titan platform. Data was processed using the Bioconductor project package, the Robust Multi-array Average method to acquire signal intensities, remapped chip definition file (CDF) to define probe sets, and the UPC algorithm to identify expressed genes. Next, cut-off values of absolute fold change compared to non-stimulated macrophages >1.8 and a false discovery rate of p<0.05 (IBMT regularised paired t-test) for at least one treatment were used to select genes for functional interpretation with Ingenuity Pathway Analysis (IPA) 3.0 (Ingenuity, Qiagen, Redwood, CA, USA). Microarray data were deposited in the Gene Expression Omnibus (GEO) database.

2.4 Kynurenine assay

Polarised, NDP-treated or medium stimulated macrophages were washed with PBS and incubated with 0.5 mM tryptophan (Sigma, St. Louis, MO, USA) for 24 h. Supernatant of macrophage cultures (100 μl) was mixed with 20 μL of 30% trichloroacetic acid and incubated at 50 °C for 30 minutes followed by centrifugation at 2500 g for 15 minutes. Subsequently, 75 μl of supernatant was added to 75 μl of Ehrlich reagent (100 mg *P*-dimethylbenzaldehyde (Sigma) in 5 mL glacial acetic acid) in 96-well plate wells and measured at 475 nm with a microplate reader (Tecan, Männedorf, Switzerland). A calibration range of kynurenine concentration (0-200 μM) was included in each plate. All assays were performed in biological triplicates and each sample analysed by technical duplicates.

2.5 Flow cytometry

IDO protein level was analysed by intracellular staining and analysed by flow cytometry. Polarised, NDP-treated or medium stimulated macrophages were washed with PBS without calcium and magnesium and detached by 10 minutes incubation with 0.25% Trypsin/EDTA (Gibco, Bleiswijk, The Netherlands) at 37 °C. Following two washes with PBS (450g centrifugation), macrophages were permeabilized using the FIX&PERM cell permeabilization kit (Invitrogen) according to the manufacturer's instructions. Cells were subsequently stained with 2 μl of IDO-APC (R&D systems) or an isotype control (BD Biosciences, Breda, The Netherlands) for 30 minutes in the dark at RT. Finally, cells were washed with PBS and analysed using flow cytometry (excitation at 635 nm, emission at 670 nm) (Accuri™ C6, BD Biosciences) with Accuri C6 software.
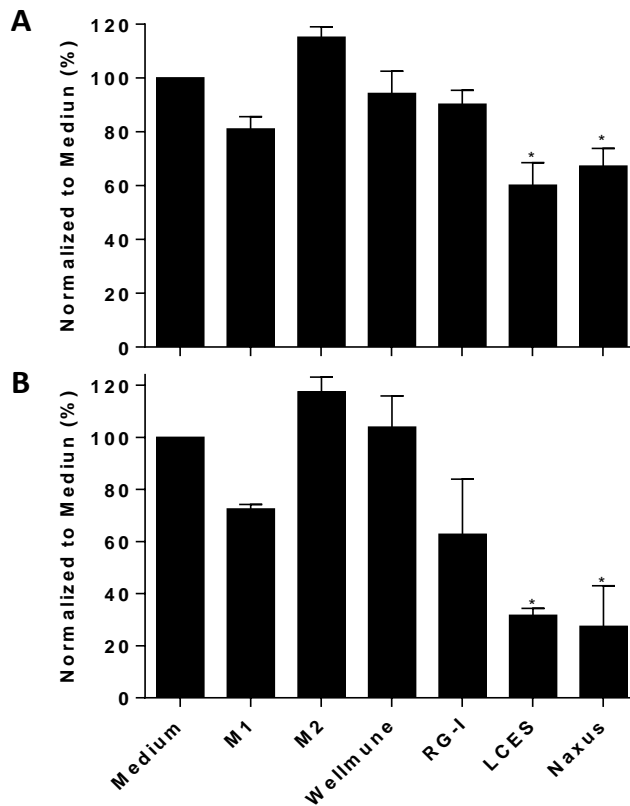
Figure 1. NDP-treated macrophages demonstrated decreased phagocytosis of *E.coli* particles. Macrophages were polarised towards M1 or M2 or treated with 500 µg/ml Wellmune Soluble, RG-I, LCES, or Naxus for 18 (A) or 48 h (B) and subsequently incubated for 1 h with fluorescent *E.coli*-bioparticles. Phagocytosis of *E.coli*-bioparticles was analysed by measuring MFI using flow cytometry. Bar charts show mean values normalised to medium stimulation (100%) ± SEM of n=5 (A) or 2 (B) different donors. Statistically significant differences were analysed compared to medium by one-way ANOVA: ***** $p < 0.05$

2.6 Phagocytosis assay and antigen processing analysis

To analyse phagocytic activity and antigen processing, polarised, NDP-treated or medium stimulated macrophages were washed with PBS and incubated for 1 h in 0.5 ml culture medium containing 2 µg/ml *E.coli* bioparticles (Molecular Probes, Leiden, The Netherlands) or 10 ng/ml DQ-OVA (D12053, Molecular Probes), respectively. Before

addition, *E.coli* bioparticles were re-suspended in non-heat-inactivated human serum (2 mg / 100 µl) and incubated for 60 minutes at 37 °C. Macrophages were detached as described above and diluted three times with PBS with 2% FBS. Cells were re-suspended in 200 µl PBS and analysed using flow cytometry (bioparticles: excitation at 495 nm, emission at 519 nm; DQ-OVA: excitation at 505 nm, emission at 515 nm) with Accuri C6 software.

## 2.7 Multiplex cytokine analysis

Secretion of cytokines and chemokines by macrophages following stimulation with medium, polarisation or treatment with NDP was measured using Bio-Plex Pro Reagent Kit (BioRad, Veenendaal, The Netherlands) according to the manufacturer's instructions. In brief, 50 µL magnetic beads were dispersed in a plate together with 50 µl of undiluted sample or standard and incubated for 1 h at RT. Subsequently, the beads were incubated with 25 µl detection antibodies for 30 min and with 50 µl streptavidin-PE for 10 min at RT. The beads were re-suspended in 125 µl assay buffer and read by Bio-Plex® MAGPIX™ Multiplex Reader (BioRad). Data processing was performed by using Bio-Plex Manager 5.0, and concentrations (in pg/ml) were interpolated from standard curves. To identify relations and to display our results most effectively, we used lower or upper limit of the standard curve for data points that were below or above the standard curve, respectively.

2.8 Migration assay

Migration assays were performed using an xCELLigence RTCA DP system (xCELLigence, ACEA Biosciences, San Diego, USA) and CIM-16 well plates, according to the manufacturer's instructions. In brief, 160 µl of macrophage-conditioned medium following polarisation, NDP-treatment or medium stimulation were added to the lower chamber of CIM-16 well plates. Fresh macrophage culture medium was used as negative control and CCL5 (400 ng/ml) as positive control. Following upper chamber attachment to the lower chamber, the upper chamber wells were filled with 50 µl THP-1 cell culture medium and equilibrated at 37 °C for 1 h which was recorded as background cell index. Next, 50 µl THP-1 cell suspension ($8x10^6$ cells/ml) was added to the upper chamber after which cells were allowed to settle for 5 minutes at RT. The cell index is a unit-less parameter used to indicate the impedance of electron flow caused by adherent cells and defined as (impedance at time point impedance - impedance in the absence of cells) / nominal impedance value. The cell index was measured every 5 min over the course of 22 h. Monocyte migration was subsequently calculated as area under the curve using Prism 6 software (Graphpad, La Jolla, USA).

2.9 Statistics

All parameters are presented as means ± SEM and differences between parameters were assessed for their significance with one-way ANOVA using Prism 6 software. Differences with $p < 0.05$ were considered significant.
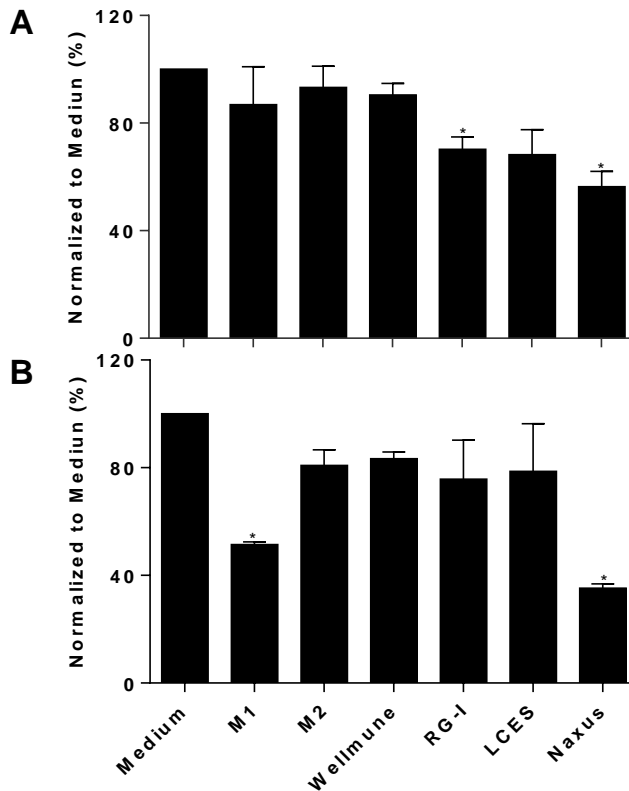
Figure 2. NDP-treatment of macrophages decreases their antigen processing capacity. Macrophages were polarised towards M1 or M2 or treated with 500 µg/ml Wellmune Soluble, RG-I, LCES, or Naxus for 18 (A) or 48 h (B) and subsequently incubated for 1 h with DQ-OVA. Processing of DQ-OVA was analysed by measuring MFI using flow cytometry. Bar charts show mean values normalised to medium stimulation (100%) ± SEM of n=4 (A) or 2 (B) different donors. Statistically significant differences were compared to medium by one-way ANOVA: * $p < 0.05$.

3 Results

3.1 NDPs LCES and Naxus reduce macrophage phagocytic capacity

Phagocytosis constitutes an important function and characteristic of macrophages [22]. In our previous study, where we investigated gene transcriptional responses of macrophages, analysis with Ingenuity Pathway analysis (IPA) revealed that genes coding for proteins involved in the pathway of phagosome formation were strongly affected by the NDPs (Supplementary Table 1) [20]. Those genes included receptors for NDP recognition (Dectin-1, MRC1, MRC2, CD302, MTERTK, TLR3, TLR4, and TLR7) and adaptors for modulating signalling pathways (SYK, PKC, PI3K, Rho-GTPase). To evaluate phagocytosis capacity of macrophages stimulated with various NDPs, we analysed uptake of *E. coli* bioparticles by flow cytometry (Figure 1). Following 18 and 48 h of incubation, M2 macrophages demonstrated an enhanced uptake of *E.coli*-bioparticles and M1 macrophages showed a reduced uptake. Interestingly, LCES and Naxus significantly reduced *E.coli*-bioparticle uptake following 18 h and, even more pronounced, following 48 h of incubation. In addition, RG-I demonstrated a reduced, albeit not significant, phagocytosis after 48 h of incubation.

3.2 NDP-treated macrophages demonstrated reduced antigen processing capacity

As phagocytosis of pathogens can lead to antigen presentation [23], we investigated whether LCES and Naxus also reduce antigen processing in macrophages. To study this, polarized or NDP-treated macrophages were incubated with DQ-OVA, a self-quenching conjugate of ovalbumin that fluoresces upon proteolytic degradation (Figure 2). Following 18 h of macrophage treatment, M1 and M2 macrophages demonstrated no change in antigen processing. However, NDPs RG-I and Naxus significantly, and LCES non-significantly, reduced antigen processing of macrophages. Of note, M1 macrophages and also Naxus-treated macrophages, but not RG-I-treated macrophages, showed significantly reduced antigen processing following 48 h of incubation.

3.3 NDPs did not induce IDO translation and activity

IDO activity is considered a key characteristic of M1 [24] and IDO1 was identified as M_{NDP} gene transcription marker [20]. To investigate IDO activity we analysed kynurenine levels in macrophage-conditioned medium following polarisation towards M1, M2, or treatment with NDPs (Figure 3A). As expected, we found high concentrations of kynurenine in M1-conditioned medium and could not detect kynurenine in M2-conditioned medium. Surprisingly, kynurenine levels in M_{NDP}-conditioned medium was also non detectable. To investigate the discrepancy between IDO gene transcription and activity in macrophages treated with NDPs, we analysed IDO protein expression
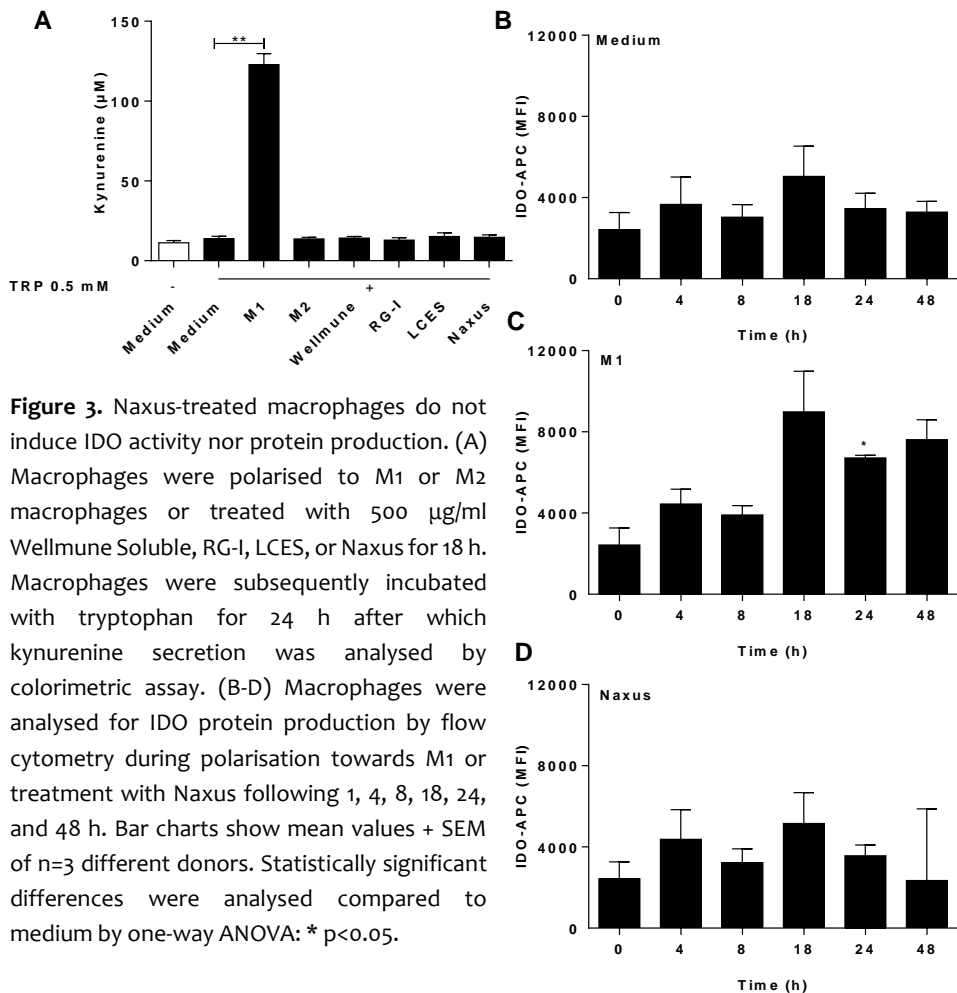
Figure 3. Naxus-treated macrophages do not induce IDO activity nor protein production. (A) Macrophages were polarised to M1 or M2 macrophages or treated with 500 µg/ml Wellmune Soluble, RG-I, LCES, or Naxus for 18 h. Macrophages were subsequently incubated with tryptophan for 24 h after which kynurenine secretion was analysed by colorimetric assay. (B-D) Macrophages were analysed for IDO protein production by flow cytometry during polarisation towards M1 or treatment with Naxus following 1, 4, 8, 18, 24, and 48 h. Bar charts show mean values + SEM of n=3 different donors. Statistically significant differences were analysed compared to medium by one-way ANOVA: * $p < 0.05$.

following 4, 8, 18, 24, and 48 h of incubation in M1- and Naxus-treated macrophages, which induced the strongest increase in IDO1 transcription among the NDPs. We used flow cytometry and analysed relative intracellular IDO protein concentrations by detecting mean fluorescence intensity (Figure 3B-D). This revealed that M1 macrophages contain significantly increased levels of IDO protein compared to medium-stimulated macrophages following 24 h of incubation. Naxus-treated macrophages, however, demonstrated no apparent increase in IDO protein level compared to medium control at any time point.

3.4 NDPs promote macrophage secretion of a unique set of signalling molecules

Analysis of gene transcriptional responses of macrophages to NDPs revealed a distinct signature profile of cytokine and chemokine gene expression when compared to M1 and M2 macrophages (Table 1). To analyse how these high levels of transcripts related to protein production, we quantified cytokines and chemokines in macrophage-conditioned medium following 18 and 48 h of incubation by multiplex cytokine analysis (Figure 4 and Supplementary Figure 1, respectively). CXCL9, CXCL10, CXCL11, and TNFα were chosen for analysis based on M1 macrophage gene expression levels, CCL24 was chosen based on M2 macrophage gene expression levels, and CCL1, CCL5, CCL20, CXCL8, IL1β, and MMP-1 were chosen based on NDP-treated macrophage gene expression levels. Most of the significant differences in gene transcription between medium stimulated and NDP-treated or polarised macrophages could not be confirmed with protein secretion. However, the multiplex results did even more strongly than gene transcription analysis demonstrate the unique profile of cytokine and chemokine production of NDP-treated macrophages when compared to medium, or M1 and M2 macrophages. M1 macrophages demonstrated a significant increase in secretion of TNFα, CXCL9, CXCL10, and CXCL11 following 18 h of polarisation and also of CCL5 following 48 h of incubation. In contrast, M2 macrophages did not show a significant change in cytokine and chemokine secretion compared to medium following either 18 or 48 h of incubation, including CCL24 which was identified as M2 macrophage marker. Naxus-treated macrophages demonstrated a significant increase in CCL1, CCL5, CCL20, CXCL8, and IL1β secretion following 18 h of incubation and also of CCL24 following 48 h of incubation. Macrophages treated with LCES significantly increased secretion of CCL1, CCL24, and CXCL8 following 18 h of incubation whereas RG-I-treated macrophages only significantly increased CXCL8 production. Finally, none of the cytokines or chemokines of which Wellmune Soluble significantly increase gene transcription revealed an increased secretion level compared to medium-stimulated macrophages.

Figure 4. Macrophages treated with NDPs secrete a unique mixture of cytokines and chemokines. Macrophages were polarised towards M1 or M2 or treated with 500 µg/ml Wellmune Soluble, RG-I, LCES, or Naxus for 18 h. Cytokine and chemokine secretion was analysed with Bio-Plex multiplex analysis. Bar charts show mean values ± SEM of n=3 different donors. Statistically significant differences were analysed compared to medium by one-way ANOVA: * $p<0.05$, ** $p<0.01$, *** $P<0.001$. (See next page)

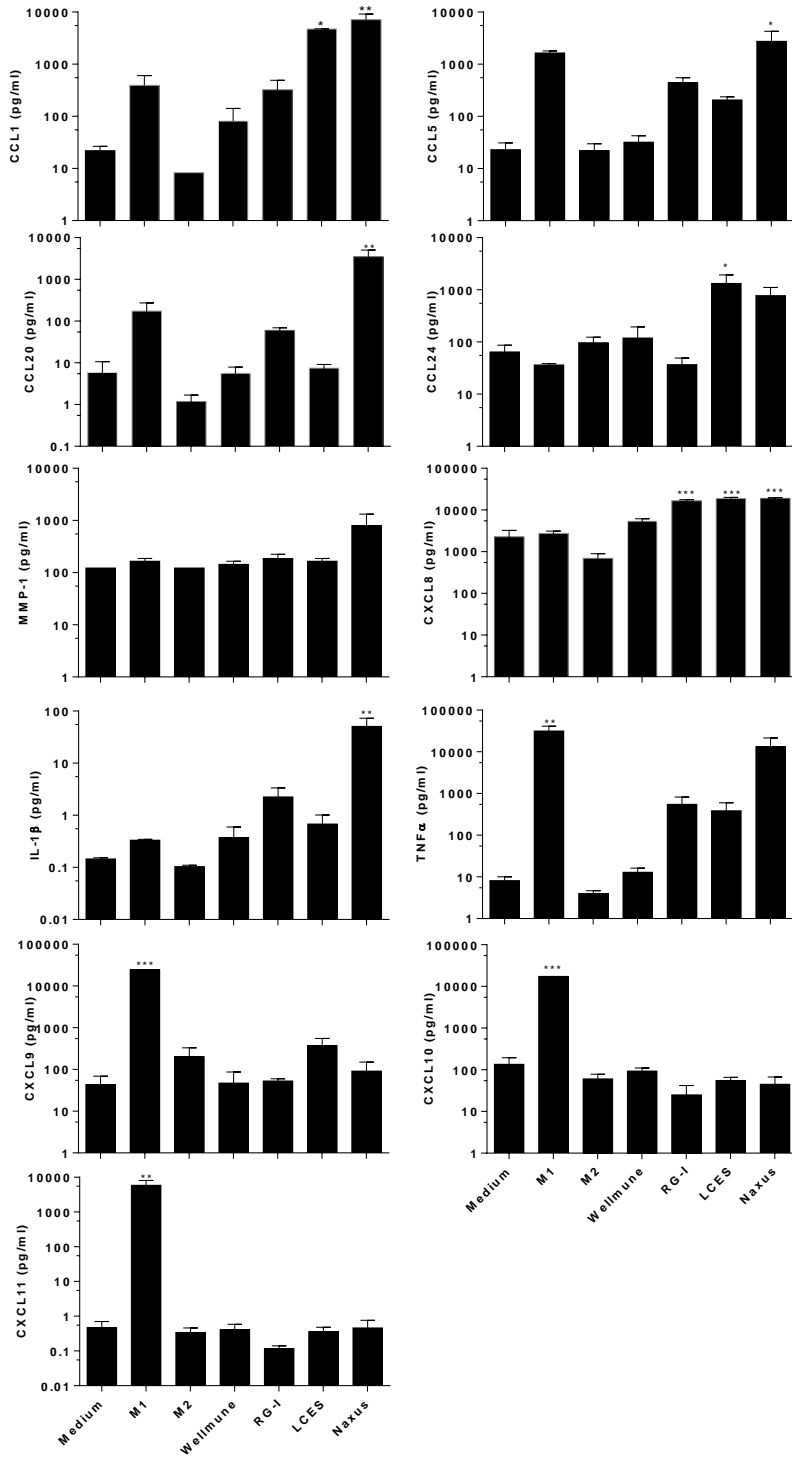Table 1. Differential expression of genes selected for Bio-Plex multiplex analysis

Genes	M1	M2	Wellmune	RG-I	LCES	Naxus	Chemoattractant functions[1]
CCL1	26.7***	-1.0	8.9*	23.8***	57.0***	238.9***	Monocytes but not neutrophils
CCL5	18.3***	1.6	2.1	9.0***	2.8*	9.6***	Monocytes, memory T-helper cells and eosinophils
CCL20	6.2**	-1.5	2.6	7.3**	4.3*	69.9***	Lymphocytes and, neutrophils, but not monocytes
CCL24	-1.7	7.3***	22.2***	18.9***	121.6***	150.1***	Resting T-lymphocytes, and eosinophils
CXCL8	1.3	-3.6**	13.3***	33.9***	17.2***	51.2***	Neutrophils, basophils, and T-cells, but not monocytes
IL1B	-1.4	-3.7**	8.8***	19.4***	10.7***	32.8***	Neutrophils and macrophages
TNF	11.4***	-1.2	1.5	1.0	2.3*	5.7***	Neutrophils and macrophages
MMP1	-1.0	1.2	3.5	19.4**	6.1*	106.5***	Muscle Cells
CXCL9	481.2***	2.3	2.5	2.2	6.6	1.6	Activated T-cells
CXCL10	239.0***	-1.5	6.1**	2.0	2.3	-2.2	Monocytes and T-lymphocytes
CXCL11	304.0***	1.1	1.9	1.7	1.3	-1.1	Interleukin-activated T-cells but not unstimulated T-cells, neutrophils or monocytes.

Values represent fold changes of gene expression, n=3 different donors. Statistically significant differences were calculated with IBMT regularised paired t-test: * FDR $P<0.05$, ** FDR $p<0.01$; *** FDR $p<0.001$
[1] Adapted from GeneCards (http://www.genecards.org/).

3.5 Naxus promotes macrophages recruitment of monocytes

M1 macrophages and NDP-treated macrophages, in particular after treatment with Naxus, demonstrated production of varying combinations of cytokines and chemokines. Based on the described activities of the signalling molecules (Table 1), we anticipated that the different combinations mainly lead to migration of monocytes and T cells. In addition, Naxus-treated macrophages might also induce migration of various granulocytes. To test whether the secreted signalling molecules of M1 and Naxus-treated macrophages indeed induce migration of monocytes and T cells, we performed a cell migration assay. To this end we used a transwell system in which the impedance of electron flow through the permeable membrane indicates cellular migration. We added monocytes or T cells to the upper compartment and CCL5 or macrophage-conditioned medium to the lower compartment. Unfortunately, the T cells were not able to impede the electric current, possible due to too small cell size, as no signal was detected whilst T cells could be detected by microscope in the lower compartment (data not shown). In contrast, monocyte migration was detected and variable depending on the medium added to the lower compartment (Figure 5). Monocytes did not spontaneously migrate as no signal was detected when fresh macrophage medium was added to the lower compartment. The presence of CCL5 or signalling molecules released by non-stimulated macrophages induced significant monocyte migration compared to fresh medium. In turn, signalling molecules released by M1 or Naxus-treated macrophages induced a significant increase in migration compared to non-stimulated macrophages.

4 Discussion

NDPs are considered immunomodulatory food components but the mechanism of action is not well understood yet. Besides that, different NDPs are expected to have different bioactive properties but there is no consensus on strategies how to properly analyse the bioactivity of NDPs [25]. Based on a large body of evidence it can be hypothesised that macrophages, as one of the key resident cell types in intestinal tissue, including Peyer's Patches, are involved in sensing NDPs in or from the lumen [26]. Based on the interaction with luminal ligands, macrophages will signal to other immune cells and hence may activate a multitude of complex downstream events. Here, we analysed four different NDPs with different biochemical characteristics for their effects on macrophage functionality in comparison to M1 and M2 macrophages. We demonstrated that an NDP such as Naxus can modulate macrophage function by decreasing phagocytosis and antigen processing capacity, which are essential innate immune
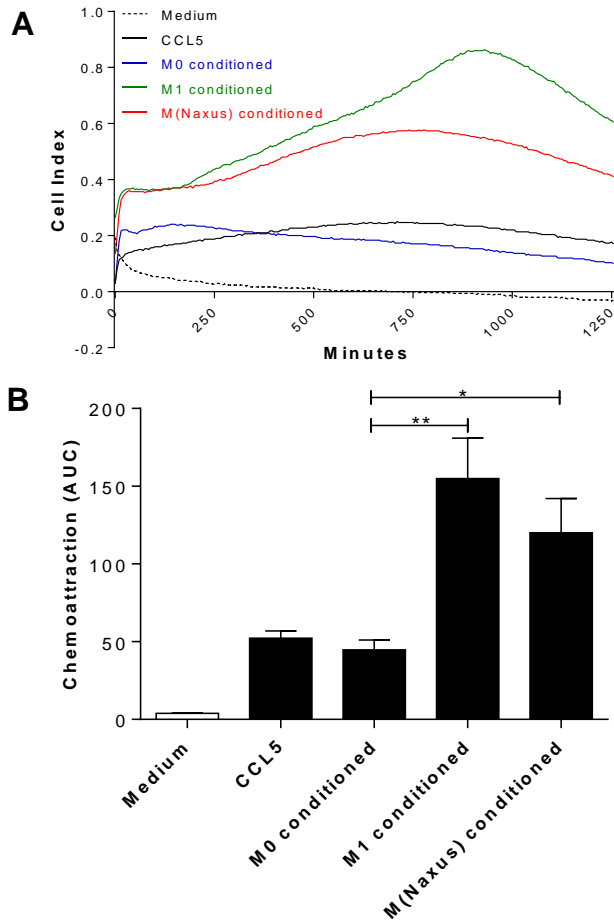
Figure 5. Naxus-treated macrophages promotes migration of monocytes. Macrophages were non-treated, polarised towards M1 or treated with 500 μg/ml Naxus for 18 h. The macrophage-conditioned medium, CCL5 or fresh medium was placed in the bottom compartment of the xCeLLigence RTCA DP system and THP-1 monocytes in the upper compartment. Migration of monocytes was determined by measuring every 5 minutes the electrical current resulting from the connection of electrodes by monocyte passage which is shown as the cell index. (A) Migration curves are shown as cell index that was recorded every 5 min. (B) Monocyte migration was compared by analysing area under curve. Bar charts show mean values ± SEM of n=3 different donors. Statistically significant differences were analysed compared to M0-conditioned medium by one-way ANOVA: * $p<0.05$, ** $p<0.01$.

functions and part of the first line of defence. At the same time, Naxus increases chemokine production and enhances monocyte recruitment by macrophages and possibly other immune cells towards the place of exposure.

A characteristic function of M1 macrophages is their IDO activity [27]. As IDO gene transcription was also strongly induced in NDP-stimulated macrophages [20], we analysed IDO protein expression and activity. M1 macrophages indeed demonstrated a strong IDO protein expression and activity, but, surprisingly, NDP-stimulated macrophages did not increase IDO protein content nor demonstrated IDO activity and kynurenine production (Figure 3). This suggests an inadequate translation of IDO mRNA or a selective inhibition of IDO protein production. Short-hairpin RNA (shRNA) was used to reduce IDO activity in tumour models which abrogated immune suppression [28]. It would therefore be of interest to analyse whether NDP-treatment of macrophages results in increases anti-IDO shRNA production as this might benefit colon cancer treatments.

M2 macrophages demonstrate increased particle uptake and processing capabilities when compared to M1 macrophages [29]. Interestingly, NDP-treated macrophages, in particular those treated with Naxus and LCES, strongly decreased phagocytosis compared to M2 and M1 macrophages. This decrease might be linked to the previously described reduction in gene expression of certain lectins such as CD302, MERTK and dectin-1. All are known to be involved in formation of the phagocytic synapse and uptake of particles [20, 30, 31].

When particle uptake is reduced one might anticipate to also observe a reduced particle processing. However, we used DQ-OVA to test particle processing and ovalbumin uptake was linked to mannose receptor (Aliases: MRC-1, CLEC13D, CD206) activity [32] which expression was not affected by NDPs [20]. Without evidence that the uptake of DQ-OVA might be impeded it appears that NDP-stimulated macrophages have a reduced capacity to process particles as well as a reduced capacity to take up particles. Indeed, the NDPs significantly downregulate gene expression of HLA-DM and HLA-DO (Supplementary Table 2) which are critically involved in loading of lysosomal antigens on HLA-class II molecules [33].

NDP-treated macrophages are equally dissimilar from M1 and M2 macrophages regarding cytokine/chemokine production as for the above described characteristics. NDP-treated macrophages, in particular Naxus, secreted a signature mix of CCL1, CCL5, CCL20, CCL24, CXCL8 and IL1β in the absence of CXCL9, 10 and 11 (Figure 4). Notably, the absence of CXCL11 as well as functional IDO protein production by NDP-treated macrophages again confirms that our NDP-preparations were devoid of functional LPS-

contaminations. We observed that chemokines produced by M1 macrophages strongly attract monocytes (Figure 5). This is in line with literature which indicates that an influx of bone-marrow-derived monocytes replenishes intestinal macrophage populations and in particular M1-like macrophages [13]. Similarly, Naxus-treated macrophages also induced migration of monocytes. The combination of chemokines produced by Naxus-treated macrophages might also attract eosinophils, neutrophils, basophils and resting T cells (Table 1). More directly, IL1β production was shown to be important for intestinal ILC3 activation [34], CCL5 mediates recruitment of CCR5-positive regulatory T cells into the intestine [35] and CCL20 is essential for migration of CCR6-positive TH17 cells towards the intestine [36]. As all these cells have demonstrated to prevent exacerbation of inflammation in the intestine, NDP-treated macrophages might strongly contribute to maintenance of intestinal homeostasis.

This broad effect on immune interaction and activity is in line with various studies describing beneficial health effects of orally administered NDPs [3, 5, 7]. The recruitment of a variety of immune cells by macrophages may be pivotal in orchestrating a full blown innate response, resulting in signalling to and, possibly, activation of the adaptive immune functions.

In conclusion, following a transcriptional analysis we here characterized M_{NDP} from a functional perspective. NDPs and especially Naxus were shown to induce an alternative functionality in macrophages compared to inflammatory or tolerant macrophages. NDP-treated macrophages were also characterized by a unique cytokine and chemokine secretion profile and prompted macrophages recruitment on monocytes. This specific NDP-induced phenotype may be relevant in regulating the physiological consequences of NDP-intake. Ex vivo analysis of macrophage phenotypes following oral intake of NDPs coupled to intestinal health and homeostasis measurement could clarify such possible relevance.

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Supplementary Table 1 Differential expression of genes in phagosome formation pathway

Gene	M1	M2	Wellmune	RG-I	LCES	Naxus
ITGA2	1.3	1.07	1.14	2.2	1.6	6.7**
FCAR	-1.7	-1.71	2.23*	3.7***	1.6	3.1***
RND3	1.4	-1.54	2.06*	2.5**	2.3***	3.1**
ITGA5	1.1	1.14	1.50**	1.7***	2.1***	2.9***
RHOH	1.3	-1.01	1.11	2.0	1.1	2.9**
FCER2	1.2	23.73***	1.82	1.6	2.5*	2.6*
PRKCH	-2.5***	-1.6*	1.1	1.2	1.7*	2.1**
PLCB1	-1.1	2.7***	1.1	1.1	1.7*	2.0**
TLR2	-1.5	-1.6*	2.0**	2.8***	-1.2	1.5
PIK3R6	1.5*	2.1***	1.3	1.4	1.3	1.3
RHOQ	2.1***	1.2	1.0	1.3*	1.4**	1.2
RHOF	-2.0***	1.5*	-1.1	-1.1	1.1	1.2
RHOU	2.0***	-1.0	1.3	1.7**	-1.4*	1.2
FCGR2A	-3.6***	1.0	1.0	1.3	-1.6*	1.1
MRC1	-4.7***	2.7***	2.1**	1.7*	1.7*	1.1
FCGR2B	-4.8**	1.8	1.7	1.6	-1.4	-1.0
PRKCA	-2.1***	1.0	-1.1	-1.4*	1.1	-1.2
FN1	-2.2*	2.0	-1.5	-1.2	-1.0	-1.4
MARCKS	1.4	1.4	-1.2	1.1	-2.8***	-1.5
Dectin-1	-1.4*	1.9***	1.3	1.3*	-1.0	-1.7**
PIK3C2B	-2.1***	1.3	-1.7*	-1.9**	-1.4	-1.7*
TLR8	2.9**	-1.4	-1.2	-1.0	-1.8*	-1.8
MSR1	-1.4	-1.3	-1.3	-1.7*	-1.2	-1.8*
PIK3CD	-1.3	1.2	-1.4	-1.3	-1.5*	-1.9***
PIK3R3	1.6	-1.1	-1.3	-1.9*	-1.7	-1.9*
PLCL2	-1.2	-1.2	-1.3*	-1.5***	-1.8***	-2.0***
TLR3	1.4	-1.1	-1.5	-1.5	-2.3**	-2.1**
TLR4	1.2	-1.3	-1.2	-1.4	-2.1***	-2.2***
ITGA3	-2.9***	1.0	-1.4	-2.0***	-1.3	-2.2***
MRC2	-2.0***	-1.2	-1.6*	-2.1***	-1.6**	-2.6***
INPP5D	1.1	1.2	-1.2	-1.6*	-1.6*	-2.7***
FCGR3A	-2.9***	-1.1	1.1	-1.0	-2.2**	-3.4***
ITGA4	-2.0***	-1.1	-1.4	-2.0***	-1.9***	-3.6***
MERTK	-5.1***	-1.8	-1.1	-1.3	-2.1*	-3.7***
TLR5	-4.7***	-1.1	-1.2	-2.4***	-2.5***	-4.3***
CD302	-3.4***	1.0	-1.5	-2.6*	-2.8**	-4.9***
TLR7	-1.0	-3.4**	-4.2**	-8.2***	-7.9***	-11.2***

Values represent fold changes of gene expression of M1 or M2 macrophages or Wellmune Soluble, RG-I, LCES or Naxus-treated macrophages compared to non-stimulated macrophages of n=3 different donors. Statistically significant differences were calculated with IBMT regularised paired t-test: * FDR P<0.05, ** FDR p<0.01; *** FDR p<0.001.

Supplementary Table 2 Differential expression of antigen processing related genes

Genes	M1	M2	Wellmune	RG-I	LCES	Naxus
CD80	11.7***	1.7*	2.4**	4.7***	2.2**	4.9***
CD83	2.9***	1.4**	1.1	1.1	1.7***	2.0***
CD274	12.8***	3.0***	2.2**	4.0***	2.5***	5.9***
HLA-DMA	1.3*	1.2	1.0	-1.5**	-1.5**	-1.9***
HLA-DMB	1.3	-1.0	-1.2	-2.2***	-1.7**	-3.1***
HLA-DOA	3.6***	1.7***	-1.2	-1.8***	-1.7***	-3.5***
HLA-DOB	5.6***	1.3	1.1	1.1	1.1	1.1
HLA-DQA2	11.4***	1.1	2.2	2.2	1.5	2.2
HLA-DQB2	3.0***	1.1	1.2	1.3	1.0	-1.1
HLA-F	2.8***	-1.0	1.1	1.2	-1.0	1.0
HLA-L	3.3***	1.1	-1.3	-1.0	-1.1	-1.0

Values represent fold changes of gene expression of M1 or M2 macrophages or Wellmune Soluble, RG-I, LCES or Naxus-treated macrophages compared to non-stimulated macrophages of n=3 different donors. Statistically significant differences were calculated with IBMT regularised paired t-test: * FDR P<0.05, ** FDR p<0.01; *** FDR p<0.001.

Supplementary Figure 1. Macrophages treated with NDPs produce a unique cytokine and chemokine profile following 48 h of incubation. Macrophages were polarised towards M1 or M2 or treated with 500 µg/ml Wellmune Soluble, RG-I, LCES, or Naxus for 48 h. Cytokine and chemokine secretion was analysed with Bio-Plex multiplex analysis. Bar charts show mean values ± SEM of n=2 different donors. Statistically significant differences compare to medium were analysed by one-way ANOVA: * p<0.05, ** p<0.01, *** P<0.001. (See next page)
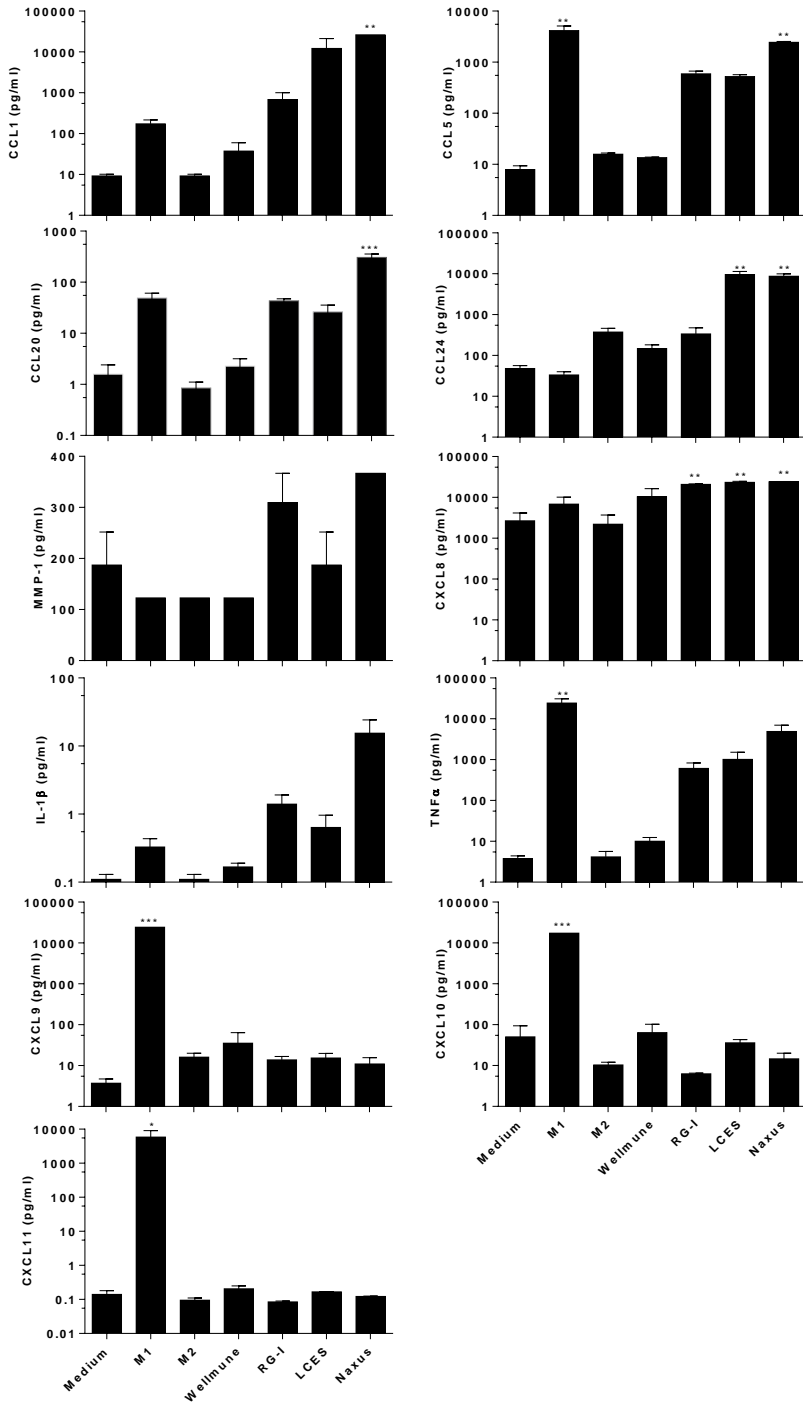
Chapter 5

Macrophage plasticity can be modulated by non-digestible polysaccharides

Yongfu Tang, Coen Govers, Ellen H. Stolte, Harry J. Wichers, Jurriaan J. Mes

Chapter 5

Abstract

Macrophage plasticity is essential for intestinal immune homeostasis. We previously reported that non-digestible polysaccharides (NDPs) skewed macrophages to an alternative subset M_{NDP} compared to inflammatory macrophages (M1) as induced by TNFα and IFNγ or tolerant macrophages (M2) as induced by IL4. Here, we analysed plasticity of M1 and M2 macrophages in response to NDPs treatment to evaluate whether phenotype and function of M1 and M2 can be further modulated. Subset specific gene expression markers were analysed together with functional properties including phagocytosis capacity, antigen processing capacity, cytokine secretion, and monocytes recruiting. Macrophage plasticity was demonstrated as M1 and M2 macrophages could be skewed to an alternative subset indicated by gene expression markers. In addition, phagocytosis and antigen processing capacity of both M1 and M2 were decreased by the NDP Naxus. Besides, cytokine secretion by both M1 and M2 macrophages were induced by Naxus. In addition, Naxus induced M2 macrophages recruiting ability, but not M1. In conclusion, we observed that NDP Naxus modulated M1 and M2 macrophage towards phenotype and function of M_{NDP}, which implies the NDP Naxus might be used for modulating intestinal immune balance.

Keywords: Arabinoxylan; Inflammatory macrophages; Macrophages function; Tolerant macrophages; Wheat

1 Introduction

Macrophages play a critical role in intestinal immunity as the largest amount of immune cells in intestine [1]. Intestinal macrophages are constantly replenished from circulating CD14+ monocytes by CCR2-dependent influx in the intestine [2]. They play a role in maintaining intestinal homeostasis by remodelling tissue [3] and clearing dying cells [4]. These functions are initiated upon activation of pattern recognition receptors (PRRs) through ligand binding [5] or as response to environmental ques in the form of cytokines or other signalling molecules [6, 7].

Macrophages respond with modulating their phenotype (also termed plasticity) upon these stimulations exemplified by skewing of tolerant (M2) macrophages to inflammatory (M1) macrophages by LPS stimulation [8]. The process of subset skewing is accompanied by metabolic reprogramming [9]. For instance, M1 macrophages polarised by LPS were modulated by IL10 and underwent metabolic reprogramming from glucose uptake and glycolysis to oxidative phosphorylation [9].

There are two main subsets of intestinal macrophages termed lamina propria macrophages (LpM) and muscularis propria macrophages (MMs), which function as M1 and M2 macrophages, respectively [10]. Those two subsets balance intestinal immunity by sensing the danger signals from the local environment and are important for wound healing and tissue repair [5]. Research has shown that microbiota and dietary components are able to direct macrophage subtypes [11]. ω-3 fatty acid exhibited anti-inflammatory effects by decreasing both gene expression and protein secretion of LPS-induced M1 markers TNFα and IL6 through PPAR4 activation [12]. In addition, yeast-derived β-glucan converted M1 to M2 as shown by an enhanced glycolysis, Krebs cycle, and glutamine utilization [13].

Non-digestible polysaccharides (NDPs) potentially simulate cell wall fragments of natural pathogens which can be recognized by receptors on the surface of macrophages. β-Glucan, as component in fungal cell walls, was proven to activate dectin-1 and trigger NFAT activation in macrophages and dendritic cells to secrete cytokines [14]. This activation initiated an innate immune response via dectin-1 activation and was accompanied with the formation of a phagocytic synapse [15]. Like the fungus-derived β-glucans, also the yeast-derived β-glucans could skew M2 to M1 through dectin-1 and subsequently the Syk-Card9-ERK pathway [13]. Also other dietary β-glucans were reported to be taken up and transported by intestinal macrophages [16]. Lentinan, the β-glucan from shiitake, upregulated pro-inflammatory cytokines (IL1α, TNFα, and IL6) through TLR4 signalling in murine macrophages *in vitro.* However, it also inhibits IL1β production through inhibiting AIM2 inflammasome activation [17]. In addition, a

randomized dietary intervention in healthy young adults demonstrated that shiitake mushrooms affected immunity by increasing IL4, IL10, TNFα and IL1α levels and decreased MIP1α/CCL3 level [18]. Oral intake of oat β-glucans activated NF-κB transactivation and increased IL12 in intestinal leukocytes in mice [19]. Immune activation by cereal glucans might also proceed through the dectin-1 pathway. For example, barley β-glucans induced IL6 secretion in murine macrophages, and this activation is mediated by the dectin-1 pathway and accompanied by phosphorylation of Syk [20]. Furthermore, we previously showed that NDPs derived from wheat, apple and shiitake skewed macrophages to an alternative subset (M_{NDP}). These M_{NDP} were characterised by increasing gene expression of IDO1, LAMP3, dectin-2, Mincle, CD80, GCH1, and decreasing gene expression of CXCL11 and MGL compared to medium control (M0) [21]. In addition, M_{NDP} showed decreased phagocytosis and antigen processing capacity (**Chapter 4**) but an increased production of chemokines and recruiting ability towards monocytes compared to M0 (**Chapter 4**).

M1 and M2 subset macrophages do play distinct roles in intestinal immunity. It is hence interesting to investigate whether NDPs can induce plasticity in these subsets and re-polarize these macrophage subsets towards the newly identified M_{NDP}. In this study, we confirmed the effects of NDPs on the phenotype and functional capacity of M1 and M2. As a result, the effects of NDPs on M1 and M2 showed a similar trend in the gene expression as primary non-polarised macrophages exposed to NDP; Naxus significantly decreased phagocytosis and antigen processing capacity of both M1 and M2 while increasing the secretion of CCL1 and other cytokines. This enhanced recruiting ability capacity of M2 but not of M1. However, these effects on M1 and M2 were blocked by a Caco-2 monolayer of small intestinal cells in a coculture system indicating that a physical interaction of NDPs and macrophages is required.

2 Materials and Methods

2.1 NDPs information

Macrophages were treated with Wellmune Soluble, RG-I, LCES, or Naxus as described previously [21]. In brief, Wellmune Soluble (referred as Wellmune in tables and figures) is a water soluble β-1,3/1,6-glucan from the yeast *Saccharomyces cerevisiae* provided by BioThera (Eagan, MN, USA). RG-I is a concentrate of rhamnogalacturonan I from apple juice provided by INRA (Paris, France). LCES (lentinan containing extract from shiitake) is a β-1,3/1,6-glucan extract from shiitake. Naxus is a polysaccharide from wheat, rich in arabinoxylan, provided by BioActor (Maastricht, The Netherlands). Before exposure to

macrophages, RG-I was treated with alkaline-ethanol at 4 °C for 5 h, and LCES and Naxus were treated with alkaline-ethanol at 56 °C for 5 h, followed by neutralization with HCl and freeze-drying to inactivate lipopolysaccharide (LPS) [22]. Resulting functional LPS contaminations of all NDPs samples was demonstrated to be below 0.01 EU/mg [22].

2.2 Primary macrophage differentiation and polarization

Primary macrophages were differentiated as previously described [21]. In brief, human monocytes were obtained from buffy coats from healthy donors (Sanquin, Nijmegen, The Netherlands) using the QuadroMACS system and CD14 microbeads according to manufacturer's protocol (Miltenyi Biotec, Leiden, The Netherlands). A written informed consent was obtained before sample collection. Monocytes were differentiated into macrophages following 7 days culturing in presence of 50 ng/ml MCSF (R&D systems, Minneapolis, USA). MCSF was replenished after 3 and 5 days of culture. On day 7, macrophages were polarised by 20 ng/ml TNFα and 20 ng/ml IFNγ (for M1 polarization), or 20 ng/ml IL4 (for M2 polarization) without MCSF for 18 h. To analyse plasticity, these M1 and M2 macrophages subsets at day 8 were then exposed to 500 µg/ml NDPs in medium without and appropriate controls for 24 h.

2.3 RNA extraction and cDNA synthesis

RNA was extracted by lysing cells with 200 µL TRIzol (Invitrogen, Bleiswijk, The Netherlands) for each well in the 24-well plates. This was followed by RNeasy (Qiagen, Venlo, The Netherlands) clean-up according to manufacturer's protocol. The integrity of the ribosomal RNA was analysed using agarose (Eurogentec, Liège, Belgium) gel electrophoresis (1%). RNA concentration and purity were checked using the Nanodrop spectrophotometer system (Nanodrop Technologies, Wilmington, DE, USA) and only samples with a ratio (Abs 260/280 nm) between 1.8 and 2.1 were used for qPCR and microarray analysis. Subsequently, cDNA was synthesised with iScript (Bio-Rad, Veenendaal, The Netherlands) according to manufacturer's protocol.

2.4 qPCR

qPCR was performed as described elsewhere [23]. In brief, 5 µl cDNA was mixed with 10 µl iQ™ SYBR Green supermix (Bio-Rad), forward and reverse primers, and milliQ water to total 20 µl. Primers were derived from the Harvard Primerbank (http://pga.mgh.harvard.edu/primerbank/) or designed using Clone Manager Professional 9 and synthesized by Biolegio (Nijmegen, The Netherlands). Primers are listed in Supplementary Table 2. The following thermal cycling conditions were used for amplifying the target sequences: 90 s at 95 °C, followed by 40 cycles at 95 °C for 10 s, 58 °C for 10 s and 72 °C for 15 s, and a final elongation step at 72 °C for 2 min and performed

on the CFX96 Touch Real-Time PCR Detection System (Bio-Rad). qPCR was performed in technical duplicate, and all samples were normalised to geometric means of PUM1 and RPLP0 expression [24] and medium-stimulated macrophages using the qBase$^+$ software (Biogazelle, Gent, Belgium).

2.5 Microarray analysis

Microarray analysis was performed as described previously [25]. Briefly, RNA quality was verified with the RNA 6000 Nano assay on an Agilent 2100 Bioanalyzer (Agilent Technologies, Amstelveen, The Netherlands). Hybridization, washing and scanning of the Affymetrix Human Gene 1.1 ST array plate was performed on an Affymetrix Gene Titan platform according to standard protocols. Scans of arrays were analysed using package from Bioconductor project. Raw signal intensities were obtained by Robust Multi-array Average method. Probe sets were defined using remapped chip definition file (CDF) based on Entrez gene database. Individual Genes were considered to be expressed if UPC algorithm values are above 0.5 on at least one array [26]. Genes were selected that meet the cut-off of absolute fold change > 1.8 and false discovery rate corrected p<0.05 (IBMT regularised paired t-test) on at least one treatment. The functional interpretation of gene signatures was executed using IPA 3.0 (Ingenuity, QIAGEN, Redwood, CA, USA). Microarray data were deposited in the Gene Expression Omnibus (GEO) database.

2.6 Phagocytosis assay and antigen processing analysis

To analyse phagocytic activity and antigen processing, M1 and M2 macrophages with or without NDP treatment were washed with PBS and incubated for 1 h in 0.5 ml culture medium containing 2 µg/ml *E.coli* bioparticles (Molecular Probes, Leiden, The Netherlands) or 10 ng/ml DQ-OVA (Molecular Probes), respectively. Before addition, *E.coli* bioparticles were re-suspended in non-heat-inactivated human serum (2 mg / 100 µl) and incubated for 60 minutes at 37 °C. Macrophages were detached as described above and diluted three times with PBS with 2% FBS. Cells were re-suspended in 200 µl PBS and analysed using flow cytometry (bioparticles: excitation at 495 nm, emission at 519 nm; DQ-OVA: excitation at 505 nm, emission at 515 nm) with Accuri C6 software.

2.7 Multiplex cytokine analysis

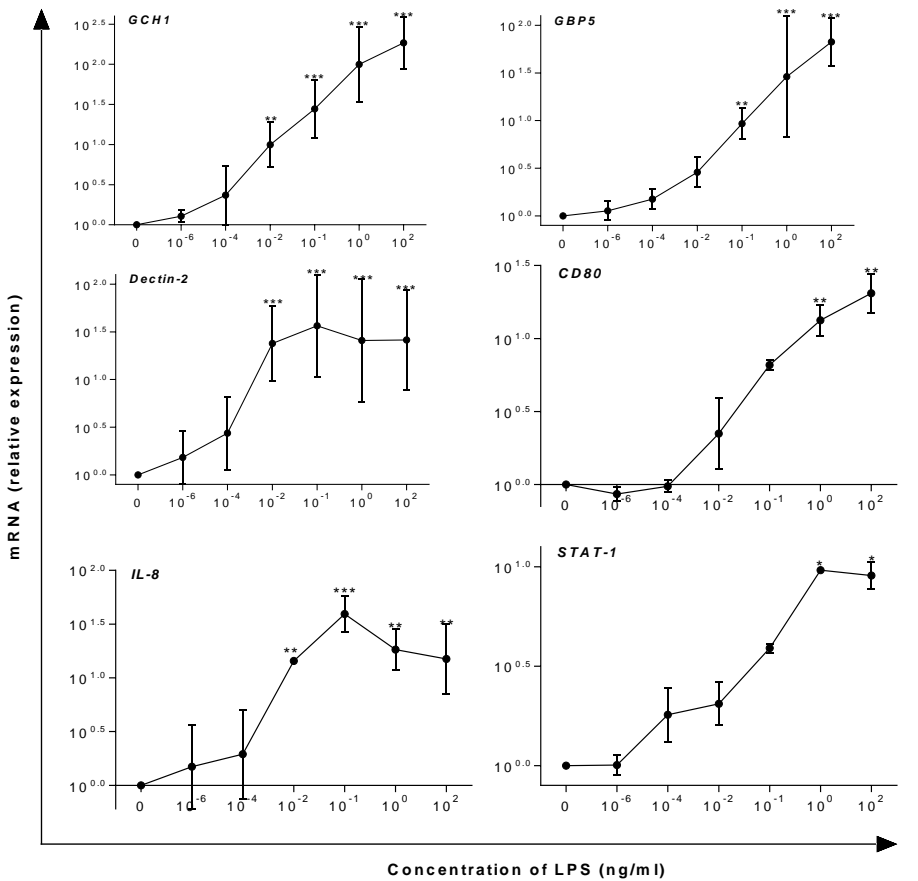Secretion of cytokines and chemokines by M1 and M2 macrophages with or without NDP stimulation were measured using Bio-Plex Pro Reagent Kit (BioRad, Veenendaal, The Netherlands) according to the manufacturer's instructions. In brief, 50 µL magnetic beads were dispersed in a plate together with 50 µl of undiluted sample or standard and incubated for 1 h at RT. Subsequently, the beads were incubated with 25 µl detection

antibodies for 30 min and with 50 µl streptavidin-PE for 10 min at RT. The beads were re-suspended in 125 µl assay buffer and read by Bio-Plex® MAGPIX™ Multiplex Reader (BioRad). Data processing was performed by using Bio-Plex Manager 5.0, and concentrations (in pg/ml) were interpolated from standard curves. To identify relations and to display our results most effectively, we used lower or upper limit of the standard curve for data points that were below or above the standard curve, respectively.

2.8 Migration assay

Migration assays were performed using an xCELLigence RTCA DP system (xCELLigence, ACEA Biosciences, San Diego, USA) and CIM-16 well plates, according to the manufacturer's instructions. In brief, 160 µl of macrophage-conditioned medium were

Figure 1A

added to the lower chamber of CIM-16 well plates. Fresh macrophage culture medium was used as negative control and CCL5 (400 ng/ml) as positive control. Following upper chamber attachment to the lower chamber, the upper chamber wells were filled with 50 µl THP-1 cell culture medium and equilibrated at 37 °C for 1 h which was recorded as background cell index. Next, 50 µl THP-1 cell suspension ($8x10^6$ cells/ml) was added to the upper chamber after which cells were allowed to settle for 5 minutes at RT. The cell index is a unit-less parameter used to indicate the impedance of electron flow caused by adherent cells and defined as (impedance at time point impedance - impedance in the absence of cells) / nominal impedance value. The cell index was measured every 5 min over the course of 22 h. Monocyte migration was subsequently calculated as area under the curve using Prism 6 software (Graphpad, La Jolla, USA).
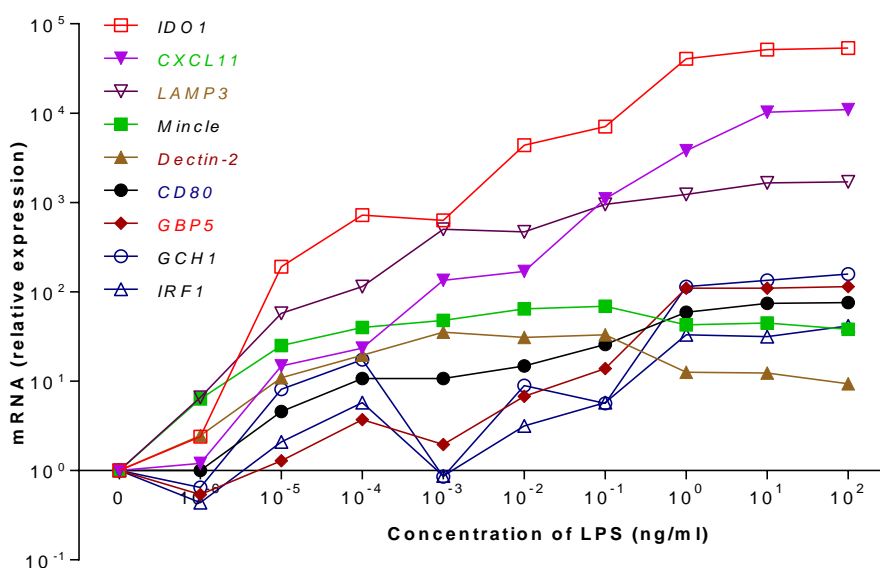
Figure 1B
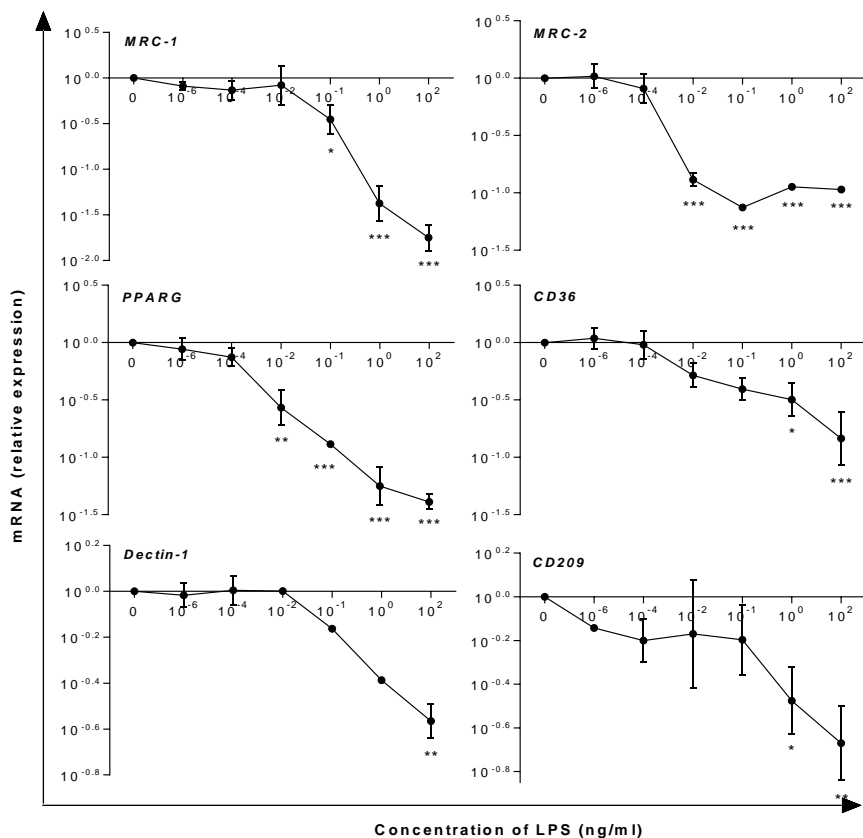
2.9 Caco-2-macrophages coculture

Macrophages were differentiated on 250 µl hydrogel (Cellendes, Reutingen, Germany) coated 24-well plate for 7 days with MCSF and differentiated to either M0, M1 or M2 for 18 h. This was followed with coculturing with 21-day transwell grown Caco-2 (**Chapter 1**) which were stimulated with either medium control, LCES (500 µg/ml) or Naxus (500 µg/ml). Macrophage-conditioned medium was collected for migration analysis, and macrophages were collected by TRizel for qPCR.

2.11 Statistics

All parameters are presented as means ± SEM and differences between parameters were assessed for their significance with one-way ANOVA using Prism 6. Differences with p<0.05 were considered significant.

Figure 1C

Figure 1. Gene expression pattern of macrophages following exposure to titrated amounts of LPS. M0 macrophages were stimulated with various concentrations of LPS (ng/ml) for 18 h and analysed for gene expression of M1 (A) and M2 (B) markers using qPCR. Results are shown as fold change compared to non-stimulated macrophages by mean values ± SEM, n=2 different donors. Statistically significant differences were analysed by one-way ANOVA: ***** p<0.05, ****** p<0.01, ******* P<0.001. C. M2 macrophages were stimulated with various concentrations of LPS (ng/ml) for 24 h and analysed gene expression of M1 markers using qPCR. Results are shown as fold change compared to non-stimulated macrophages, n=1 donors.

3 Results

3.1 Macrophages plasticity characterised by responding to LPS

To study the plasticity of macrophages, first the response to a strong immune potent agent was analysed by exposure to different concentrations of LPS. M1 (GCH1, GBP5, dectin-2, CD80, IL8, and STAT1) and M2 (MRC1, MRC2, PPARG, CD36, dectin-1, and CD209) specific gene expression markers were subsequently analysed by qPCR. Results clearly showed that gene expression of M1 markers on M0 were increased in an LPS concentration dependent manner (Figure 1A). Expression of GCH1, dectin-2, and IL-8 showed more sensitivity than other M1 makers and already significant changes in expression were found at 0.01 ng/ml LPS. On the other hand, gene expression of M2 markers on M0 were decreased in an LPS concentration dependent manner (Figure 1B). MRC1, MRC2, and PPARγ were more sensitive than other M2 markers, and MRC1 responded stronger to the highest experimental concentration (100 ng/ml) than other markers.

To investigate plasticity of our macrophages, we analysed gene expression of some M1 markers in M2 cells exposed to LPS (Figure 1C). The expression of all of the genes in the list was increased and showed to be concentration dependent. IDO1, CXCL11, and LAMP3 showed more sensitively in responding to LPS than other genes. This gene expression trend of M1 markers shows the inherent ability of M2 macrophages to alter their phenotype in our culturing and polarization method.

3.2 NDPs can re-polarise both M1 and M2 macrophages towards an M$_{NDP}$ subset based on gene expression analysis

Our previous research showed that NDPs can also polarise M0 towards a phenotype alternative to pro-inflammatory and tolerant macrophages which was termed M$_{NDP}$. This could be demonstrated most clearly based on gene expression of IDO1, LAMP3, CD80, Mincle, dectin-2, CXCL11, MGL, and IRF1 [21]. Through microarray analysis, the marker panel is expanded with CCL20, IL1β, IL8, LOX1, TNF, CCL13, and TLR7 (Supplementary Table 1) which all specifically responded to NDP-stimulations. To investigate whether NDPs can also re-polarise M1 and M2 polarised macrophages, we stimulated M1 with four different NDPs (i.e. Wellmune soluble, RG-I, LCES, and Naxus) and analysed gene expression (Figure 2). Naxus treatment increased gene expression of IL1B, IL8, TNF, CD80, dectin-2, Mincle, LOX1, CCL20, and LAMP3, while it decreased gene expression of MGL, TLR7, and CXCL11. Similarly, RG-I increased gene expression for IL1B, IL8, CD80, dectin-2, Mincle, LOX1, CCL20, and LAMP3, while it decreased gene expression for MGL, TLR7, and CXCL11. LCES increased IL1B, IL8, and CCL20 gene expression, while it
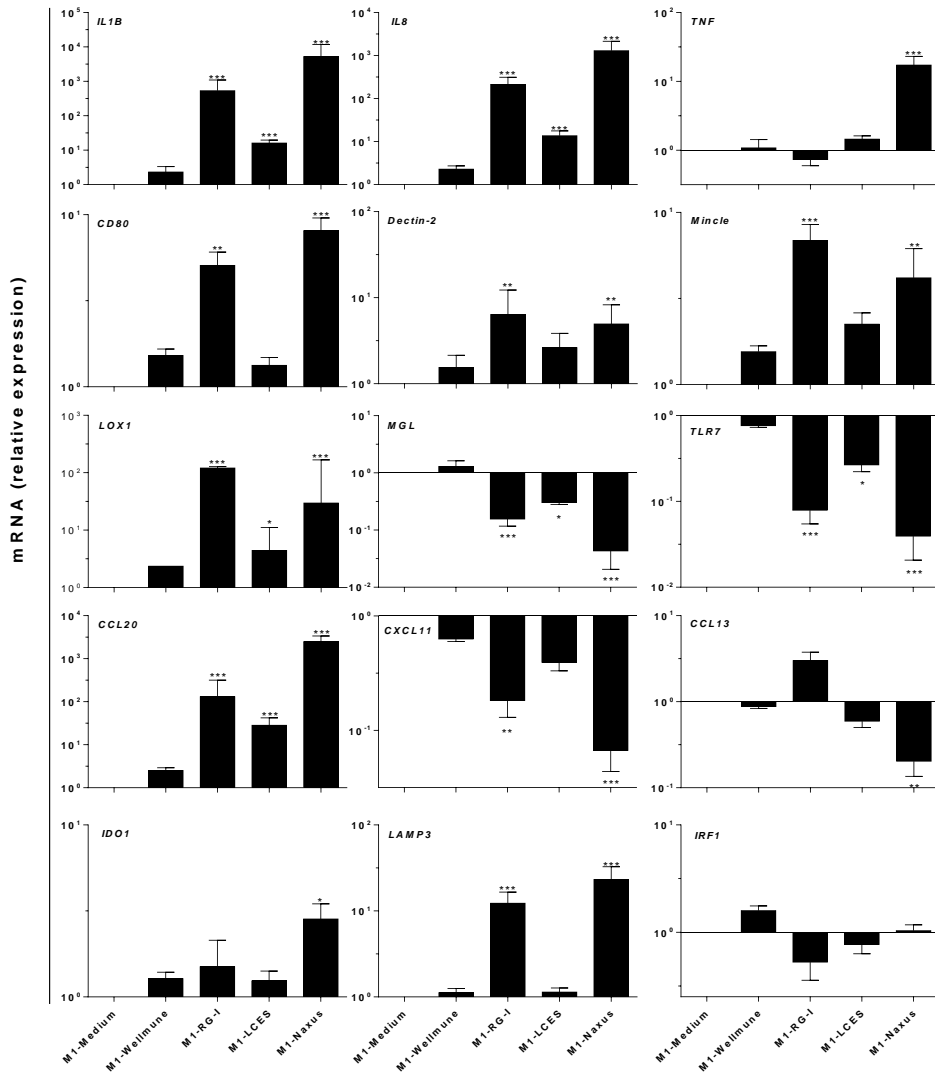
Figure 2. NDPs promote re-polarisation of M1 to an M$_{NDP}$ phenotype. M1 polarised macrophages were stimulated with Wellmune, RG-I, LCES, and Naxus at 500 µg/ml for 24 h and analysed for gene expression using qPCR. Results are shown as fold change by mean values ± SEM in a bar chart, n=3-4 different donors. Statistically significant differences compared to medium control were analysed by two-way ANOVA: * p<0.05, ** p<0.01, *** p<0.001.
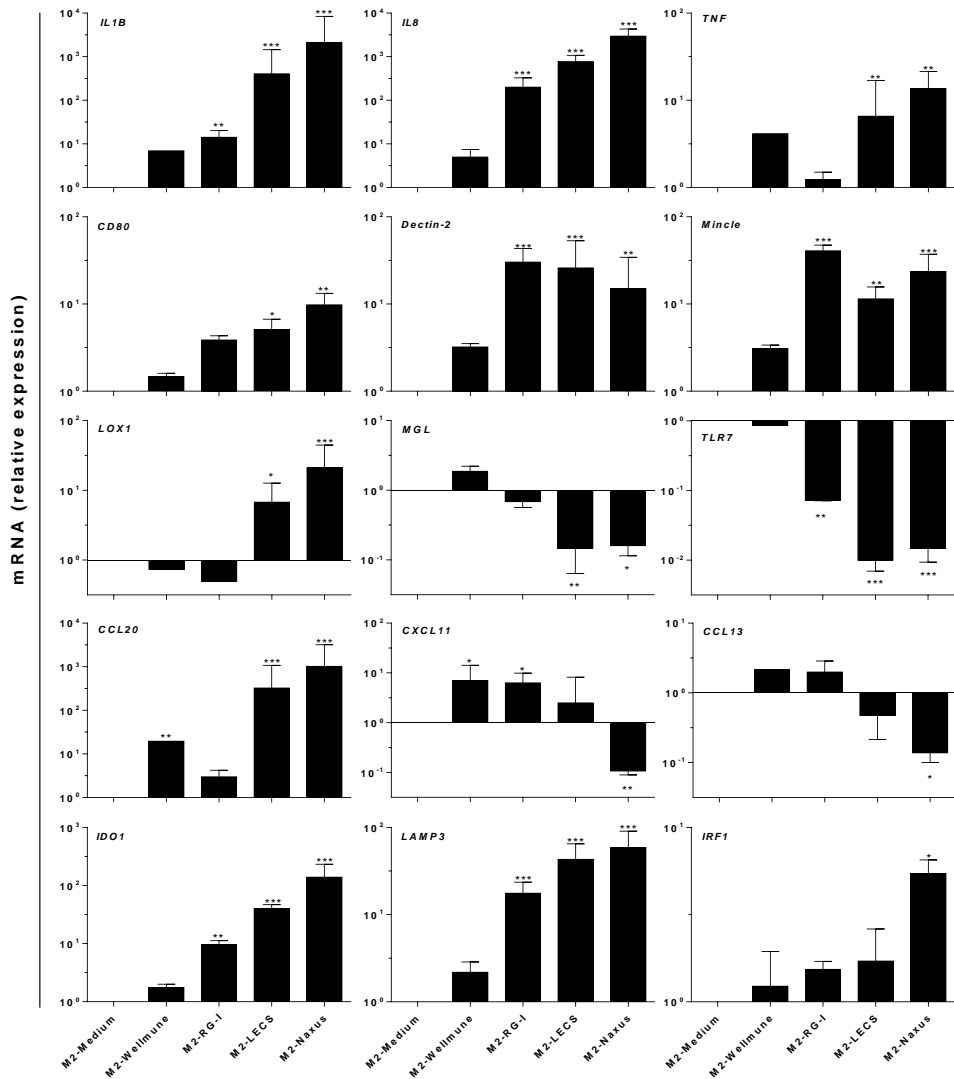
Figure 3. NDPs promote re-polarisation of M2 polarised macrophages towards a M$_{NDP}$ phenotype. M2 polarised macrophages were stimulated with Wellmune, RG-I, LCES, and Naxus at 500 µg/ml for 24 h and analysed for gene expression using qPCR. Results are shown as fold change by mean values ± SEM in a bar chart, n=2-3 different donors. Statistically significant differences compared to medium control were analysed by two-way ANOVA: * p<0.05, ** p<0.01, *** p<0.001.

decreased gene expression of MGL. Wellmune Soluble did not modulate gene expression of these genes, similar as we had seen when M0 were exposed to Wellmune Soluble [21].

Similar as for the plasticity of M1 macrophages we also studied the plasticity of M2 macrophage subsets when exposed to the four NDPs and analysed gene expression (Figure 3). Interestingly, NDPs triggered a similar gene expression trend in M2 macrophages as they did in M1 (Figure 9). Naxus increased gene expression of IL-1B, IL-8, TNF, CD80, dectin-2, Mincle, LOX1, CCL20, IDO1, LAMP3, and IRF1, while it decreased gene expression of MGL, TLR7, CXCL11, and CCL13. Again, also LCES increased gene expression for IL-1B, IL-8, TNF, CD80, dectin-2, Mincle, LOX1, CCL20, IDO1, and LAMP3, while it decreased gene expression for MGL, TLR7, and CXCL11. RG-I increased gene expression for IL-1B, IL-8, dectin-2, Mincle, IDO1, and LAMP3, while it decreased gene expression for TLR7. Wellmune Soluble only increased gene expression of CCL20.

These profiles following stimulation of M1 or M2 polarised macrophages indicate that NDPs have the capacity to change the macrophage phenotype and skew them towards an M$_{NDP}$ subset (Figure 9 and Supplementary Table 1).
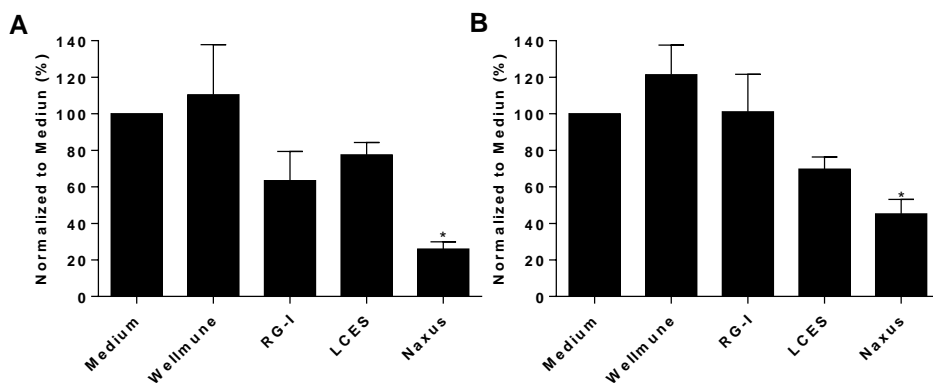
Figure 4. NDP decreased phagocytosis function of M1 and M2. M1 (A) and M2 (B) were stimulated with Wellmune, RG-I, LCES, and Naxus at 500 µg/ml for 24 h followed by 1 h incubation with *E.coli* bioparticles and analysis with flow cytometry. The bar chart shows mean fluorescence intensity (MFI) values ± SEM, n=3 different donors. Data was normalized to medium control. Statistically significant differences compared to medium control were analysed by one-way ANOVA: * p<0.05.

3.3 NDPs decrease phagocytosis capacity of both M1 and M2 polarised macrophages

We previously showed that LCES and Naxus decreased the phagocytosis capacity of M0 macrophages both after 18 h and 48 h stimulation (**Chapter 4**). Similarly, Naxus significantly decreased the uptake of *E. coli* bioparticles for M1 polarised macrophages (Figure 4A) and at the same time it also deceased phagocytosis capacity of M2 polarised macrophages, although this was not significant (Figure 4B). LCES showed a lowering, but insignificant trend in phagocytosis capacity for both M1 and M2 polarised macrophages. The other NDPs did not modulate uptake of *E. coli* bioparticles by neither M1 nor M2 polarised macrophages. This was also found for M0 macrophages exposed to these same NDPs.

3.4 NDPs decrease antigen processing capacity of both M1 and M2 polarised macrophages

Our previous research proved that both RG-I and Naxus decreased antigen processing capacity of M0 (**Chapter 4**). To analyse whether NDPs modulate antigen processing capacity of inflammatory or tolerant macrophages, M1 and M2 were stimulated by NDPs and antigen processing was analysed by using DQ-OVA. Naxus significantly decreased processing of DQ-OVA for M1 polarised macrophages but other NDPs did not show any effect on this activity (Figure 5A). Again, only Naxus changed antigen processing capacity of M2 polarised macrophages (Figure 5B).
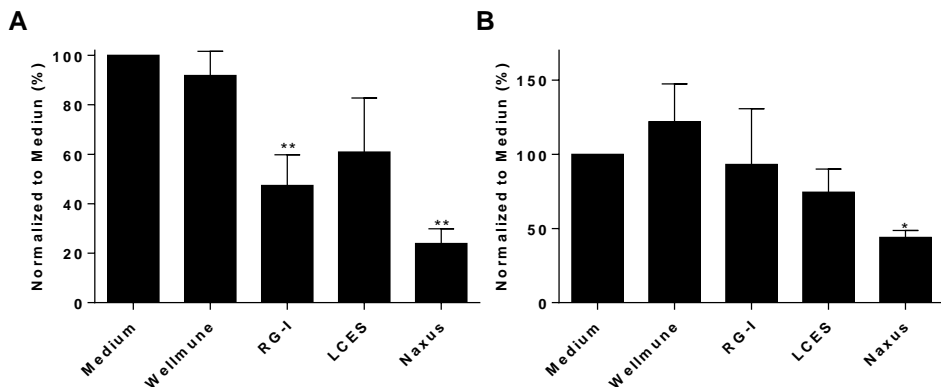
Figure 5. NDP decreased antigen procession function of M1 and M2 polarised macrophages. M1 (A) and M2 (B) polarised macrophages were stimulated with Wellmune, RG-I, LCES, and Naxus at 500 µg/ml for 24 h followed by 1 h incubation with DQ-OVA and analysed by flow cytometry. The bar chart shows MFI values ± SEM, n=3 different donors. Data was normalized to medium control. Statistically significant differences compared to medium control were analysed by one-way ANOVA: * p<0.05, ** p<0.01.

3.5 NDPs activated M1 and M2 to produce CCL1

A significantly increased CCL1 production was previously found when Mo cells were exposed to Naxus and LCES, while M1 or M2 polarised macrophages showed no significant CCL1 production. Here, we analysed whether NDPs are capable of modulating CCL1 production in M1 and M2 polarised macrophages. Indeed, Naxus increased production of CCL1 in both M1 and M2 polarised macrophages, albeit only significant for M1 polarised macrophages (Figure 6). In addition, we analysed other cytokines and chemokines (Table 1). M1 polarised macrophages were stimulated with Naxus which resulted in increased IL8, CCL5, CCL20, CCL24, IL1β, and TNFα production, while stimulation with RG-I increased IL8, CCL5, CCL20, CCL24, and TNFα production and LCES stimulation increased IL8 production. M2 polarised macrophages stimulated with Naxus or LCES increased IL8, CCL5, CCL20, CCL24, IL1β, and TNFα production, while stimulation with RG-I increased IL8, CCL5, CCL20, and TNFα production. All tested NDPs had no effects on secretion of MMP-1, CXCL9, CXCL10, and CXCL11 by M1 or M2 polarised macrophages.

Table 1. NDPs modulate cytokines M1 and M2 macrophage cytokine secretion.

	IL8	CCL5	CCL20	CCL24	IL1β	TNFα	MMP-1	CXCL9	CXCL10	CXCL11
M1-Medium	921.0	916.3	17.8	14.9	0.2	203.4	122.7	24198.6	224944.3	1188.0
M1-RG-I	15864.9	1897.8	110.3	54.1	0.3	3035.3	251.9	24198.6	2603.3	36.6
M1-LCES	4335.5	704.9	13.2	16.4	0.2	284.0	122.7	24198.6	699198.1	409.5
M1-Naxus	19590.0	4961.2	1783.8	647.7	3.1	30330.8	122.7	24198.6	17327.2	698.2
M2-Medium	298.4	9.9	0.8	352.1	0.1	2.3	122.7	25.2	13.5	0.1
M2-RG-I	21968.3	375.4	18.3	271.5	0.2	549.0	251.9	37.7	18.7	0.1
M2-LCES	23591.8	889.6	223.4	1969.8	1.3	10011.8	251.9	37.7	28.8	0.2
M2-Naxus	23213.1	1928.8	858.7	1784.3	4.1	23877.0	367.0	126.2	64.8	0.4

Values represent concentration of cytokines/chemokines (pg/ml) analysed by Bio-Plex multiplex, n=1 donor.
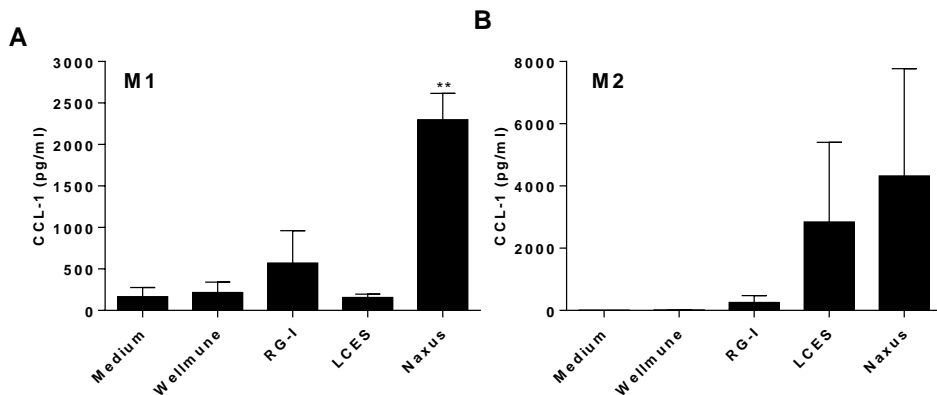
Figure 6. Naxus induced CCL1 production in M1 and M2 polarised macrophages. M1 (A) and M2 (B) polarised macrophages were stimulated with Wellmune, RG-I, LCES, and Naxus at 500 μg/ml for 24 h. CCL1 secretion was analysed by Bio-Plex multiplex analysis. The bar chart shows mean values ± SEM, n=2 different donors. Statistically significant differences compared to medium control were analysed by one-way ANOVA: ** $p<0.01$.
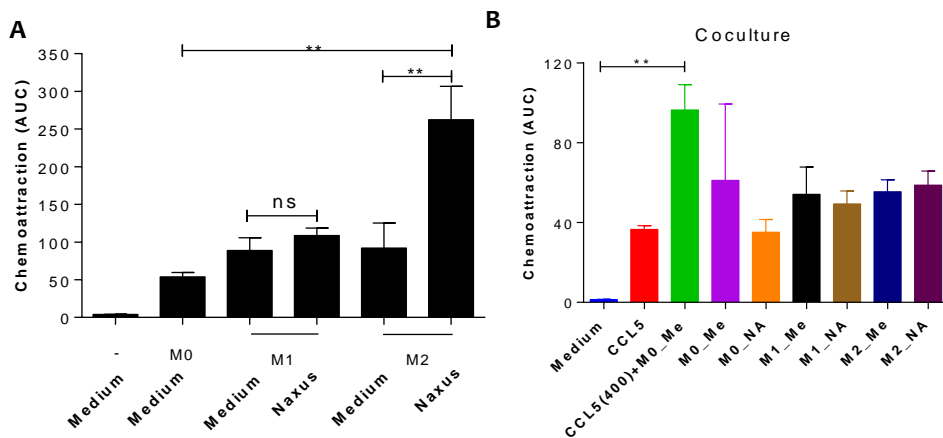
Figure 7. Naxus enhanced M2 macrophages recruiting ability which can abolished by Caco-2 barrier in coculture. M0, M1, and M2 macrophages were stimulated with Naxus (NA) at 500 μg/ml or medium control (Me) for 24 h either direct interact with macrophages by single culture (A) or indirect interact by adding to Caco-2 in coculture (B). Chemoattraction were analysed by THP-1 monocytes cell migration induced by macrophages conditioned medium. The bar chart shows mean values ± SEM, n=3 different experiments (A) or n=2 technique replicates (B). Statistically significant differences were analysed by one-way ANOVA: ** $p<0.01$.

3.6 NDPs promote recruiting ability for M2 polarised macrophages but not for M1

In comparison to M1 and M2 polarised macrophages, M$_{NDP}$ had shown increased chemokine secretion. Therefore, we analysed migration of THP-1 monocytes by conditioned medium from M1 or M2 macrophages that had been exposed to Naxus (Figure 7A). M0 conditioned medium showed increased recruiting ability compared to fresh medium (background control) and CCL5 (the positive control). M1 and M2 polarised macrophage conditioned medium demonstrated a slight increase in monocyte recruitment compared to M0 conditioned medium. Naxus exposure did not change THP-1 monocyte recruiting ability by M1 polarised macrophages. However, Naxus exposure did significantly increase recruiting ability by M2 polarised macrophages.

3.7 Intestinal immune system *in vitro* model

To mimic the intestinal immune barrier *in vitro*, an intestinal epithelial cells-macrophages coculture system was used. As a first test, it was studied whether cocultivation of 21 days differentiated small intestinal-like Caco-2 grown on the transwell with M1 or M2 polarised macrophages in the basolateral compartment affected specific gene expression markers of the polarised macrophages. Neither expression of the M1 gene markers (CXCL11, IDO1, dectin-2, and CXCL9), the M2 markers (CCL13, MGL, CCL18, and IL17RB) nor the MNDP gene marker (CCL-1, IL8, CCL24, and CXCL5) were significantly changed in M1 or M2 polarised macrophages following coculture (Figure 8). Subsequently, we analysed whether the NDPs Naxus or LCES could directly or indirectly influence macrophage polarization when applied in the apical compartment. Therefore, we analysed changes in macrophage polarisation again using the gene expression markers (Figure 8). M1 markers CXCL11, IDO1, dectin-2, and CXCL9 were similarly high expressed in M1 polarised macrophages in coculture, following apical exposure to both LCES and Naxus, as in single cultures. The same was found for the M2 specific gene markers CCL13, MGL, CCL1B, and IL17RB which were also not changed in the coculture compared to the single cultured cells. CCL1, IL8, CCL24, and CXCL5 gene expression, indicators for NDPs recognition and responses by macrophages in single cultures, neither changed. In addition, we analysed recruiting ability of THP-1 monocytes by basolateral medium from the M0, M1, and M2 coculture system with and without apical exposure of Naxus (Figure 7B). The results indicate that the apical exposure of Naxus did not influence the recruitment capability of the M0, M1, or M2 polarised macrophages.
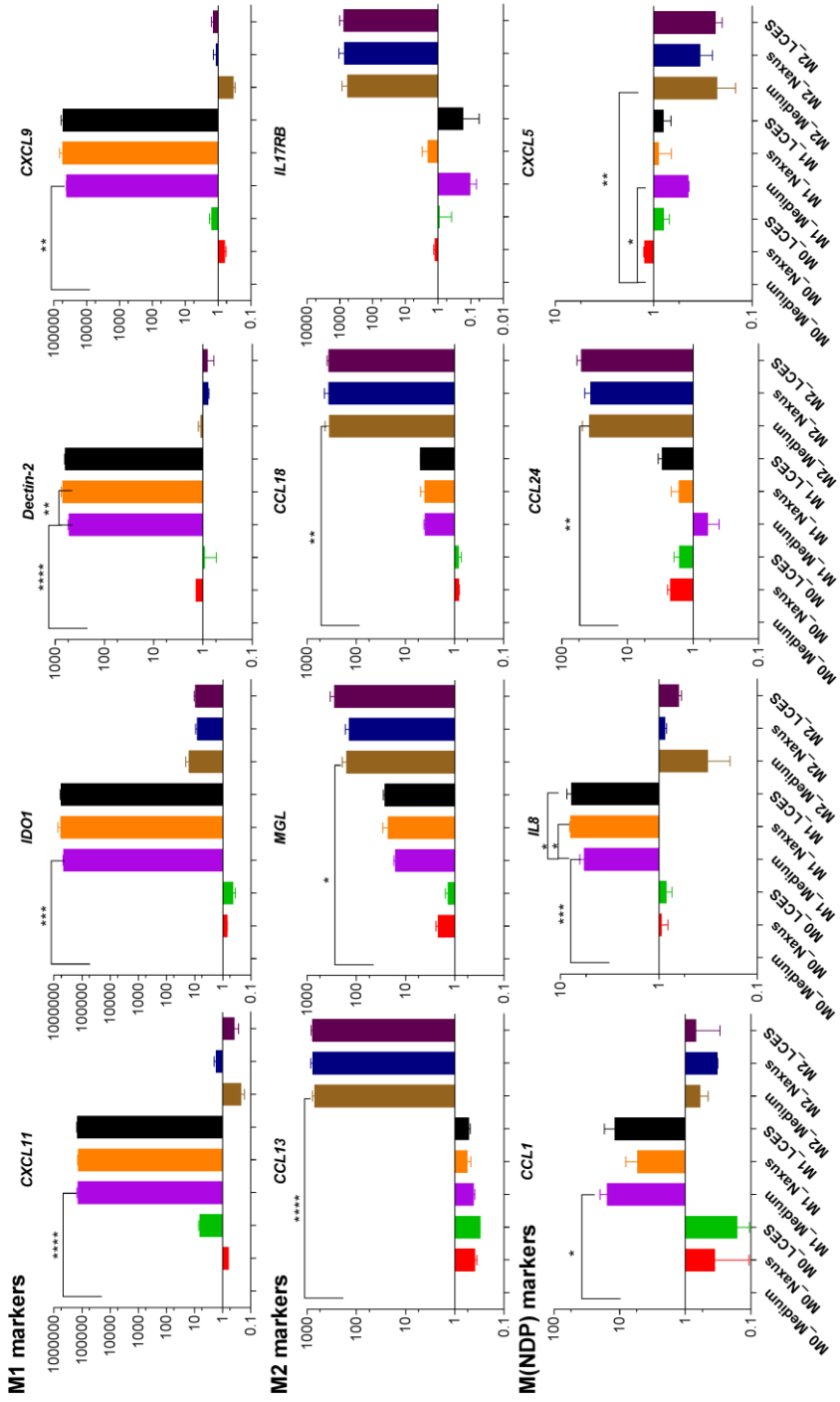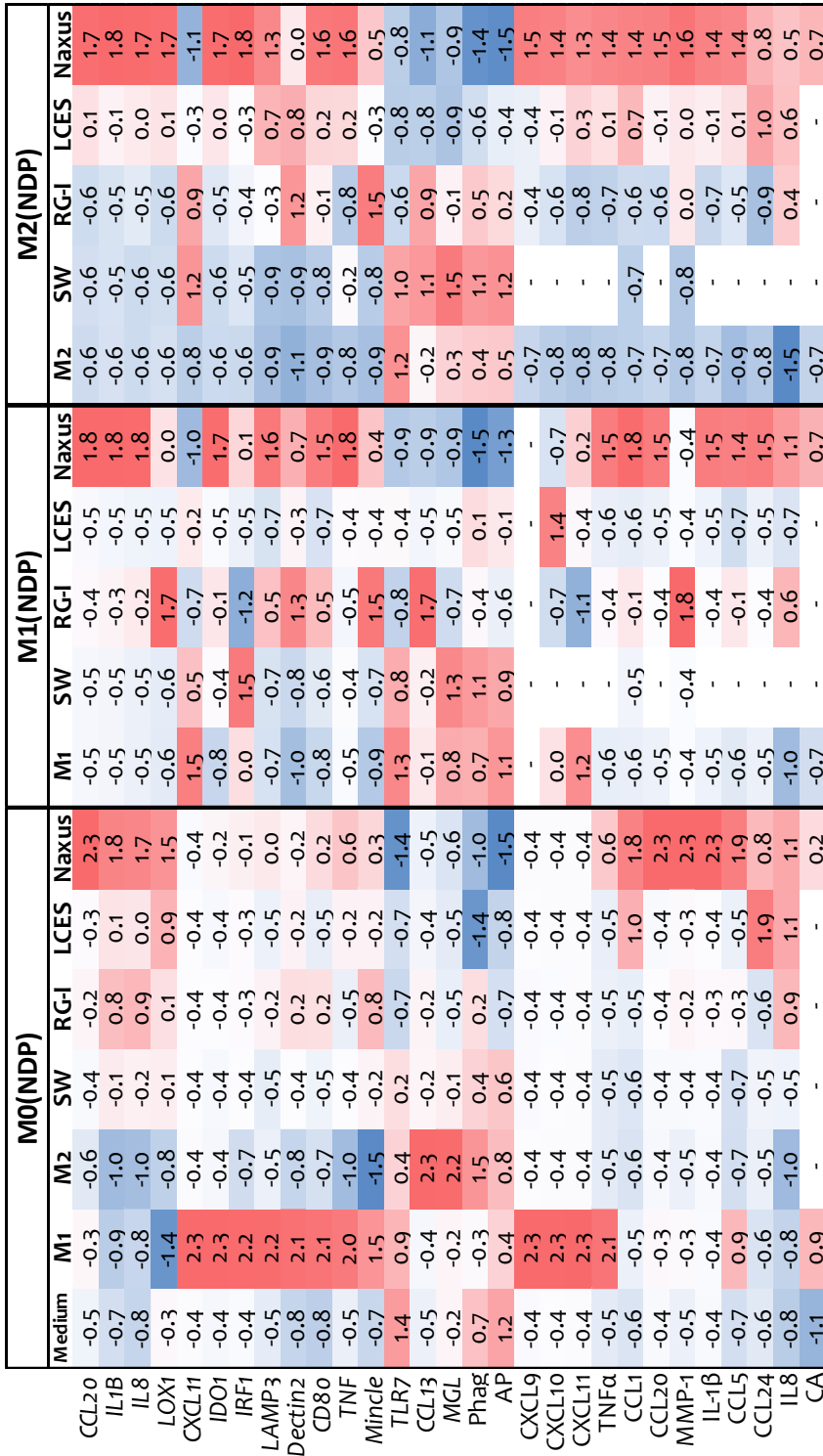
4 Discussion

The intestine contains the largest macrophage population in the body [1]. Within this population, a continuous spectrum of subsets exists, with inflammatory (M1) and tolerant macrophages (M2) as extremes [10]. Plasticity of macrophages is one of the functional properties to balance the intestinal immune status. Macrophages are polarised towards the M1 subset upon inflammatory stimulation such as the sensing of danger signals resulting in macrophages capacity to kill microorganisms and increase cytokine secretion in the host [27]. The M2 subset is formed after IL-4, and IL13 stimulation, and functions in wound healing, tissue repair and remodeling [6]. Food components that impact the macrophage phenotype may lead to the formation of inflammatory or tolerant macrophages or to an alternative status. We previously found that NDPs skewed macrophages to an alternative subset compared to M1 and M2, based on gene expression markers [21].

In this study, we analysed the immunomodulatory effects of NDPs on M1 and M2 by analysing phenotype and functionality after NDP-stimulation. We used cocultures of intestinal epithelial cells and macrophages *in vitro* to mimic the intestinal immune barrier and to evaluate the effects of NDPs on this model. Previous studies revealed that Caco-2 spent medium enhanced cytokine production by dendritic cells which was attenuated by dietary fibers [28]. However, the direct effects of NDPs on macrophages were blocked by the Caco-2 layers, for both gene expression and monocyte recruitment. This might be due to the lack of direct physical interaction of NDPs with immune cells, since the Caco-2 layer can block passage and signals from the apical side. This might be supported by our previous finding that NDPs reduced FD4 translocation by Caco-2 (**Chapter 2**). To investigate the importance of direct contact between Caco-2 and macrophages, the up-side-down culture model might be applied [29]. In the coculture system, macrophage phenotype and recruiting ability were not influenced by Caco-2. Also, M cells might be interesting to be included in such studies, since M cells might sample NDPs to macrophages.

Figure 8. Naxus and LCES do not affect macrophages gene transcription in coculture with Caco-2. Transwell grown 21 days differentiated Caco-2 were placed in wells with M0, M1 or M2 polarised macrophages for 24 h. On the apical side, Caco-2 were stimulated with either medium control, 500 µg/ml LCES or Naxus. M0, M1, and M2 polarised macrophages located at the basolateral side were analysed for gene expression using qPCR. The bar chart shows fold change by mean values ± SEM, n=2 different donors. Statistically significant differences were analysed by one-way ANOVA: ***** $p<0.05$, ****** $p<0.01$, ******* $p<0.001$, ******** $p<0.005$. (Legend for page 126)

	M0(NDP)							M1(NDP)					M2(NDP)				
	Medium	M1	M2	SW	RG-I	LCES	Naxus	M1	SW	RG-I	LCES	Naxus	M2	SW	RG-I	LCES	Naxus
CCL20	-0.5	-0.3	-0.6	-0.4	-0.2	-0.3	2.3	-0.5	-0.5	-0.4	-0.5	1.8	-0.6	-0.6	-0.6	0.1	1.7
IL1B	-0.7	-0.9	-1.0	-0.1	0.8	0.1	1.8	-0.5	-0.5	-0.3	-0.5	1.8	-0.6	-0.5	-0.5	-0.1	1.8
IL8	-0.8	-0.8	-1.0	-0.2	0.9	0.0	1.7	-0.5	-0.5	-0.2	-0.5	1.8	-0.6	-0.6	-0.5	0.0	1.7
LOX1	-0.3	-1.4	-0.8	-0.1	0.1	0.9	1.5	-0.6	-0.6	1.7	-0.5	0.0	-0.6	-0.6	-0.6	0.1	1.7
CXCL11	-0.4	2.3	-0.4	-0.4	-0.4	-0.4	-0.4	1.5	0.5	-0.7	-0.2	-1.0	-0.8	1.2	0.9	-0.3	-1.1
IDO1	-0.4	2.3	-0.4	-0.4	-0.4	-0.4	-0.2	-0.8	-0.4	-0.1	-0.5	1.7	-0.6	-0.6	-0.5	0.0	1.7
IRF1	-0.4	2.2	-0.7	-0.4	-0.3	-0.3	-0.1	0.0	1.5	-1.2	-0.5	0.1	-0.6	-0.5	-0.4	-0.3	1.8
LAMP3	-0.5	2.2	-0.5	-0.5	-0.2	-0.5	0.0	-0.7	-0.7	0.5	-0.7	1.6	-0.9	-0.9	-0.3	0.7	1.3
Dectin2	-0.8	2.1	-0.8	-0.4	0.2	-0.2	-0.2	-1.0	-0.8	1.3	-0.3	0.7	-1.1	-0.9	1.2	0.8	0.0
CD80	-0.8	2.1	-0.7	-0.5	0.2	-0.5	0.2	-0.8	-0.6	0.5	-0.7	1.5	-0.9	-0.8	-0.1	0.2	1.6
TNF	-0.5	2.0	-1.0	-0.4	-0.5	-0.2	0.6	-0.5	-0.4	-0.5	-0.4	1.8	-0.8	-0.2	-0.8	0.2	1.6
Mincle	-0.7	1.5	-1.5	-0.2	0.8	-0.2	0.3	-0.9	-0.7	1.5	-0.4	0.4	-0.8	-0.8	1.5	-0.3	0.5
TLR7	1.4	0.9	0.4	0.2	-0.7	-0.7	-1.4	1.3	0.8	-0.8	-0.4	-0.9	1.2	1.0	-0.6	-0.8	-0.8
CCL13	-0.5	-0.4	2.3	-0.2	-0.2	-0.4	-0.5	-0.1	-0.2	1.7	-0.5	-0.9	-0.2	1.1	0.9	-0.8	-1.1
MGL	-0.2	-0.2	2.2	-0.1	-0.5	-0.5	-0.6	0.8	1.3	-0.7	-0.5	-0.9	0.3	1.5	-0.1	-0.9	-0.9
Phag	0.7	-0.3	1.5	0.4	0.2	-1.4	-1.0	0.7	1.1	-0.4	0.1	-1.5	0.4	1.1	0.5	-0.6	-1.4
AP	1.2	0.4	0.8	0.6	-0.7	-0.8	-1.5	1.1	0.9	-0.6	-0.1	-1.3	0.5	1.2	0.2	-0.4	-1.5
CXCL9	-0.4	2.3	-0.4	-0.4	-0.4	-0.4	-0.4	-	-	-	-	-0.7	-0.7	-	-0.4	-0.4	1.5
CXCL10	-0.4	2.3	-0.4	-0.4	-0.4	-0.4	-0.4	0.0	-	-0.7	1.4	0.2	-0.8	-	-0.6	-0.1	1.4
CXCL11	-0.4	2.3	-0.4	-0.4	-0.4	-0.4	-0.4	1.2	-	-1.1	-0.4	-	-0.8	-	-0.8	0.3	1.3
TNFα	-0.5	2.1	-0.5	-0.5	-0.5	-0.5	0.6	-0.6	-	-0.4	-0.6	1.5	-0.8	-	-0.7	0.1	1.4
CCL1	-0.6	-0.5	-0.6	-0.6	-0.5	1.0	1.8	-0.6	-0.5	-0.1	-0.6	1.8	-0.7	-0.7	-0.6	0.7	1.4
CCL20	-0.4	-0.3	-0.4	-0.4	-0.4	-0.4	2.3	-0.5	-	-0.4	-0.5	1.5	-0.7	-	-0.6	-0.1	1.5
MMP-1	-0.5	-0.3	-0.5	-0.4	-0.2	-0.3	2.3	-0.4	-0.4	1.8	-0.4	-0.4	-0.8	-0.8	0.0	0.0	1.6
IL-1β	-0.4	-0.4	-0.4	-0.4	-0.3	-0.4	2.3	-0.5	-	-0.4	-0.5	1.5	-0.7	-	-0.7	-0.1	1.4
CCL5	-0.7	0.9	-0.7	-0.7	-0.3	-0.5	1.9	-0.6	-	-0.1	-0.7	1.4	-0.9	-	-0.5	0.1	1.4
CCL24	-0.6	-0.6	-0.5	-0.5	-0.6	1.9	0.8	-0.5	-	-0.4	-0.5	1.5	-0.8	-	-0.9	1.0	0.8
IL8	-0.8	-0.8	-1.0	-0.5	0.9	1.1	1.1	-1.0	-	0.6	-0.7	1.1	-1.5	-	0.4	0.6	0.5
CA	-1.1	0.9	-	-	-	-	0.2	-0.7	-	-	-	0.7	-0.7	-	-	-	0.7

Figure 9. Heatmap showing effects of NDPs on M0, M1, and M2 polarised macrophages. Data were collected from gene expression, cytokines secretion, phagocytosis (Phag), antigen processing (AP), and chemoattraction (CA) analysis. Results were analysed by cumulative gaussian distribution as [(X-Xmean)/SD]. Dark red indicates a high value, while dark blue indicates a low value.

Upon direct exposure of M1 and M2 polarised macrophages to Naxus we observed a skewing to a subset similar as found for M0 exposed to the same NDP. This intermediate M$_{NPD}$ could be hypothesised to be an intermediate and resolving type of macrophage that in case of inflammation can prohibit overstimulation and damage of tissue. Similar conclusions on anti-inflammatory activity have been drawn previously for several polysaccharides based on inhibiting LPS-induced nitric oxide production in macrophages, including arabinoxylans and lentinan containing extracts [30, 31]. Some studies hypothesized that this effect could be caused by blocking of the TLR4 receptor as some of the molecular structures of polysaccharides show similar structures with LPS [32]. Our experimental setup, however, is free of LPS as we used TNFα and IFNγ, instead of LPS, to induce M1 polarisation and our polysaccharides were devoid of functional levels of LPS contaminations [22]. Instead of blocking the TLR4 receptor, the NDPs might signal via other receptors to exert their effect. Volman and colleagues proposed other TLRs such as TLR2 and TLR6 for recognition of β-glucan [33], while others suggested the involvement of CR3 in arabinoxylan-mediated immune responses [34] or identified dectin-1 to be involved in recognition of arabinoxylan [35]. Alternatively, our arabinoxylan preparation has been demonstrated to contain β-glucans and mannose (**Chapter 3**), potentially for stability, and these structures might also provide signalling towards the macrophages [36].

Naxus significantly decreased phagocytosis and antigen processing capacity for both M1 and M2 polarised macrophages. This is in contrast to arabinoxylan from rice and finger millet that both increased phagocytosis of yeast by macrophages [37, 38]. These contradicting results might stem from non-detected LPS contaminations or might be due to the use of arabinoxylans derived from various sources with different molecular weights and degree of branching [36, 39].

Our results imply that Naxus might be a potential candidate for modulating intestinal macrophages and immunomodulatory activity as also pointed out in several studies and reviews [36, 40]. In particular the capability to reduce inflammatory macrophages can be an interesting direction for future research as several intestinal diseases have shown to involve inflammation as a common factor like crohn's disease, ulcerative colitis and irritable bowel syndrome (IBS). Interestingly, research with polysaccharides, including lentinan and arabinoxylan arrived at similar conclusions as oral administration of lentinan significantly ameliorated DSS-induced colitis [41] and arabinoxylan has been reported to significantly decrease diarrhoea, constipation and CRP values in IBS patients [42].

Taken together the plasticity of macrophages is demonstrated by showing that the NDP Naxus can skew both inflammatory and tolerant macrophages to an alternative subset defined previously as M$_{NDP}$ which warrant further in depth in vivo studies using this NDP.

Y.T., C.G., and J.J.M. conceived and designed the experiments. Y.T. performed the experiments. Y.T., C.G., and J.J.M. analysed the data. E. H. S., contributed Multiplex cytokine analysis. Y.T., C.G., J.J.M., and H.J.W. wrote the manuscript.

The authors have declared no conflicts of interest.

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Supplementary Table 1. Gene expression profile on M0 determined by microarray.

Genes	M1	M2	Wellmune	RG-I	LCES	Naxus
IL1B	-1.4	-3.7	8.8	19.4***	10.7**	32.8***
IL8	1.3	-3.6	13.3	33.9***	17.2**	51.2***
TNF	11.4***	-1.2	1.5	1.0	2.3	5.7**
CD80	11.7***	1.7	2.4	4.7***	2.2	4.9***
Dectin-2	24.5***	1.0	4.3	8.6*	5.7*	5.3*
Mincle	12.5***	-3.0	3.8	9.0**	3.9	6.4**
LOX1	-4.6*	-1.6	1.9	3.1	7.0**	9.9***
MGL	1.0	19.8***	2.0	-1.1	-1.4	-1.9
TLR7	-1.0	-3.4	-4.2	-8.2**	-7.9**	-11.2***
CCL20	6.2*	-1.5	2.6	7.3	4.3	69.9***
CXCL11	304.0***	1.1	1.9	1.7	1.3	-1.1
CCL13	3.4	94.1***	10.3	10.8*	2.9	1.5
IDO1	799.7***	1.2	2.7	15.8***	4.1*	54.0***
LAMP3	83.0***	2.2*	1.4	12.1***	2.8**	16.0***
IRF1	22.0***	-1.3	1.2	1.5	1.5	3.1***

Values represent fold changes of gene expression, n=3 donors. Statistically significant differences were calculated with IBMT regularised paired t-test: * FDR $p<0.05$, ** FDR $p<0.01$; *** FDR $p<0.001$.

Supplementary Table 2. Primer details

Genes	Forward (5'-3')	Reverse (5'-3')
PUM1	TGAGGTGTGCACCATGAAC	CAGAATGTGCTTGCCATAGG
RPLP0	GCAATGTTGCCAGTGTCTG	GCCTTGACCTTTTCAGCAA
IL8	CTGATTTCTGCAGCTCTGTG	GGGTGGAAAGGTTTGGAGTATG
CD80	ACCATCCAAGTGTCCATACC	CACGTGGATAACACCTGAAC
STAT1	CTGGCACCAGAACGAATG	GCTGACGTTGGAGATCAC
TNF	GGCGTGGAGCTGAGAGATAA	GATGGCAGAGAGGAGGTTGA
CD209	AGGAGCAGAACTTCCTAC	GCTCTCCTCTGTTCCAATAC
MRC1	CAGCGCTTGTGATCTTCATT	TACCCCTGCTCCTGGTTTTT
MRC2	GGAAACTCCCACGGAAAG	GTCTCGCAGTCGTTACTC
PPARG	TCTAAAGAGCCTGCGAAAGC	GCGGTCTCCACTGAGAATAATG
CD36	GCCAAGGAAAATGTAACCCAGG	GCCTCTGTTCCAACTGATAGTGA
IDO1	GCCAGCTTCGAGAAAGAGTTG	ATCCCAGAACTAGACGTGCAA
GBP5	ACATTAGTTCTGCTTGACACCG	GCTGCTCAGTAAGAGTGCCAG
GCH1	ACGAGCTGAACCTCCCTAAC	GAACCAAGTGATGCTCACACA
LAMP3	ACTACCCCAGCGACTACAAAA	CTAGGGCCGACTGTAACTTCA
CXCL11	GACGCTGTCTTTGCATAGGC	GGATTTAGGCATCGTTGTCCTTT
IRF1	GCAGCTACACAGTTCCAGG	GTCCTCAGGTAATTTCCCTTCCT
Mincle	CTGAAACACAATGCACAGAGAGA	AAAGATGCGAAATGTCACAACAC
Dectin-1	AACCACAGCTACCCAAGAAAAC	GGGCACACTACACAGTTGGTC
Dectin-2	GCTTTCAGACCCACAAGGTAAT	GCAGAATGATTGGGCTCACCTA
MGL	AGCAACTTCACCTCAAACACTG	AGATGCTATCGTTTCTTCCAAGC

Chapter 6

General discussion

Non-digestible polysaccharides (NDPs), gain attention for their immunomodulatory activity. The mechanism of the immunomodulatory effects of NDPs on the intestinal immunity remained largely unknown. Some research resulted in the hypothesis that the health effects would result from fermentation products of NDPs, such as short chain fatty acids which are subsequently recognized by immune cells, leading to regulation of intestinal immunity [1]. Others studies demonstrated a direct interaction of NDPs with immune cells aligning the intestine. It is clear that the intestinal immune system involves an abundant set of immune cells both in types (include dendritic cells, macrophages, T cells, B cell as well as innate lymphoid cells (ILCs)) and in number, as it contains approximately 70% of all immune cells [2]. This high abundance and complexity of the intestinal immune system on the one hand and the presence of many different NDPs that are or could be part of our diet raised the interest to gain more knowledge on which NDPs interact with these intestinal immune cells and how, and to what immune effects that leads. As the variation of NDPs in our diet is almost endless it is hard to image that all these different structures can be recognized by immune cells or lead to similar effects. Our research presented in this thesis focused on this direct interaction between NDPs and intestinal immune cells and has formed a basis to start in differentiating these NDPs based on immunomodulating effects. Our research included the interaction between NDPs and intestinal epithelial cells (IECs), macrophages, and macrophage subsets like the inflammatory macrophages and tolerant macrophages. NDPs could reduce FD4 translocation across the epithelial cells through physical interaction. Microarray analysis revealed characteristics of NDPs activation on gene expression of surface markers, NF-κB family members, and cytokines. Based on transcriptome and functional analysis of macrophages, we revealed that NDPs can direct macrophages towards a specific subset for which we used the term M_{NDP}, a separate group compared to inflammatory macrophages or tolerant macrophages. Comparing the effects provoked by the different NDPs that we had to our disposal, we have to conclude that the wheat-derived arabinoxylan-rich preparation Naxus revealed the strongest activation of this M_{NDP} subset and related responses. Therefore, we advocate that this product should be further studied *in vivo* for its immunomodulatory effect in order to link the observed *in vitro* activity to biological relevance for the *in vivo* situation. The models developed and used in this thesis and the studied components like Naxus could lead to their development in a functional supplement that supports a healthy human gut and immune status.

Intestinal immune barrier

Studying the function of human small intestinal tissue and the responses to luminal factors is not easy. Small intestinal biopsy material is difficult to sample and *ex vivo* stimulations in Ussing chambers have shown that incubation time should be short (preferable <1h), that RNA after such incubation is hard to obtain and cytokine expression often too low to detect due to large buffer volumes required (John-Peter Ganda Mall, personal communication). An alternative research tool to study the responses of small intestinal tissue that is currently developed is based on organoids, either derived from human inducible pluripotent stem cell lines [3, 4] or from intestinal crypt cells derived from biopsies or surgery [5]. Both strategies have high potency for future research, each having its advantages and disadvantages. However, a robust protocol for generating 2D multicellular human tissue with a reproducible make up of cell types from these 3D spheres still needs further optimisation before it can be applied on a routine base. Especially, as foods are likely to generate only mild changes, methods applied should be highly reproducible.

In literature several intestinal epithelial *in vitro* cell models were described which included various cell lines as HT29, T84, and Caco-2. These cell lines have shown such a reliable performance, although also for these cell systems lab-to-lab and experiment-to-experiment variation is not uncommon. Since many labs are using Caco-2 as a model, data can be integrated and used for more systematic comparison and effects evaluation like we did in our lab [6]. Therefore we chose Caco-2 as IECs model for NDP evaluation. Twenty-one-days differentiated Caco-2 cells, grown in transwells, have shown to resemble small intestinal cells. These epithelial layers can build up a transepithelial electrical resistance (TEER) between 800 and 1200 ($\Omega*cm^2$) per transwell in 24 well plates. TEER values are both used to check for the quality of the insert tissue but also can be used to analyse the effect of a treatment on intestinal integrity. Our data are based on single measurements on specific time points. However, continuous measurements of TEER by *e.g.* cellZscope or similar methods can even be used for a more detailed time-dose response on TEER [7]. But only when grown on membranes like in the transwell system TEER measurements can be combined with other measurements to study integrity *e.g.* by analysing translocation of markers like FD4. Low layer integrity is correlated with high translocation of FD4, but due to a fine regulation of cell-cell junctions a high layer integrity does not always correlate with low FD4 translocation.

In **Chapter 2**, we found that NDPs do not affect TEER, and physically block FD4 transport. This physical blocking could potentially also occur by other molecules in the lumen and therefore it can be hypothesised that NDPs could inhibit uptake of nutrients but also of

risk factors resulting in positive health effects. A protected barrier function can therefore be defined in different ways; an inhibition of translocation by maintaining cell integrity or support in blocking translocation of undesired components which both can have a physiological background (active response of cells) and a physical background (direct binding). In fact, a dietary fibre like β2/1→fructan was found to exert a polysaccharide length-dependent protection of barrier function on T84 IECs [8]. This protection involved TLR-2 activation as the effects were inhibited by a TLR-2 blocking antibody [8]. In practise also other mechanisms can have an effect on barrier function, *e.g.* mucus (not produced in Caco-2 in biologically relevant amounts [9]) have the capability of trapping luminal factors and food based treatments that can stimulate this mucus production [10]. Besides that, many cellular factors will be involved in maintenance of tissue integrity including the energy supply and mitochondrial function, as it was reported that an increased permeability linked to mitochondrial inhibition [11].

Intestinal integrity is very relevant for gut health and is correlated with tight junction (TJ) proteins. These TJ proteins are composed of transmembrane proteins (occludins and claudins) and intracellular scaffold proteins (ZO-1, ZO-2, and ZO-3). Some reports claim that tight junction proteins are upregulated by polysaccharides [12]. However, we found no change in TJ-gene expression after NDP treatment in Caco-2. We did find that our 21-day differentiated Caco-2 exhibited high TEER values and low permeability. This suggests that TJ proteins are abundantly expressed but not changed by NDPs. We, however, did not study intracellular protein translocation and protein folding of TJ proteins which could also be effected by exposures without changes in *de novo* synthesis. The Caco-2 layer could also block LPS and NDP translocation, prohibiting to detect changes in gene expression by macrophages (**Chapter 5**).

Caco-2 challenge model could support research

As discussed above, 21-days differentiated Caco-2 form an intact epithelial layer for which we could not detect FD4 passage and for which we found that it blocked the potency of NDPs to signal towards macrophages in the basolateral department (chapter 5). Similar results were observed for LPS transport over Caco-2 which was fully blocked as studied with basolateral THP-1 macrophages which are able to respond to LPS even at very low concentrations of LPS [13].

M cells and goblet cells function as transporters of luminal antigens or food components to underneath located immune cells such as macrophages and DCs [14]. Twenty-one-days differentiated Caco-2 might lack M cells and goblet cells or do not have the full

functional capacity to transport NDPs or LPS. *In vitro* models of IECs therefore should make more use of methods to introduce these transporter cells, *e.g.* by inducing M cells via co-culturing with Raij B cells [15]. Also other immune cells are likely to signal to IECs to steer differentiation and activation towards these transport cells and structures [16].
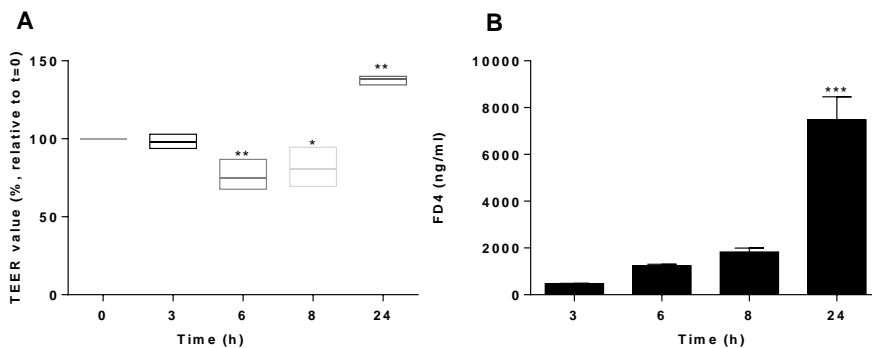
Figure 1. TEER and barrier integrity of Caco-2 with ethanol challenge. Twenty-one-day differentiated Caco-2 cells were stimulated with 15% ethanol for 3, 6, 8, and 24 h. Cells were monitored by TEER (A) and FD4 translocation (B). N=3 different experiments. Statistically significant differences were analysed compared to medium (A) or baseline (B) by one-way ANOVA: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Another approach we and others followed to study effects of treatments towards IECs was based on challenge models. The general idea is that the intestinal system has to counteract challenges and that the time needed to restore the intestinal barrier and homeostasis is an indication for health. Also for IECs several of these challenge models have been developed including bacterial derived toxins (*e.g. C. difficile* Tox A and B), DON (mycotoxin), melittin (bee venom), lectins like WGA, glycoalkaloids (*e.g.* from the *Solanum* family), and mixtures of inflammatory cytokines (such as TNFα and INFγ) [17]. We also challenged Caco-2 with ethanol as model for the effect of alcohol consumption and found that the TEER decreased within the first 6 h and then starts to recover. TEER reached even higher values than control levels after 24 h (Figure 1A) indicating that cell morphology or connections between cells (over)compensate for the loss of integrity. This whole process was also monitored with FD4 translocation and basolateral accumulation, indicating that even after 8 hours, when the TEER started to restore, translocation was still proceeding (Figure 1B). The ethanol-induced decrease in TEER is

also dose-dependent. Changes in TJ protein (ZO-1 and claudin-1) [18] and activation of the mitogen activated protein kinase pathway might be involved [19]. Therefore, this ethanol method, or any other model mentioned earlier, could be used to further test the ability of NDPs to support recovery of intestinal homeostasis.

Immunomodulating signals of IECs

Besides forming a physical barrier, IECs also signal towards neighbouring cells and associated immune cells to warn, recruit, and instruct other immune cells [20]. IECs produce TSLP, TGFβ, IL25, and APRIL that activate intestinal immune cells to limit inflammation and direct appropriate immune responses [21]. For instance, after helminth infection, IECs produce TSLP and IL25 to drive T_h2 cells to promote the development of type 2 cytokine [22, 23]. To give an example for *in vitro* results, 21-days differentiated Caco-2 respond to LPS when applied apically at high concentration. LPS is also abundantly present in the intestine. However, as these IECs should not continuously be triggered by luminal factors like LPS, they have a higher threshold/tolerance level than immune cells. For Caco-2 this tolerance of direct apical LPS stimulation was determined to be 1 μg/ml *E.coli* LPS, at which level no significant difference in gene expression of inflammatory markers was detected (**Chapter 2**). This threshold level of LPS is around 10^6 higher than for THP-1 macrophages [13] and 10^3 higher than or primary macrophages [24]. The relatively high tolerance of Caco-2 to LPS compared to macrophages might be due to less TLR4 expression on the apical side of Caco-2 [25]. Twenty-one-days Caco-2 exposed to NDPs showed a mild but significant increase in gene expression and protein secretion of CXCL10 and CCL20 (**Chapter 2**). Similar results were previously reported for IEC18 cells secreting CCL2, CXCL1, and CXCL2 when incubated with oligosaccharides as FOS, GOS, GMOS, and inulin [26]. HT-29, another IEC cell line used *in vitro*, was reported to respond to zymosan and curdlan by secretion of IL8 in a dose-dependent manner, but not other cytokines (CCL2, IL1β, TNFα, IL6, IL10, or IL12p70) [27]. In addition, cereal β-glucan was reported to be unable to directly activate IL8 secretion in both Caco-2 and HT-29 [28]. In fact, an animal study showed that cytokine secretion from enterocytes might be due to cellular interaction with intestinal leukocytes that are activated by dietary β-glucan [29]. These immune responses could be activated by TLR4 and are dependent on activation of NF-κB and Myd88 signalling [26], or activated by dectin-1 and dependent on activation of SyK signalling [27]. Our data revealed that the expression of cytokines induced by NDP exposure was routed via the NF-κB pathway and likely via the TLR2 receptors (**Chapter 2**).

Sampling in intestinal immunity

The classical view to study risk related factors in relation to intestinal immunity is based on a passive response to invading pathogens when they have entered the tissue [30, 31]. However, recent studies based on high resolution imaging revealed that dendrites of APC like macrophages can cross the intestinal barrier to sample pathogens [32]. The macrophages that reside in the intestine are the largest population of macrophages in the body [33]. They are continuously replenished by recruiting Ly6Chi monocytes [34]. A subset of mononuclear cells, including macrophages, extended dendrites across the intestinal barrier into the intestinal lumen in a CX3CR1 dependent manner, as demonstrated based on transgenic mice [35]. CX3CR1 is a GPCR transmembrane protein expressed by monocytes, macrophages, dendritic cells and T-cells. The CX3CR1 positive cells showed a response to *Salmonella enterica in vivo*, which was absent in transepithelial dendrite-deficient (CX3CR1$^{GFP/GFP}$) mice [35]. In fact, CX3CR1$^+$ cells are non-migratory intestine-resident cells with slow turnover rates and characterized by a poor response to FLT-3L and MCSF [36]. Within CX3CR1$^+$ cells, 70% of these cells were macrophages expressing the CD64 marker [37]. In contrast to CX3CR1$^+$ cells, CD103$^+$ DCs migrate into the intestine draining mesenteric lymph nodes at both steady state and in inflammatory conditions with high T cell stimulatory capacity [36]. Interaction between CX3CR1$^+$ macrophages and CD103$^+$ DCs further revealed the role of CX3CR1$^+$ macrophages in taking up soluble antigens and quickly transferring them to CD103$^+$ DCs in a Connexin 43-dependent manner and requiring membrane transfer [38]. The starting point for our research was therefore that the cells in the intestine that interact with NDPs should be resident macrophages. Intestinal resident macrophages are highly phagocytic and in the steady-state show no enhanced production of inflammatory cytokines as IL1, IL6, and TNFα in response to ligation of TLR [39], but they do produce substantial amounts of IL10 [34]. By exposing the macrophages to NDPs, we could use the data to better predict and explain how NDPs impact human health.

Macrophage based research

To investigate effects of food components on intestinal immunity *in vitro,* models preferably should mimic both the intestinal epithelial layers and should contain the sampling immune cells as explained above. In literature, several models are described such as Caco-2 and HT-29 as IECs in coculture and with THP-1 macrophages or RAW 264.7 macrophages as immune cells. However, the IEC-barrier could also abolish the response by macrophages as we found, since the dendrites of immune cells do not cross the IEC barrier in the *in vitro* co-culture model. We chose to evaluate the direct interaction of

NDPs and macrophages, which is still valuable to evaluate NDPs immunomodulatory activity.

In general, there are two ways to obtain macrophages. The first is based on a human cell line like THP-1 monocytes which can be differentiated into macrophages by PMA. THP-1 cells are a human leukemia cell line broadly used for *in vitro* assays to evaluate immunomodulatory effects of food components [40], since it is sensitive and robust. THP-1 macrophages have been used to study immunomodulating effects of quercetin, citrus pectin, and barley glucan [41]. This macrophage model was further used to compare the immunomodulating properties of three β-glucans derived from oat, barley, and shiitake [42]. THP-1 macrophages respond to LPS as low as 1 pg/ml [13], while the primary M0 macrophages threshold is higher than THP-1 macrophages (1 ng/ml; data not shown). THP-1 macrophages can be further polarized to M1 or M2 subsets that express specific marker genes and show specific functionality [43]. This model also has shown to be capable of identifying food components with macrophage-polarizing potential.

The second strategy for obtaining human macrophages, which was used in this thesis, is based on primary human monocytes isolated from blood buffy coat from which macrophages can be differentiated by stimulation with MCSF *in vitro*. Human macrophages obtained from healthy donors have the advantage that they reflect the human steady status. However, the disadvantage is that this also influences the responses to stimuli and therefore research should always be based on at least two or three donors. Again, these macrophages can be further polarized to inflammatory or tolerant macrophages with specific stimulations. We used TNFα together with INFγ instead of LPS (in order to discriminate between effects of NDPs and those that might be generated by LPS contaminations in the NDP samples) to polarize to inflammatory macrophages. At the start of our research no fixed set of gene expression markers was available to characterize these subsets of macrophages. Therefore, we invested in selecting and validating a marker gene set (**chapter 3**). These sets of gene markers we setup were well established and could be useful for food components evaluation. We also studied the macrophage functionality by several assays such as phagocytic assay, antigen processing assay, and immune cell recruitment assay (**chapter 4**). Next, we used such human macrophages, both the non-differentiated M0 and the M1 and M2 subsets, to study the effect of NDPs on their differentiation. Furthermore, we studied the effects that macrophage-NDP interactions could have on their functionality and on immunological processes in the intestine. This revealed that NDPs can direct macrophages towards an alternative subset which was termed M_{NDP} (**chapter 3**) and that this effect is strong enough to redirect macrophages that already were differentiated in a specific M1 or M2 subset (**chapter 4**). Characterization of this M_{NDP} subset showed a

specific secretion profile of cytokines, reduced phagocytosis and antigen processing capacity, and increased recruiting ability towards at least monocytes. The composition of the produced chemokines, which we showed in **Chapter 4**, suggested that more cell types could be interesting to be tested in future such as eosinophils, neutrophils, basophils, and resting and activated T cells. Our current results could be interpreted as that intestinal macrophages (those sampling from the lumen or situated in Peyer's patches in which NDPs were transported across the intestinal epithelium), whatever their differentiation stage or subsets, are (re-)directed towards a somewhat tolerant subset that is more active than intestinal residing tolerant macrophages in attracting other immune cells to the relevant location. This could result in a higher degree of infiltration of intestinal tissue with immune cells and therefore in a high immune capacity to respond in case of emergency (like wound/damage or infection).

Continuum, flexibility and plasticity of macrophages

As explained above, macrophage phenotypes appear not to be restricted to only a few sharply defined subsets. Instead, there appears to be a continuum of phenotype and function. The M_{NDP} also do not have sharply defined characteristics and, more importantly, also not all NDPs appear to have the same capacity in directing this differentiation. Based on the diversity of health effect described for NDPs (*e.g.* some have shown cholesterol lowering effects while others have not [44]), variety in capacity of NDPs could also be expected. As described in **chapter 3** and **4** it is very likely that specific (C-type lectin) receptors expressed on the surface of macrophages are involved and therefore the NDP should be able to correctly bind to the receptors and induce receptor clustering, crosslinking or (hetero)dimerization and subsequently activation, polarization, internalization as part of this differentiation activity [45, 46]. Not knowing the exact activity, it will be difficult to study this activation by specific receptor based assays, which as a consequence can be better performed by cells that express the full receptor panel.

The differences in potency of NDPs to direct macrophage subset differentiation should be further studied *in vivo* to develop this model to a more validated prediction strategy. This implies that *in vivo* research should be performed also with potentially less potent NDPs to be able to rank the full palette of bioactive NDPs. In our set of NDPs, Wellmune Soluble showed the least activity towards macrophage differentiation which might be in agreement with a previous report that Wellmune Soluble has reduced receptor crosslinking for dectin-1 activation [46]. We did not directly compare with the non-solubilized Wellmune, the particulate WGP, for which a strong bioactivity has been

reported previously, both based on *in vitro* and *in vivo* studies [46, 47]. When completing a validation of the macrophage polarization approach and aligning it with *in vivo* effects the method could pave the way for more fundamental studies towards efficient NDP structure-function relations, effects of processing and preparations, matrix effect in food formulation and many more.

Macrophages respond stronger to nutrients than IEC

As discussed above, Caco-2 resembling IECs could produce cytokines and chemokines in response to food exposure. However those responses were weak compared to the reactions we observed for macrophages. This may relate to the fact that exposure to luminal compounds is continuously changing with our diet and IECs major function is maintaining barrier integrity, while at the same time absorbing nutrients. Next comes support to immune cells by signalling molecules, in particular when the intestine is challenged. Although responses of IEC towards the NDPs were mild, these NDPs showed a strong immune regulating effect, with Naxus inducing the strongest responses in both Caco-2 (**Chapter 2**) and macrophages (**Chapter 4 and 5**). Unfortunately, results from the human trials with these same fibres have not been published yet. However, from results that have been communicated, among the tested NDPs Naxus also showed the strongest support of the immune system based on a flu vaccination model including elderly subjects. In this model, a 2 week NDP intervention was given prior to the seasonal flu vaccination and during the following 3 weeks. After this exposure, antibody titres against all three flu strains present in the vaccine were tested. Naxus showed a trend (though not significant) towards an increased antibody titre and increased serum protection levels for all three strains, which was higher than for any other NDP included in the study (J. Schrezenmeir, personal communication). This indicates that Naxus indeed can be the most potent NDP in our set of NDPs tested and therefore correlates surprisingly well with the immune responses found, both on Caco-2 and macrophages.

Trained immunity vs Tolerance

Immune cells are often divided as being either innate immune cells or adaptive immune cells. The innate immune system, also known as non-specific or in-born immune system, provides an immediate defence against pathogens based on general recognition and defence responses. The adaptive, or acquired, immune system, is based on specialised cells and processes to eliminate pathogens which is based on immunological memory. Upon an initial response by a pathogen the memory enables an enhanced response in

case of re-exposure. Recently it was shown that innate immune cells also possess memory. This memory by innate immune cells has been named trained immunity which is characterized as an intensified response to a secondary infection of the same pathogen but also to a different one [48]. Trained immunity has been shown for instance for monocytes primed by β-glucans [49]. This trained immunity strongly responded to TLR2 agonists like Pam3Cys resulting in an enhanced TNFα and IL6 secretion which supports long term immune activation [49]. Trained immunity likely is mediated via an epigenetic reprogramming as demonstrated by the changes in expression of three histone markers (H3K4me1, H3K4me3, and H3K27ac) [49]. We found that NDPs (RG-I, LCES, and Naxus) uniquely downregulated the expression of HIST1 family genes [24] which suggested that these NDPs might also function as stimulators of the trained immunity with epigenetic regulation. Actually, NDP treated macrophages showed an increased secretion of IL1β, TNFα, and IL8, and also a group of chemokines (**Chapter 4**). Besides, trained monocytes have been characterised for their high glucose consumption, high lactate production and activation of mTOR through a dectin-1-AKT-HIF-1α pathway [50]. Our preliminary data on macrophages exposed to Naxus also identified a high lactate production (data not shown). Although we detected NDP responses by macrophages after 18 hours and training of monocytes was performed over 6 days, the responses are in line which leads us to hypothesize that NDPs like Naxus could train macrophages resulting in a broad improved innate defence against pathogens.

In contrast to β-glucan-trained monocytes, LPS-primed monocytes are characterized by a long-term tolerance with low levels of secreted proinflammation cytokines (TNFα and IL6) [49]. Endotoxin-induced tolerance is a phenomenon in which cells arrive in a transient unresponsive state and are resistant to further challenges with endotoxin [51]. An example is presented where dendritic cells, after a long term LPS stimulation, attained a self-tolerant status, which revealed IDO to be involved in intracellular signalling and maintenance of a stable regulatory phenotype [52].

Trained immunity and tolerance can therefore be seen as opposing functional programs which greatly depend on the type and concentration of pathogen-associated molecular patterns (PAMPs) [53]. A strong infection could lead to fast resolution and to a tolerant activation state. However, a low grade of infection/food based stimulation can support trained immunity, leading to a broad enhanced defence and support of the development of memory of the immune system.

Food and trained immunity

Based on the previous discussion, the question raises whether dietary compounds can train this innate immunity and whether 'functional' foods, containing these bioactive compounds, can be used as an effective strategy to treat immune deficiency status or modulating exaggerated inflammation [54, 55]. Several NDPs were reported to have immunomodulatory effects, including yeast/fungal and cereal β-glucans which share common structural features with β-glucans as present in pathogens. It is therefore not unlikely that food, containing these β-glucans, can train our immune system, especially when intestinal cells can recognize these β-glucans and pass this 'training' signal systemically. The macrophages that cross the intestinal barrier for sampling could also initiate the interaction of immune cells and β-glucans [32]. The results on vaccination efficacy increase by Naxus, as explained earlier, also resulting in a reduced incidence of common cold and respiratory tract infection (unpublished data), are therefore in line. These NDPs, as a food product or supplement, are therefore worth to study for this trained immune effects. Optimal dosing and duration of intervention need to be fine-tuned. Also, duration of maintained trained status and the cross protection against a large array of pathogens need to be established.

Techniques to prove immunomodulatory function of food components

To study the immunomodulatory or training activity of NDPs and other food compounds we need efficient methods to study the status and function of immune cells. In the early stage of our research, at the time we developed the methods to polarise macrophages to M1 and M2, we applied common methods like flow cytometry based on established surface markers (like HLA2, CD80, and CD83 as M1 markers and CD209, CD163 as M2 markers). This resulted in only slight differences between these subsets. The reason for this could be that macrophages, differentiated by MCSF for 6-days, push macrophages to express a high background level of those markers. We next applied qPCR to check gene expression of IL8, IL1β, IL12p40, TNFα as M1 markers, and dectin-1, CD209, MRC1, MRC2, PPARγ, and CD36 as M2 markers. These showed to provide more information on differential status of the subsets and the marker list could be easily expanded based on microarray analysis. Gene expression markers are therefore preferred over surface markers for this type of *in vitro* analysis although some of the newly identified markers like the C-type lectins could be valuable surface markers, especially as it is likely that they are capable of identifying a highly variable set of carbohydrate structures. However, *in vitro*, changes in gene expression can be followed but not necessarily describe the true physiological status of the immune cell. So the challenge will be how to apply gene

expression markers more self-explanatorily in *in vivo* situations. Also here the best strategy might be to apply a challenge (like PAMPs) on whole blood, PBMCs or separately on isolated monocytes or macrophages and to analyse the responses by gene expression and cytokine analysis.
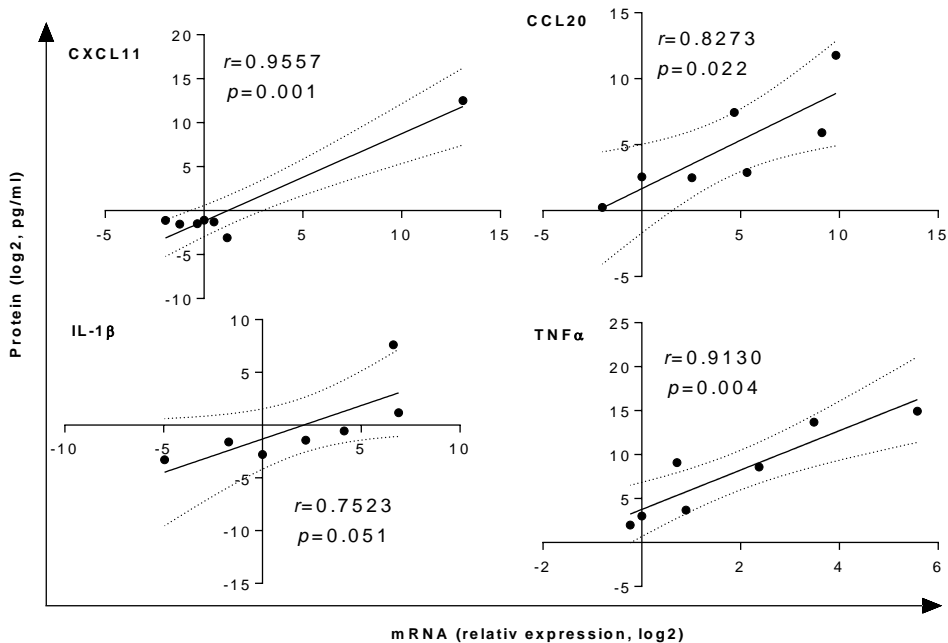
Figure 2. Correlation between gene expression and excreted protein for macrophages stimulated with NDPs. Gene expression data and cytokines protein production were analysed for the linear regression analysis by GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA).

Gene expression predicts protein level

The gene expression markers set that we developed to distinguish M1 and M2 and to characterise the newly identified M_{NDP} subset can have interesting applications in future research. However, as current research makes us of cytokines instead of gene expression markers, we studied the correlation between expression of cytokine genes and excreted proteins in our macrophage model. Here we include some additional data, analysed by linear regression analysis, to the data presented in the separate chapters

(Figure 2). These data indicate that mRNA expression is significantly correlated with protein production for CXCL11 (p=0.001), CCL20 (p=0.022), and TNFα (p=0.004). Only IL1β did not reach significance (p=0.051), which might be due to the low expression of both gene and protein or the sampling time point used for the gene expression analysis was not optimal. Overall, from our experience we believe that gene expression analysis in cell models is a rapid and sensitive tool.

Future perspectives

A macrophage polarisation model has been developed and is descried in this thesis, together with the effects of NDPs studied based on this macrophage model. The study revealed that M_{NDP} could be characterized by gene expression of a panel of markers, by secretion of cytokines and chemokines, and that M_{NDP} induce functional changes in phagocytosis, antigen processing, and recruiting ability. Furthermore the gene expression of a group of C-type lectin receptors were changed by NDPs [24]. To be more specific, we found that dectin-2 and Mincle are both increased in expression when macrophages are incubated with NDPs. Those two receptors also have been shown to be involved in α-mannose recognition, both independently. These two receptors also can form a heterodimer with different sensitivity and diversity for recognizing microbiota [45]. It would be interesting to study these receptors in more detail, specifically in relation to NDP recognition. In addition, it would be worthwhile to study the intercellular signalling in macrophages and to analyse whether receptor activation leads to similar or divergent signals that eventually regulate immune functionality. It would be also interesting to use this *in vitro* model as selection tool for other NDPs and to correlate to *in vivo* data. By using these analyses, possibly we can select and target the NDPs as functional food ingredients to support maintenance of the intestinal immune barrier, support the immune system, or support both.

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Summary

Non-digestible polysaccharides (NDPs) are considered as important ingredients to support health. Among these health effects, immunomodulatory effects raised interests in the past decade. The intestine is the primary organ that interact with NDPs. The intestinal epithelial cells (IECs) form a dynamic physical barrier and together with associated immune cells determine for a large part our immune homeostasis. Studying the direct interaction between NDPs and intestinal and immune cells could help us to uncover the mechanism by which NDPs exert immunomodulatory effects and how NDPs can differ in this activity. In this thesis, we investigated the immunomodulatory effects of NDPs through interaction with intestinal immune cells using in vitro methods in order to characterise the NDPs and preselect NDPs with differential activity for further in vivo evaluations.

The intestinal immune barrier is formed by various IECs and immune cells, which are introduced and their specific functions discussed in **Chapter 1**. NDPs could interact directly with both IECs and immune cells that sample in or from the lumen. The majority of IECs are enterocytes and most relevant immune cells responsible for sampling in the lumen have been characterised as macrophages, which leads us to focus on these cell types by *in vitro* approaches. In addition, basic information on NDPs and current status on health effects of NDPs both *in vitro* and *in vivo* are discussed.

In **Chapter 2**, the direct response of IEC to NDPs stimulation was investigated. IECs form the largest surface of the body that, with a crucial role as barrier also, perform a role in signalling towards immune cells. We used 21-day transwell cultured Caco-2 to resemble the small intestinal enterocytes that form largest part of this intestinal layer. We first characterized the chemical composition of five NDPs which revealed different mono sugar composition, linkages of backbone and side chains and a wide range of MW (from 17 KDa to 2100 KDa). The NDPs could reduce translocation of FITC-Dextran of 4 kDa across the epithelial layer, potentially through physical interference. Gene expression analysis indicated the induction of unique gene expression characteristics in Caco-2 cells upon exposure to different NDPs. An arabinoxylan preparation from wheat and a lentinan-containing extract from shiitake mushrooms showed upregulation of gene expression of the NF-κB family and chemokines CCL20 and CXCL10. Besides these immune related changes by some NDPs, we also observed changes in receptor expression (like TLR2, CD14 and GPCRs) and other pathways, amongst which the cholesterol biosynthesis pathway.

Macrophages, as the resident population of immune cells penetrating between or associating with close contact with the IECs, are generally classified as inflammatory (M1) or as tolerant (M2) macrophages. In **Chapter 3**, we set up a macrophage differentiation

method based on primary blood cells and selected and validated M1 and M2 specific gene expression markers. Next, we analysed the effect when macrophages are exposed to NDPs and compared the resulting macrophages with M1 and M2 macrophages. Based on M1 and M2 markers we identified an alternative subset that we named M_{NDP}. This M_{NDP} was further studied by microarray analysis and revealed a commonly modulated set of genes, involved in migration, metabolic processes, cell cycle, and inflammatory immune function.

In **Chapter 4**, we further functionally characterize these M_{NDP} in comparison to M1 and M2 macrophages based on a set of functional assays. NDP-treated macrophages showed no IDO activity and showed an inhibited antigen uptake and processing capacity compared to M1 and M2 macrophages. Also their phagocytic capacity was reduced compared to both M1 and M2 macrophages. Furthermore, the alternative expression pattern for NDP-treated macrophages, as demonstrated by gene expression, was confirmed by protein measurements. The signature mix of the chemokines CCL1, CCL5, CCL20, CCL24, CXCL8, and IL1β secreted by M_{NDP}, and in particular when macrophages were treated with Naxus, was shown to induce a recruitment of monocytes.

As macrophage plasticity could be essential for intestinal immune homeostasis, resolving activity of inflammatory responses upon a challenge is important. Besides, redirecting differentiation and function of tolerant macrophages can also be beneficial to the intestinal immune status. In **Chapter 5**, we analysed plasticity of M1 and M2 macrophages to NDPs exposure. Macrophage plasticity was demonstrated as M1 and M2 could be skewed to an alternative subset indicated by a dedicated set of gene expression markers, selected to characterize M1, M2 and M_{NDP} macrophages. In addition, phagocytosis and antigen processing capacity of both M1 and M2 were decreased by the NDP Naxus. Besides, Naxus could change the secretion of cytokines by macrophages that previously were differentiated towards M1 and M2. For M2, this resulted in an increase of recruitment of monocytes by M2 macrophages.

In **Chapter 6**, we discussed the important findings in each chapter of this thesis together with current literature, and gave a general perspective on this research line focussing on the immunomodulating activity of NDPs and the direction for future research. We suggested NDPs in terms of Naxus as candidate for guiding investigations in *ex vivo* and *in vivo* studies for immunomodulation of intestinal disease.

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Acknowledgement

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To House 84 at Van Uvenweg. This is a wonderful place for me and my family. It is the home for all of the friends that have lived there and friends we have met there. So many happy memories in this place, which will stay with me my whole life. We used to clean rooms together, cook together, and eat together for dinner. The topics of our talks were shopping, planning for travel, campus, new PhDs, and definitely the research and future planning. Wenbiao, you made everything happen. You were the director who made those beautiful memories happen. You were enthusiastic to go to so many places to buy everything we needed to settle "our home". Yu Zhu, you were supposed to stay a month, however, you stayed more than a year. You were our chef since you can make any dish no matter Chinese food, curried potatoes, or Burrito. However, I must complain about your playing so late and puzzled about the interest in playing games even though I have tried to learn by observing. Jing and Rong, you are amazing friends. I miss the time we were traveling together. Let us talk about those memories together by reading the photos one day. Wenbo Jiang, you made me realize that a man who loves research can have a peaceful heart. Roy Zhang, you used your actions to change my notion and be open to accept differences. Best wishes for you and Timen. Thank you Huajie, Menyuan, Yutian, Yang Zhang, Rui, and Michele, I am very happy to have met you in House 84.

About the Author

Curriculum vitae

Yongfu Tang was born on January 13, 1982, in Shandong, China. In 2001, he started his Bachelor's degree in Shandong Agricultural University (Shandong, China). After receiving a bachelor degree in Food Science and Technology, he continued with the Master degree in Nutrition and Food Hygiene in Nanchang University (Jiangxi, China) in 2005. He specialized in Nutrition and Health with a project of immunomodulatory effects of polysaccharides isolated from *Plantago asiatica L* on dendritic cells under the supervision of Prof. Mingyong Xie in State Key Laboratory of Food Science and Technology (Nanchang University). In 2008, he worked as a research assistant on health effects of tropical agricultural products in Chinese Academy of Tropical Agricultural Sciences (Guangdong, China). In 2012, he started his PhD project to investigate how non-digestible polysaccharides support intestinal immune barrier with *in vitro* macrophage and epithelial cell models under the supervision of Prof. Harry Wichers, Dr. Jurriaan Mes, and Dr. Coen Govers in Wageningen University and Research. The results of his PhD research are presented in this thesis. He will continue his research in Nutrition and Health to prevent brain disease and help brain disease recovery by nutrient intake.

List of selected publications

Yongfu Tang, Coen Govers, Luc Saulnier, Svein H. Knutsen, Harry J. Wichers, Jurriaan J. Mes. Macrophages treated with non-digestible polysaccharides reveal a transcriptionally unique phenotype. *Journal of functional foods*, 2017 (36): 280-289.

Yongfu Tang, Coen Govers, Sophie Le Gall, Luc Saulnier, Anna Rieder, Svein H. Knutsen, Monic M.M. Tomassen, Nicole de Wit, Harry J. Wichers, Jurriaan J. Mes. Gene expression analysis characterizes Caco-2 responses to non-digestible polysaccharides: a first step to a systematic comparison of dietary fibres. *Submitted*.

Yongfu Tang, Coen Govers, Ellen Kranenbarg-Stolte, Harry J. Wichers, Jurriaan J. Mes. Human macrophages stimulated with non-digestible polysaccharides are in function and chemokine production different from inflammatory and tolerant macrophages. *In preparation*.

Yongfu Tang, Coen Govers, Ellen Kranenbarg-Stolte, Harry J. Wichers, Jurriaan J. Mes. Non-digestible polysaccharides mediate macrophage plasticity by function change on macrophage subsets. *In preparation*.

Malgorzata Teodorowicz , Olaf Perdijk, Iris Verhoek, Coen Govers, Huub F. J. Savelkoul, **Yongfu Tang**, Harry Wichers, Kerensa Broersen. Optimized Triton X-114 assisted lipopolysaccharide (LPS) removal method reveals the immunomodulatory effect of food proteins. *PLOS one*, 2017 12(3): e0173778.

Danfei Huang, **Yongfu Tang**, Shaoping Nie, Yin Wan, Mingyong Xie, Xiaomei Xie. Effect of phenylethanoid glycosides and polysaccharides from the seed of Plantago asiatica L. on the maturation of murine bone marrow-drived dendritic cells. *European Journal of Pharmacology*, 2009, 620 (1-3): 105-111.

Danfei Huang, Mingyong Xie, Junyi Yin, Shaoping Nie, **Yongfu Tang**, Xiaomei Xie, Chao Zhou. Immunomodulatory activity of the seeds of Plantago asiatica L. *Journal of Ethnopharmacology*, 2009, 124 (3): 493-498.

Overview of completed training activities

Discipline specific activities (courses, workshops, symposia, summer schools, conferences etc.)

2nd progress meeting of FibeBiotics. Kiel, Germany, 2013
3rd progress meeting of FibeBiotics and 2nd InP meeting. Bologna, Italy, 2014
7th International Immunonutrition Workshop, "Eating for Preventing". Bari, Italy, 2014
4th progress meeting of FibeBiotics. Oslo, Norway, 2014
12th China Nutrition Science congress. Beijing, China, 2015
6th progress meeting of FibeBiotics and 4th InP meeting. Paris, France, 2015
17th Gut day symposium. Rotterdam, NL, 2015
Carbohydrate Competence Center symposium. Zwolle, NL, 2015
3rd Wageningen PhD Symposium "Diversity in Science", Wageningen, NL, 2016
7th progress meeting of FibeBiotics. Ede, NL, 2016
19th International Society for Mushroom Science (ISMS). Amsterdam, NL, 2016
The 5th Beneficial Microbes Conference. Amsterdam, NL, 2016
Microglia phenotype and function analysis in stroke. Houston, USA , 2017

General courses

VLAG PhD week VLAG. Baarlo, NL 4, 2013
Philosophy and Ethics of Food Science & Technology. VLAG /WGS, Wageningen, NL, 2015
WGS PhD Workshop Carousel. WGS, Wageningen, NL, 2015
Techniques for writing and presenting a scientific paper. WGS, Wageningen, 2016
Reviewing a Scientific Paper. WGS, Wageningen, NL, 2016
Scientific Publishing. WGS, Wageningen, NL, 2016
Career Perspectives. WGS, Wageningen, NL, 2016

Optional courses and activities

Preparation of research proposal. 2012
Science meeting of FQHE, weekly
Journal club of FQHE, weekly

Abbreviations:
FQHE: Food Qulity and Health Effect, Food and Biobased Research; VLAG: Graduate School for nutrition, Food Technology, Agrobiotechnology and Health Science; WGS: Wageningen Graduate School.