DIETARY RNA (*S. cerevisiae*) STIMULATED CYTOKINE SECRETION IN HUMAN IMMUNE CELLS: INVOLVEMENT OF P38 MAPK, AND CO-STIMULATION WITH LPS OR PHA

Master thesis in human nutrition

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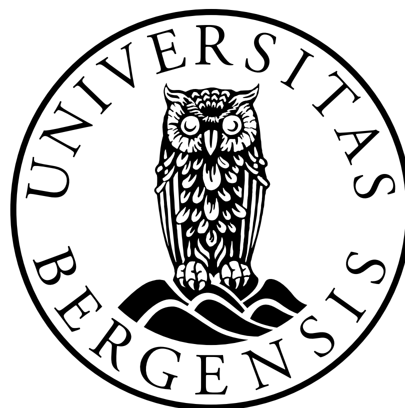

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ABSTRACT

Dietary nucleotides and nucleic acids are known to affect immune functions in animals and humans, both *in vitro* and *in vivo.* In this thesis I wanted to investigate the effect of dietary RNA from baker's yeast on cytokine secretion from human peripheral blood mononuclear cells (PBMS). We co-cultured PBMCs from 11 subjects with RNA, LPS, PHA, SB 202190 and combinations of these, and analyzed supernatants for cytokines using ELISA kits. RNA induced secretion of IL-1α, IL-1β, IL-6, IL-8, IL-10, TNF-α and GM-CSF, co-stimulation of RNA increased secretion in PHA stimulated GM-CSF only. No secretion of IL-2, IL-4, IL-12 or IL-17α was detected. Inhibition of p38 MAPK with SB 202190 decreased RNA especially, but also LPS and PHA induced cytokine secretions. These results indicate that RNA induce cytokine secretion alone, but has little additive effect on secretion by LPS and PHA stimulation, except for GM-CSF; and the effect of RNA is partly mediated by p38 MAPK. The stimulatory effect on GM-CSF might indicate a role for RNA in priming of innate immune response.

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INTRODUCTION

It is known that nucleotides have a modulating effect on immune function, both *in vivo* and *in vitro* (Nagafuchi 2007). Studies on neonatal nutrition have revealed several immunostimulating substances in human milk, of which nucleotides are included (Niers 2007). Also, several murine feeding experiments have shown a reduction of a number of immune parameters caused by a nucleotide-free diet (Nagafuchi 2007). The precise mechanism of the immune stimulating effect of nucleotides is not known, but it has been suggested that certain tissues/cells have a limited ability for *de novo* synthesis, especially during rapid growth or stress, and may need exogenous supply for optimal functioning. It is also possible that the effect is mediated through activation of purinergic-receptors or Toll-like receptors by nucleotides/nucleic acids (Holen and Jonsson 2004).

Nucleotides are non-protein, nitrogen containing molecules that have a variety of functions, of which the most prominent is their role as building blocks of DNA and RNA. Nucleotides have three components: a nitrogenous base, a pentose sugar and a phosphate. If there is no phosphate attached the molecule is a nucleoside. The nitrogenous bases are derivates of pyrimidine and purine.

Besides being building blocks for nucleic acids, nucleotides are important in energy transfer. The ribose of nucleotides can have from one to three phosphates attached; these are labelled α, β and γ (from the ribose). The α,β and the β,γ phosphates are linked by phosphoanhydride bonds, and hydrolysis of these yield more energy (30 kJ/mol) than the ester bond between the α and the ribose (14 kJ/mol). The most abundant nucleotide used for energy is adenosine 5’-triphosphate (ATP).

Adenosine is a component of cofactors and enzymes (e.g. NAD, FAD and CoA); it is not directly involved in the primary function of these, but helps facilitate binding of the substrate to the cofactor. Nucleotides also function as second messengers (e.g. cAMP and cGMP) involved in a range of regulatory functions, and as carriers of intermediates (e.g. UDP-glucose) in metabolic processes such as synthesis of glycogen, glycoproteins and phospholipids. Purinergic nucleotides can also act as ligands for nucleotide-receptors (Carver and Allan Walker 1995; Gabel 2007). Leukocytes express several purinergic receptors of both the P2X and the P2Y families. Activation of these receptors can modulate cytokine production (Gabel 2007).

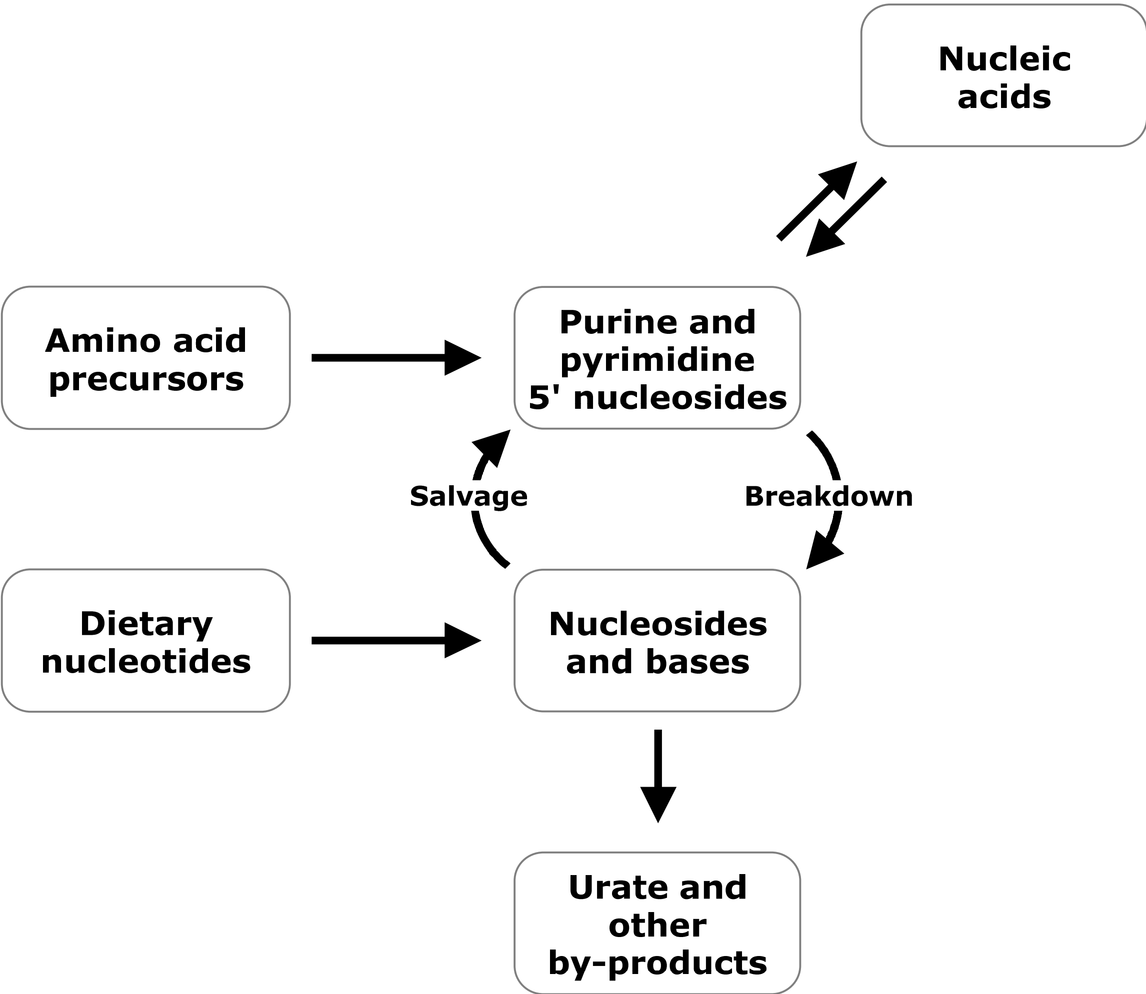


Figure 1. Model of de novo and salvage pathways in nucleotide metabolism (Modified from Rudolph 1994).

Nucleotides can be synthesized *de novo* or from salvage of nucleotides and nucleosides from degradation of DNA, RNA or other substances (figure 1). Preformed purines and pyrimidines from dietary sources and from cellular turnover provide a source for nucleotide synthesis. This salvage conserves energy by avoiding the costly *de novo* synthesis. Preformed dietary nucleosides can also directly be incorporated into nucleic acids.

Most dietary nucleic acids and nucleotides are hydrolyzed to nucleosides and nucleobases and readily absorbed in the small intestine (Ho, Miller et al. 1979; Uauy, Quan et al. 1994; Carver and Allan Walker 1995), but some 5 to 10 % appears intact as nucleotides inside mucosal cells (Berthold 1995). Nucleotides are transported into enterocytes with passive diffusion and Na+ dependent carrier mediated mechanisms (Holen and Jonsson 2004). Investigations regarding the contribution of dietary nucleotides to nucleic acids have found that it is low, approximately 2-5 %, more when the intake increase, food is limited or during rapid growth (Carver and Allan Walker 1995, Carver 1999). Some pyrimidines are incorporated, but purines are almost exclusively catabolised and excreted, a proposed explanation is that the importance of ATP and GMP (e.g. cAMP and cGMP) has lead to a strict regulation to avoid any dietary fluctuations (Uauy, Quan et al. 1994, Berthold 1995). Of the retained nucleotides a significant portion is incorporated in gastrointestinal tissue, and lymphoid tissue in the gut may activate and regulate T-cell development and peripheral immunity may be mediated via this tissue (Carver 1995).

Fairbanks et al (1995) found that for rapidly proliferating cells such as PHA stimulated T-lymphocytes there is an increased *de novo* nucleotide synthesis, especially pyrimidine synthesis for membrane biosynthesis, but also the NAD pool was expanded, e.g. for repair of DNA breaks. They also found that resting T-lymphocytes met their requirement by nucleotide salvage alone.

When inhibiting *de novo* synthesis or depleting culture medium glutamine, the addition of exogenous nucleosides attenuate the reduction of cell proliferation, as measured by thymidine incorporation into DNA in rat lymphocytes (Szondy and Newsholme 1990). This may be relevant in situations where glutamine may be limited, such as injury or sepsis.

The effect of nucleotides on DNA repair was shown by Salobir and Rezar et al (2005), who fed pigs with a polyunsaturated fatty acid rich diet to increase oxidative stress, by adding nucleotides to the diet the DNA damage of lymphocytes was reduced compared to those fed a nucleotide free diet. Similarly, in mice DNA damage to thymocytes induced by cyclophosphamide was reduced by dietary nucleotide supplementation (Wang 2008).

Because of *de novo* synthesis of nucleotides, they are not considered essential nutrients. But for some rapidly dividing cells such as in intestinal mucosa and lymphoid tissue, the *de novo* synthesis may not be adequate, and exogenous nucleotides may be required (Maldonado et al. 2001). In conditions such as disease, rapid growth and stress nucleotides may become essential (Wang 2008). They can thus be classified as conditionally essential.

When animals are fed a nucleotide free diet, a reduction of a number of immune functions occurs, a decrease in hepatic and intestinal protein synthesis has also been found (Lopez-Navarro, Ortega et al. 2004), addition of nucleotides to the diet quickly restores the immune responses (Jyonouchi 1994).

Maturation of the immune system occurs during the first year in infants. Numerous studies show that infants fed human milk are more resistant to various infectious diseases than infants fed formula (Buck 2004; Schaller, Kuchan et al. 2004; Schaller, Buck et al. 2007). This effect of milk is caused by numerous biological active substances such as immunoglobulins (notably IgA), antimicrobial proteins, leukocytes, cytokines

and nucleotides (Niers 2007). Most infant formulas are cow's milk based, and have a much lower nucleotide level than human milk, which is about 72 mg/L (Leach, Baxter et al. 1995). Supplementing infant formulas with nucleotides have successfully improved immune function markers such as increased antibody response to vaccines and increased T-cell maturation, and reduced diarrhea incidence (Schaller, Buck et al. 2007).

The mechanisms behind the immunomodulating effect of nucleotides and polynucleotides are not yet known. Earlier hypothesis was based on the need for nucleotides for nucleic acid synthesis, and the reduced ability for de novo synthesis in various tissues or under various conditions. In the recent years several studies of the Toll-like receptors (TLRs) and other pattern recognition receptors have elucidated their role in recognizing invading pathogens and the subsequent initiating of the immune response (Sioud 2006). The role of nucleic acids and nucleotides as ligands for TLRs and in signalling tissue damage and infection has been studied extensively (Ishii and Akira 2008).

RECEPTORS

TLRs recognize pathogen associated molecular patterns (PAMPs), but also various endogenous molecules such as heat shock proteins, uric acid and DNA. These endogenous ligands of TLRs have been called danger associated molecular patterns (DAMPs), and the release of these from necrotic cells may serve as a general danger signal to the immune system whether the tissue damage is cause by pathogens, tumors or transplanted tissue (Kono and Rock 2008).

The main TLR-expressing cells are macrophages, dendritic cells (DCs), neutrophils and monocytes (Sioud 2006). Several types of TLRs have been identified; TLR2 recognize peptidoglycan, TLR3 recognize double stranded RNA (dsRNA), TLR4 recognize LPS and envelope glycoproteins, TLR7 and TLR8 recognize single stranded RNA (ssRNA) and TLR9 recognize DNA with hypomethylated CpG motifs (Sioud 2006; Ranjith-Kumar, Duffy et al. 2008).

Also the purinergic receptor families, P2X and P2Y play a role in danger signalling and the shaping of immune responses through dendritic cells (Ferrari, Gorini et al. 2007). These receptors are activated by nucleotides such as ATP and UDP. The cytoplasm of most cells contains nucleotide concentrations in the 1-10 mM range, whereas the extracellular fluid has a concentration in the 1-10 nM range. A sudden increase in extracellular nucleotide levels is associated with tissue stress or damage and this in turn act as a danger signal. Depending on whether the danger signal is endogenous or exogenous, DCs will be activated to initiate the appropriate reaction (Ferrari, Gorini et al. 2007; Kono and Rock 2008).

STIMULATORS

Cells are regulated by a multitude of extracellular signals which are transmitted through trans-membrane receptors, activation of these leads to intracellular signals in which the mitogen activated protein kinases (MAPK) are a primary component (Kumar, Boehm et al. 2003). There are five groups of MAPK pathways identified in mammals; MAPK/ERK, c-Jun N-terminal kinases (JNKs), p38 MAPKs, ERK3 and ERK5 (Brown and Sacks 2009), of these, p38 is the best characterized and probably the most relevant in mediating inflammatory responses (Kumar, Boehm et al. 2003).

Cytokines are a large and diverse class of cellular signalling molecules involved in a series of processes, especially in immune activation and regulation. There is no clear distinction between hormones and cytokines, but generally cytokines are in lower concentrations and secreted from many cell types in contrast to the specialised glands for classical hormones. Cytokines can act in an autocrine, paracrine or endocrine manner. Their effects on cell include proliferation, differentiation, activation and motility (Cohen and Cohen 1996). There are many ways to classify cytokines, e.g. their cell origin or targets, category of activity they induce or their structural homology. The regulation of cytokines is important for proper immune response and to avoid unwanted activation leading to autoimmune and allergic diseases.

Phytohemagglutinine (PHA) is a lectin found in plants, and is a strong mitogen and haemagglutinator, that exercises its immunostimulating effect by activation of T-cells through the T-cell receptor (Chilson, Crumpton et al. 1984). PHA is a mixture of proteins with different actions, and can be divided into L-PHA and E-PHA, which effects lymphocyte and erythrocyte respectively. PHA stimulation of lymphocytes induces a strong inflammatory reaction, with secretion of several cytokines.

Lipopolysaccharides (LPS) are a component of the outer membrane surface in all gram-negative bacteria; it is an essential constituent which mediates membrane transport and interaction with host cells (Rietschel, Kirikae et al. 1994; Eggesbø, Hjermann et al. 1996). LPS consist of a hydrophilic heteropolysaccharide and a covalently bound lipid A, which is the immunostimulatory part (Rietschel, Kirikae et al. 1994). LPS activates the innate immune system with potent inflammatory responses, through activation of several intracellular signaling pathways, including the MAPKs. The intracellular signals activate transcription factors which coordinate genes encoding inflammatory and anti inflammatory cytokines. LPS bound with CD14 is recognized by toll-like receptor 4 (TLR4) which requires MD2 for activation. LPS binding with TLR4 activates TIRAP-MyD88 dependent pathway, which is responsible for early phase NFkB and MAPK activation, and TRIF-TRAM (MyD88 independent) pathway which activates late phase NFkB and MAPK activation (Mogensen 2009). These pathways lead to activation of transcription factors which drive expression of inflammatory cytokines (Saitoh 2008, Verstrepen, Bekaert et al. 2008). TRIF, but not TRAM, is also associated with TLR3, which recognize dsRNA (Mogensen 2009).

PURPOSE OF THE STUDY

With this study we wanted to investigate the role of RNA in cytokine secretion by human peripheral blood mononuclear cells (PBMC), in conjunction with and compared with, stimulation with T-cell dependent or independent mitogens. Because RNA has showed a good immunostimulatory effect (Holen, Bjørge et al. 2005), this was chosen as the single nucleic acid stimulator for my thesis. PBMC are blood cells with a round nucleus such as monocytes and lymphocytes, these have a density differing from erythrocytes and granulocytes, which allows for easy separation by density gradient centrifugation. Monocytes originate from bone marrow monoblasts, and their primary role is to replenish macrophages and dendritic cells. Lymphocytes stem from bone marrow precursor cells, but migrate to the thymus for maturation. Lymphocytes include T-cells (helper T-cells, cytotoxic T-cells, regulatory T-cells), B-cells and natural killer (NK) cells.

We also used SB 202190, a specific inhibitor of MAPK p38, which is involved in stress stimuli, cell differentiation and cytokine secretion (Kumar, Boehm et al. 2003), to see if this could give an indication of the pathways involved in RNA stimulation. We chose to examine the pattern of cytokine secretion as a marker of immune activation; former studies have focused mostly on cell proliferation and antibody production. To my knowledge there are no studies on the action of RNA combined with LPS of PHA on human PBMC.

The purpose of this thesis was to investigate how RNA compared with, and in combination with LPS and PHA, affected the quantitative and qualitative PBMC cytokine secretion from healthy humans, and if p38 MAPK was involved in a potential stimulatory effect of RNA.

MATERIALS AND METHODS

This project (nr 268.08) involving human volunteers was approved by an ethical committee (REK-Vest). See appendix for further details.

GUIDELINES FOR HUMAN SAMPLES AT NIFES

Projects involving human biological material must be authorized and risk assessed by the contamination risk prevention group (smitteverngruppe) led by Marian Malde. Everyone working with human biological material need authorization from this group and must have practical and theoretical training. Working with human materials is regulated by the working environment act (Forskrift om vern mot eksponering for biologiske faktorer [bakterier, virus, sopp m.m.] på arbeidsplassen).

SUBJECTS

Healthy volunteers, six females and five males, between ages 24 and 35, where recruited and given written information about the study and about personal information protection and their right to withdraw from the study at any time. Subjects were asked to sign a form declaring their consent (appendix).

STERILE TECHNIQUE

All work was done in a laminar flow cabinet, with UV-C germicidal lamp, except drawing the blood which was done in the “human room”. Rubber gloves were used at all times. Glass pipettes were autoclaved, and seal in bags before use, auto-pipettes where disinfected in 70 % ethanol before use. Work area and equipment used were marked with yellow tape to signal human biological material and contamination risk. Sample transport between labs was done using a designated case for human samples marked with yellow tape. Centrifugation of blood was done in the human-room using aerosol-

lids covering the tubes. All tubes and pipettes used for human samples were disposed of in autoclave-bins, and sharp (needles and glass pipettes) waste in designated container.

PREPARATION OF MEDIUM

The cell culture medium was made with 450 mL RPMI-1640 (Sigma, R0883), 5 mL Glutamax™ (Invitrogen35050), 5 mL PSA (Penicillin-Streptomycin-Amphotericin B (100X) from BioWhittaker®), and 50 mL fetal bovine serum (FBS, BioWhittaker®) to a total of 510 mL, and stored at ~5° C.

PREPARATION OF LPS, PHA, RNA AND SB202190

1 mg of LPS from Escherichia coli 026:B6 (L2654, Sigma) were dissolved in 1 mL of medium, and stored at ~5° C. 5 mg of PHA (L8754, Sigma) from *Phaseolus vulgaris* was dissolved in 1 mL medium and stored at ~5° C. 250 mg RNA (R6750, Sigma) from baker's yeast (*S. cerevisiae*) was mixed with 10 mL of medium and stored at ~5° C. 5 mg SB202190 (S7067, Sigma) were dissolved in 1 mL DMSO (D8418, Sigma) and stored at ~5° C. All solutions were sterile.

BLOOD SAMPLING

Venous blood was drawn into two 10 mL EDTA tubes (BD vacutainer K2E 18mg, Ref 367525, BD Plymouth, UK)) by a bioengineer. The blood was diluted 1:4 with sterile DPBS (Dulbecco's phosphate buffered saline (D8357, Sigma).

PBMC EXTRACTION BY PERCOLL AND LYMFOPREP METHOD

Blood was carefully pipetted on top of 5 mL Lymphoprep (Lymphoprep™) density 1.077, Axis Shield PoC AS, Oslo, Norway) in 50 mL sterile cytotubes (Cellstar, cat no 227261, Greiner bio-one). These were centrifuged at 800 G at 20° C for 20 minutes. For some samples Percoll (P4937, Sigma) density centrifugation were used. Percoll solutions we

prepared in two densities, 1.06 and 1.08, by mixing SIP solution (90mL percoll and 10 mL 1,5M NaCl) with sterile buffer B (9 g NaCl, 7 g EDTA-citrate, total volume 1L Milli Q-water, pH 7.02); 1.06 g/mL: 29.15 mL Buffer B + 20.85 mL SIP and 1.08 g/mL:21.45 mL buffer B + 28.55 mL SIP, according to the manufacturer's instructions. Gradients were made by layering the low density Percoll on top of the high density, and diluted blood (1:4 in DPBS) were layered on top, and centrifuged at 400 G at 20° C for 20 minutes. The PBMC layer was collected by pipette from gradient interfaces.

CELL WASHING

The PBMC layer was pipetted out and centrifuged at 3500 rpm, 20° C for 6 minutes. The cell pellets where then mixed with DPBS and centrifuged at 3500 rpm, 20° C for 6 minutes, this washing with DPBS was done twice. Finally the cell pellets were mixed with ca 20 mL medium, and the cells counted.

CELL COUNTING

Counting of PBM cells was done using Trypan blue stain solution 0,4 % (BioWhittaker, cat no 17, 942E), a Bürker chamber and a microscope (Olympys BX4). The chamber is 1 mm^2 (a-square) divided into 16 smaller b-squares (1/16 mm^2). The depth of the chamber when a cover slip is on top is 0.1 mm making the volume of the a-square (1mm^2 x 0.1mm^2) 0.1 mm^3. All cells in each of the 16 b- chambers were counted.

CELL CULTURES

The concentrations of the stimulants and inhibitor used were chosen based on previous literature and dose response curves from work done at NIFES. A relatively high dose of RNA was chosen to ensure a significant response. The calculated volume that gave ~1 x 10^6 cells was added to each well (Sterile Cell Culture Clusters, 12 well Costar Multiwell plate, Corning® CellBind® surface, Corning, NY, USA), together with medium to a final volume of approximately 2 mL. The average number of cells added to each well was 8.8

× 10^5 (± 3.1 × 10^5). SB202190 was added at two concentrations, 1 µM (SB1) and 5 µM (SB2). The cell cultures were left to incubate at ~37 °C and 5 % CO_2 (Thermo Forma Steri Cycle CO_2 –incubator) for two hours before LPS (1 µg/mL), PHA (5 µg/mL) and RNA (500 µg/mL) were added. Cell cultures where incubated at ~37 °C and 5 % CO2 for 72 hours. After incubation, the cell culture solutions were centrifuged 3500 rpm, 20° C for 6 minutes and the supernatant collected and stored at -20 °C.

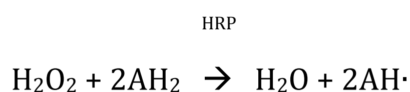
GIEMSA STAINING

Cell suspension 25000-1x 10^6 c/mL was added to the cell-funnel device in Termac cytospin rotor JC101, in a Hettich centrifuge (Universial 320R) and centrifuged 100xg for 2 min. PBMCs on the glass-slide were fixed using 4 % paraformaldehyd. After washing with DPBS the cells were stained adding a drop of Giemsa stain (48900 Giemsa stain, modified solution, Fluka) in 5 % methanol to the cells for 10 minutes. The slide was rinsed with milli-Q water and photographed using and invert microscope NIKON TMS. Cell cultures were photographed with the same microscope setup.

Principle of the assay

The microplate is pre-coated with antibody specific for cytokines (table 1). Assay buffer is added to all wells. Negative control (BSA), samples and positive controls are added to their respective wells, and cytokines bind to the antibodies. After incubation unbound substances are washed away and detection antibodies (with biotin) specific for each cytokine are added to their respective wells and incubated. Unbound substances are washed away and the secondary enzyme-linked antibody, avidin-HRP is added to each well. The avidin-HRP conjugate binds to the biotin of the detection antibodies. After incubation unbound substances are washed away and development solution is added to each well and color develops in proportion to the amount of cytokines bound. The development solution contains a substrate for the HRP linked to the secondary antibody. HRP catalyzes the oxidation of this substrate by hydrogen peroxide. The substrate changes color/absorbance and this is measured by spectrophotometry. The substrate (not specified in the manual) is most likely 3.3', 5.5'-Tetramethylbenzidine (TMB), which change color to green-blue when reacting with HRP and hydrogen peroxide and to yellow when stop solution is added. After incubation stop solution is added to stop the color development, and microplate is read at 450 nm within 30 minutes.

General oxidation reaction catalyzed by HRP, where AH_2 is the reducing substrate and AH· is the radical product of the reducing substrate (Veitch 2004):



$$H_2O_2 + 2AH_2 \xrightarrow{HRP} H_2O + 2AH\cdot$$

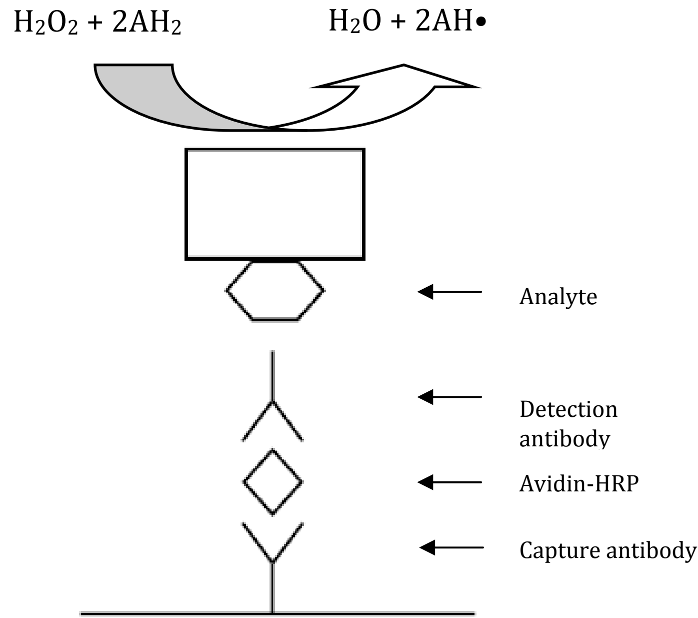


Figure 2. Solid phase sandwich immunoassay; the capture antibody is fixed to a surface, and the analyte is "sandwiched" between two antibodies. The avidin-HRP binds to the detection antibody and HRP catalyzes the oxidation of the substrate (AH_2).

Cytokine screening assay

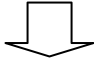
For a screening of the cytokines present in the supernatants, Multi-Analyte ELISArray™ Kits (SABioscience™) where used. This screening was done with a mix of supernatants from all eleven individuals. This was thought to give a reasonable representation of the mean cytokine concentration from the subjects.

Table 1. Microplate setup, samples are in wells B through G, A is the negative control and H is the positive control. Each well in the numbered vertical strips have the same capture antibody.

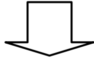
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	IL1α	IL1β	IL2	IL4	IL6	IL8	IL10	IL12	IL17α	IFNγ	TNFα	GM-CSF
B	IL1α	IL1β	IL2	IL4	IL6	IL8	IL10	IL12	IL17α	IFNγ	TNFα	GM-CSF
C	IL1α	IL1β	IL2	IL4	IL6	IL8	IL10	IL12	IL17α	IFNγ	TNFα	GM-CSF
D	IL1α	IL1β	IL2	IL4	IL6	IL8	IL10	IL12	IL17α	IFNγ	TNFα	GM-CSF
E	IL1α	IL1β	IL2	IL4	IL6	IL8	IL10	IL12	IL17α	IFNγ	TNFα	GM-CSF
F	IL1α	IL1β	IL2	IL4	IL6	IL8	IL10	IL12	IL17α	IFNγ	TNFα	GM-CSF
G	IL1α	IL1β	IL2	IL4	IL6	IL8	IL10	IL12	IL17α	IFNγ	TNFα	GM-CSF
H	IL1α	IL1β	IL2	IL4	IL6	IL8	IL10	IL12	IL17α	IFNγ	TNFα	GM-CSF

Assay procedure summary

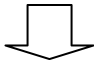
1. Samples and reagents are prepared
2.
3. Samples and reagent buffer is added to the wells on the microplate and incubated for two hours at room temperature



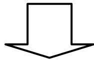
4. Wash the wells on the microplate 3 times



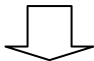
5. Add detection antibody solution to all wells and incubated for one hour at room temperature



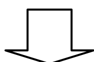
6. Wash the wells on the microplate 3 times



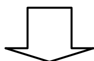
7. Add avidin-HRP to all wells and incubated for 30 min in the dark at room temperature



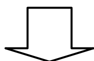
8. Wash the wells of the microplate 4 times



9. Add development solution to all wells and incubate for 15 min in the dark at room temperature



10. Add stop solution to all wells



11. Read microplate at 450 nm within 30 minutes

QUANTITATIVE DETERMINATION OF IL-1 , IL-10, TNF- AND GM-CSF

Quantitative sandwich enzyme immunoassay kits (Quantikine®, R&D Systems) specific for each of the cytokines, IL-1β (DLB50), IL-10 (D1000B), TNF-α (DTA00C) and GM-CSF (DGM00) where used for the quantitative determination. This quantitative determination analysis was done on two groups: mixed supernatants from males (n = 5) and mixed supernatants from females (n = 6).

Detection limits:

IL-1β	8.0 pg/mL
IL-10	3.9 pg/mL
TNF-α	1.6 pg/mL
GM-CSF	3 pg/mL

Minimum detectable dose is determined by adding two standard deviations to the mean optical density value of twenty zero standard replicates and calculating the corresponding concentration, according to the manufacturer.

Principle of the assay

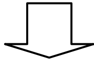
A monoclonal/polyclonal antibody is specific for the cytokine is pre-coated to a microplate. Samples and standards are pipetted into the wells (Table 2) and any cytokines present is bound to the antibody. Unbound substances are washed away and enzyme-linked polyclonal antibody specific for the cytokine is added. After washing away unbound substances a substrate solution is added and color develops in proportion to the amount of cytokine bound in the initial step. The color development is stopped and the intensity of the color is measured.

Table 2. Microplate setup for Quantikine® kits. Std = standard. Columns 4-6 are male samples, columns 7-9 are female samples.

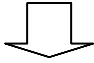
	1	2	3	4	5	6	7	8	9	10
A	Std	Std	Control	Control	LPS+RNA	LPS+RNA	Control	Control	LPS+RNA	LPS+RNA
B	Std	Std	RNA	RNA	PHA+RNA+ SB1	PHA+RNA+ SB1	RNA	RNA	PHA+RNA+ SB1	PHA+RNA+ SB1
C	Std	Std	SB1	SB1	PHA+RNA+ SB2	PHA+RNA+ SB2	SB1	SB1	PHA+RNA+ SB2	PHA+RNA+ SB2
D	Std	Std	RNA+ SB1	RNA+SB1	LPS+RNA+ SB1	LPS+RNA+ SB1	RNA+SB1	RNA+SB1	LPS+RNA+ SB1	LPS+RNA+ SB1
E	Std	Std	RNA+ SB2	RNA+SB2	LPS+RNA+ SB2	LPS+RNA+ SB2	RNA+SB2	RNA+SB2	LPS+RNA+ SB2	LPS+RNA+ SB2
F	Std	Std	PHA	PHA	DMSO1	DMSO1	PHA	PHA		
G	Std	Std	LPS	LPS	DMSO2	DMSO2	LPS	LPS		
H	Std	Std	PHA+ RNA	PHA+RNA			PHA+RNA	PHA+RNA		

Assay procedure summary

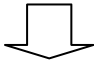
1. Preparation of reagents and standards.



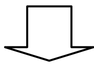
2 Add assay diluents to each well.



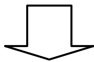
3 Add standard, sample or control to each well within 15 minutes. Incubate for 2 hours at room temperature.



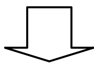
4 Aspire and wash wells.



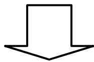
5 Add conjugate to each well, incubate for 1 or 2 hours at room temperature



6 Aspire and wash wells.

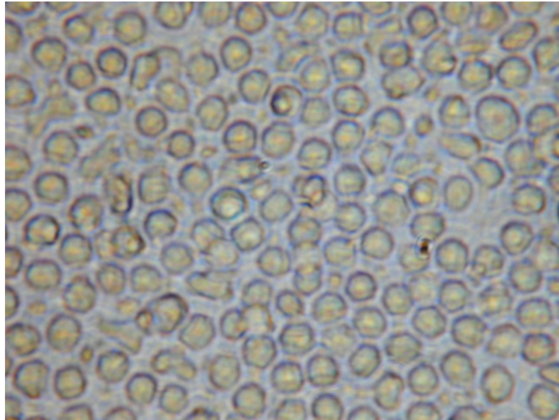


7 Add substrate solution to each well, incubate for 20 or 30 minutes at room temperature, protected from light.

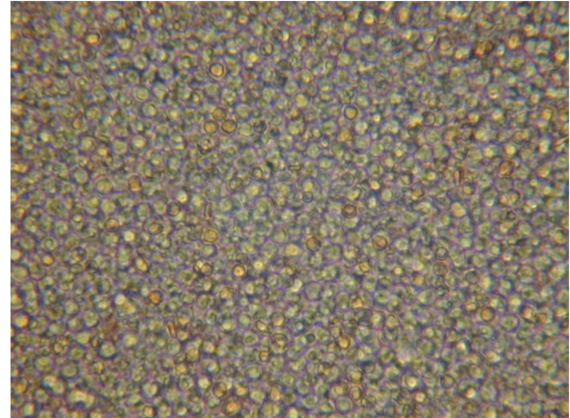


8 Add stop solution to each well, read at 450 nm (correction 540 nm or 570 nm) within 30 minutes.

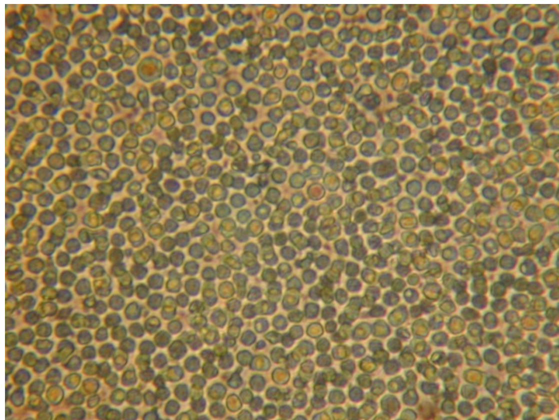
CELL CULTURE PICTURES



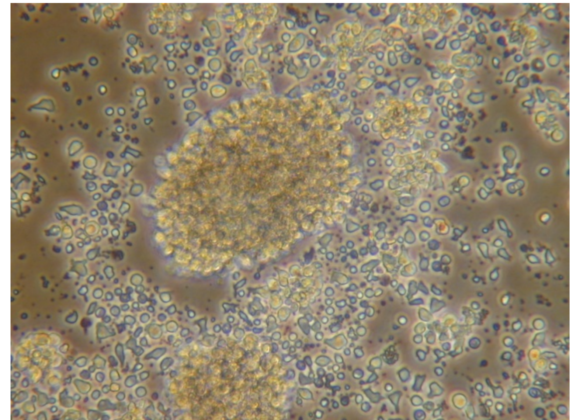
1. Control x400



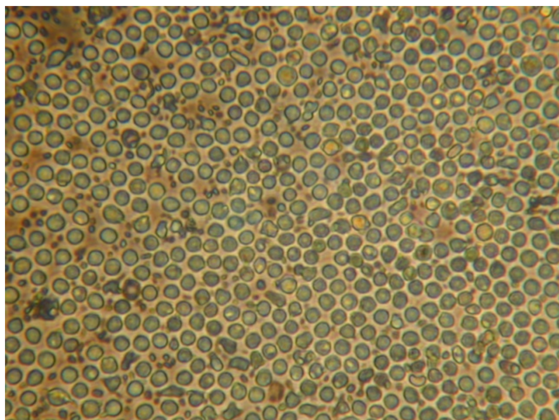
2. SB1 x200



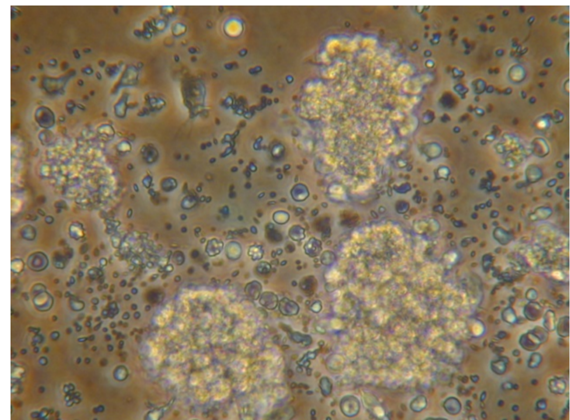
3. RNA x200



4. PHA x100



5. LPS+RNA x200



6. PHA+RNA+SB2 x200

Figure 3. Examples of cell culture treatments; 1) Untreated control , 2) Treated with 5 μM SB202190 3) treated with RNA 4) treated with PHA 5) treated with LPS and RNA 6) treated with PHA, RNA and 1 μM SB202190. Pictures were taken on second day of incubation.

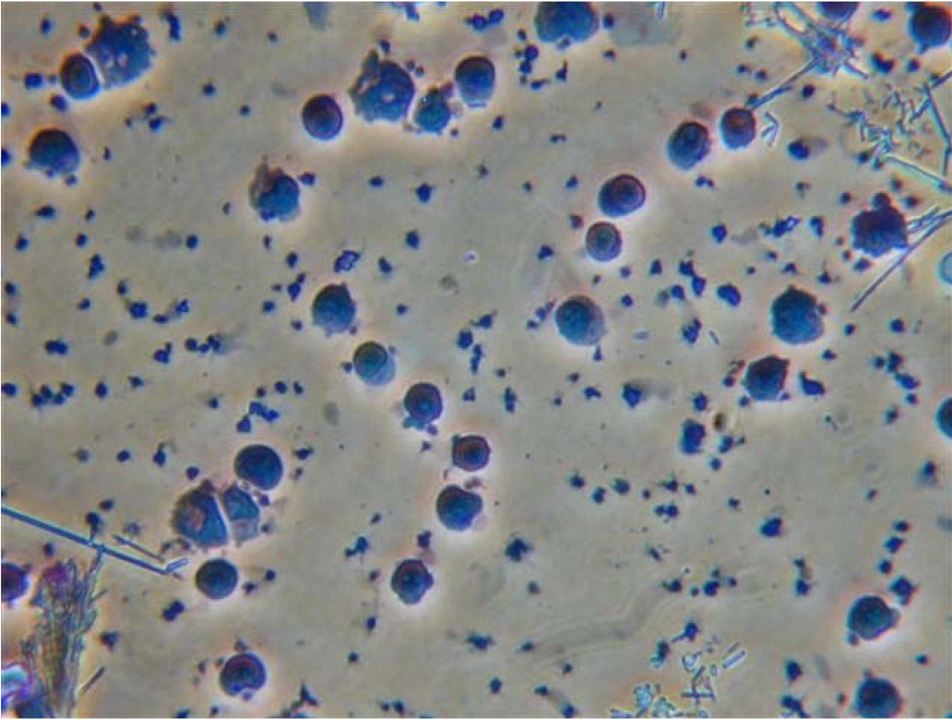


Figure 4. Peripheral blood mononuclear cells stained with Giemsa.

CYTOKINE SCREENING RESULTS

The results from the cytokine profiling are in given as optical density only (table 3); the concentration of the positive controls was 1000 pg/mL.

Table 3. Optical density (450 nm) results of cytokine screening with ELISArray® kits. The negative control (not shown) has been subtracted from all values. The last row shows positive controls (1 ng/µL). The cytokines that were selected for further analysis are in black text. Red numbers indicates values higher than positive control or 2.5.

Supernatants:	IL1A	**IL1B**	IL2	IL4	IL6	IL8	**IL10**	IL12	IL17α	**IFNγ**	**TNFα**	**GM-CSF**
Control	0,082	0,034	0,000	0,001	3,443	1,872	0,229	0,004	0,003	0,025	0,037	0,013
RNA (n=2)	0,223	0,139	0,000	0,000	3,737	4,169	0,456	0,015	0,000	0,000	0,240	0,121
SB1	0,035	0,010	0,000	0,007	2,994	1,475	0,175	0,007	0,022	1,133	0,101	0,024
RNA+SB1 (n=2)	0,008	0,000	0,000	0,001	0,908	4,085	0,020	0,006	0,000	0,004	0,023	0,007
RNA+SB2	0,026	0,000	0,000	0,003				0,010	0,000	0,000	0,028	0,022
PHA	0,293	0,215	0,000	0,003	4,090	1,962	0,760	0,024	0,089	3,243	0,634	0,076
PHA+SB1	0,051	0,016	0,204	0,011	2,854	1,772	0,090	0,011	0,018	1,750	0,481	0,042
RNA+PHA	0,299	0,387	0,011	0,005	4,167	4,322	0,847	0,010	0,108	1,945	0,735	0,286
RNA+PHA+SB1	0,011	0,060	0,068	0,003	2,715	4,087	0,067	0,003	0,011	0,593	0,244	0,036
LPS	0,323	0,233	0,000	0,005	4,183	1,866	0,775	0,025	0,019	0,040	0,444	0,094
LPS+SB1	0,038	0,026	0,000	0,000	3,877	1,735	0,124	0,018	0,011	0,009	0,097	0,041
RNA+LPS (n=2)	0,398	0,287	0,000	0,000	3,913	3,341	0,666	0,010	0,000	0,008	0,335	0,156
RNA+LPS+SB1 (n=2)	0,075	0,049	0,000	0,003	3,624	3,827	0,106	0,009	0,001	0,006	0,066	0,082
RNA+LPS+SB2	0,071	0,000	0,000	0,013				0,022	0,000	0,017	0,134	0,057
Pos. cont. (n=3)	2,133	1,476	0,857	0,931	1,302	1,232	1,585	1,942	0,940	1,474	1,996	0,611

Table 3 shows that there was no induction of IL-2, IL-4, IL-12 or IL-17α secretion by any of the treatments, and these cytokines will not be discussed further.

RNA (500 μg/mL), LPS (1 μg/mL) and PHA (5 μg/mL) induced secretion of IL-1α, IL-1β, IL-6, IL-8, IL-10, TNFα and GM-CSF. There seems to be a small amount of IFN-γ and TNF-α secretion induced by the p38 inhibitor alone. Secretion induced by RNA was inhibited by SB in all cytokines except IL-8. Secretion induced by PHA was inhibited by SB in all cytokines; the reduction was modest for IL-8. Secretion induced by LPS was inhibited by SB in all cytokines; the reduction was modest for IL-8.

After contacting SABioscience™ about the high IL-6 results, more than twice the positive control, we were told that IL-6 antibody became unstable after three months, and that they were reconsidering the expiry date of the kits. For this reason IL-6 were not considered for further analysis, also IL-8 were not chosen for further analysis because it was not substantially inhibited by SB and had inconsistent and high results, far above the positive control. Also IFN-γ gave some inconsistent results. These results were equal in three different kits and showed little interkit variation. The cytokines chosen for further analysis were IL-1β, IL-10, TNF-α and GM-CSF, the reason being that these, with the exception of IFN-γ, had responses within the range of the positive standards.

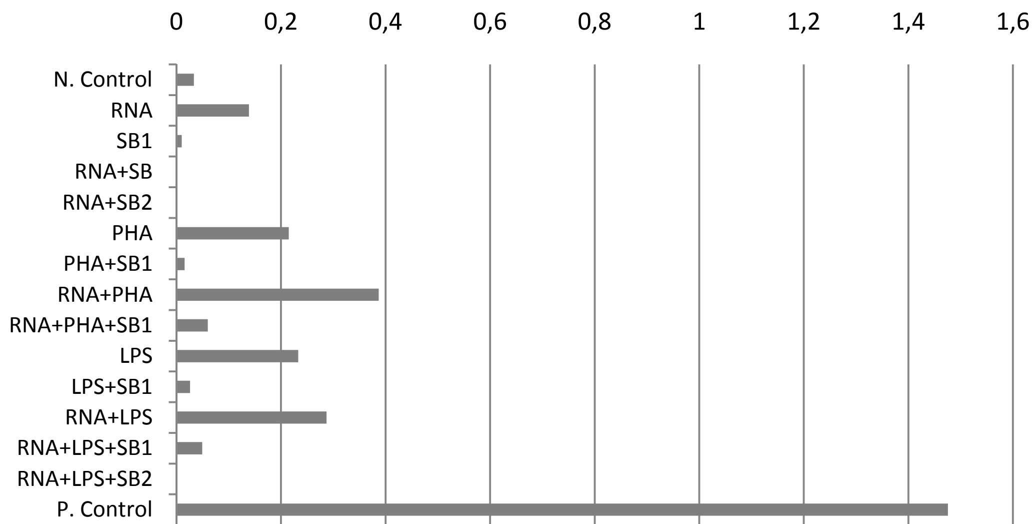


Figure 5. Optical density (450 nm) results for IL-1β screening with ELISArray kit, negative control have been subtracted from all values, n=11.

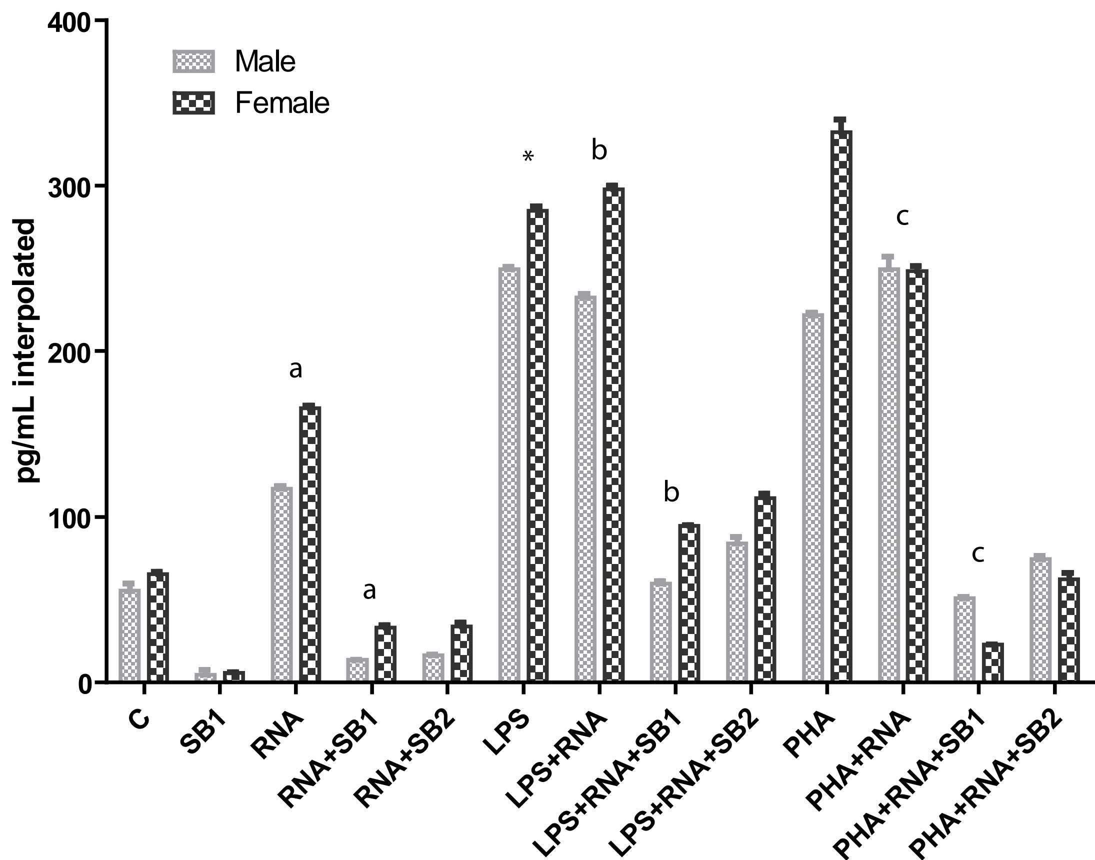


Figure 6. IL-1β concentrations (interpolated) for male (n=5) and female (n=6) subjects. Asterixes (*) indicate significant difference from control ($p<0.05$), letters (a, b, c) indicate significant difference between treatment groups ($p<0.05$). Error bars are difference in two parallel analyses. High standard concentration was 250 pg/mL.

RNA (500 µg/ml), LPS (1 µg/ml) and PHA (5 µg/ml) increased secretion of IL-1β compared to controls (fig 5, 6). LPS and PHA induced the highest IL-1beta concentration of 250-285 pg/mL and 222-333 pg/mL respectively. RNA induced IL-1β in the range 117-166 pg/mL.

The LPS and PHA induced IL-1β secretion was not increased by RNA. The RNA, PHA and LPS induced secretion of IL-1β was significantly decreased by the MAPK p38 inhibitor SB202190. There seemed to be a small spontaneous IL-1β secretion in the control (56-66 pg/mL). Females seemed to have a higher cytokine secretion than males.

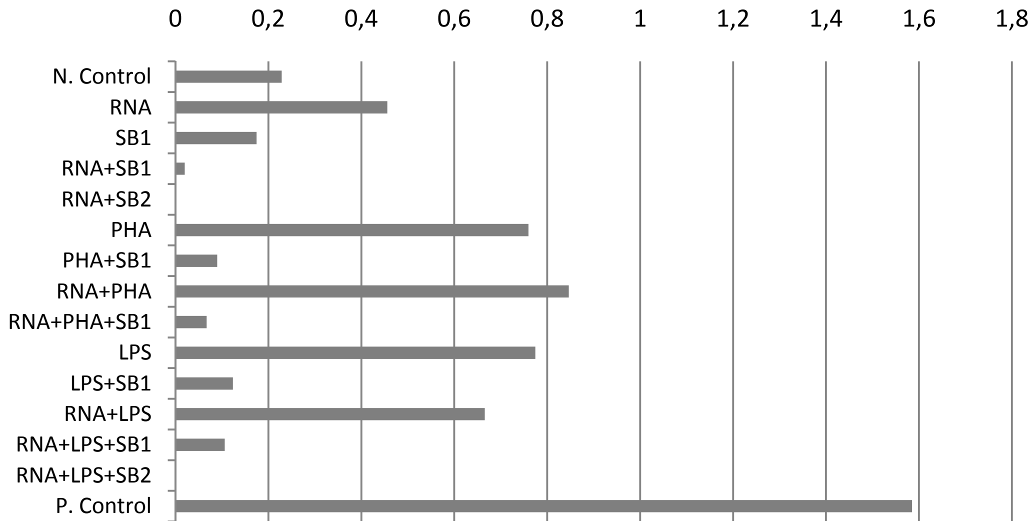


Figure 7. Optical density (450 nm) results for IL-10 screening with ELISArray kit, negative control have been subtracted from all values, n=11.

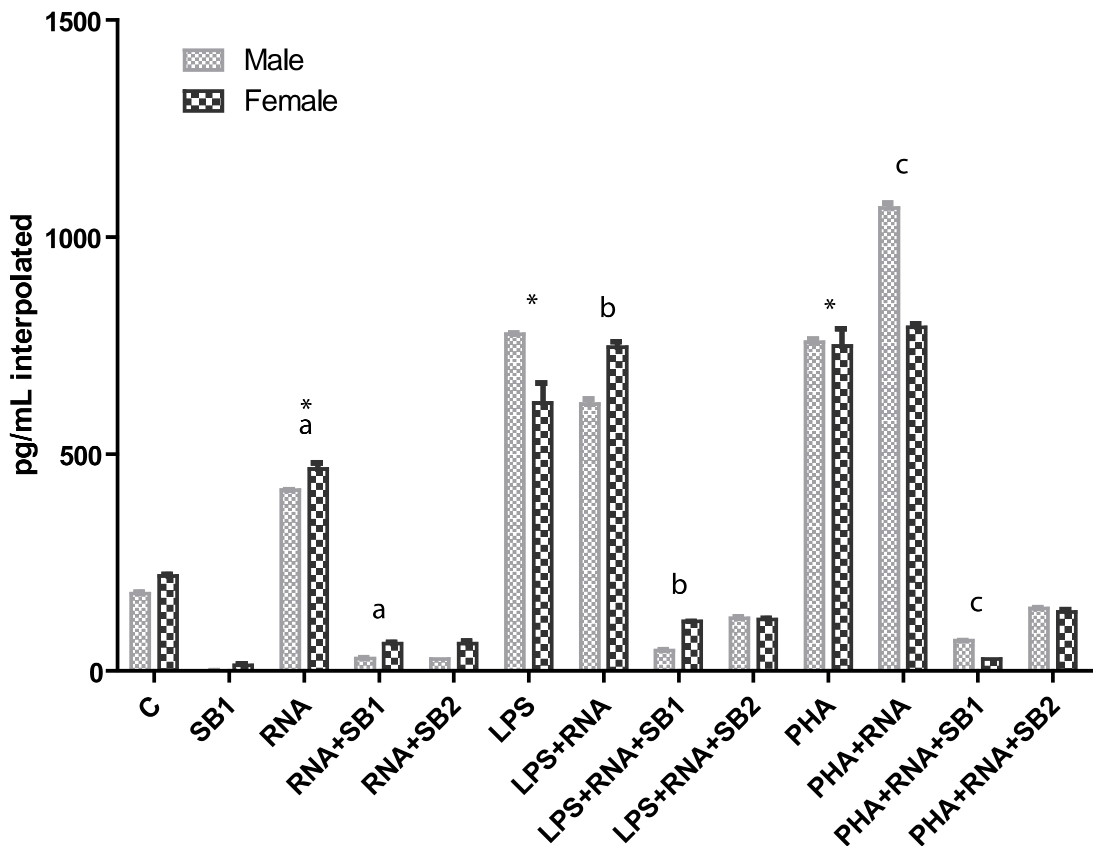


Figure 8. IL-10 concentrations (interpolated) for male (n=5) and female (n=6) subjects. Asterixes (*) indicate significant difference from control ($p<0.05$), letters (a, b, c) indicate significant difference between treatment groups ($p<0.05$). Error bars are difference in two parallel analyses. High standard concentration was 500 pg/mL.

RNA (500 µg/ml), LPS (1 µg/ml) and PHA (5 µg/ml) increased secretion of IL-10 compared to controls (fig 7, 8). PHA and PHA+LPS induced the highest IL-10 secretion, with the concentrations 715-724 pg/mL and 756-1010 respectively. RNA induced IL-10 in the range 404-451 pg/mL.

There are no apparent difference between the treatments LPS and LPS+RNA, or between treatments PHA and PAH+RNA. The RNA, PHA and LPS induced secretion of IL-10 was substantially decreased by SB202190. Spontaneous IL-10 secretion in the control group was in the range 178-215 pg/mL.

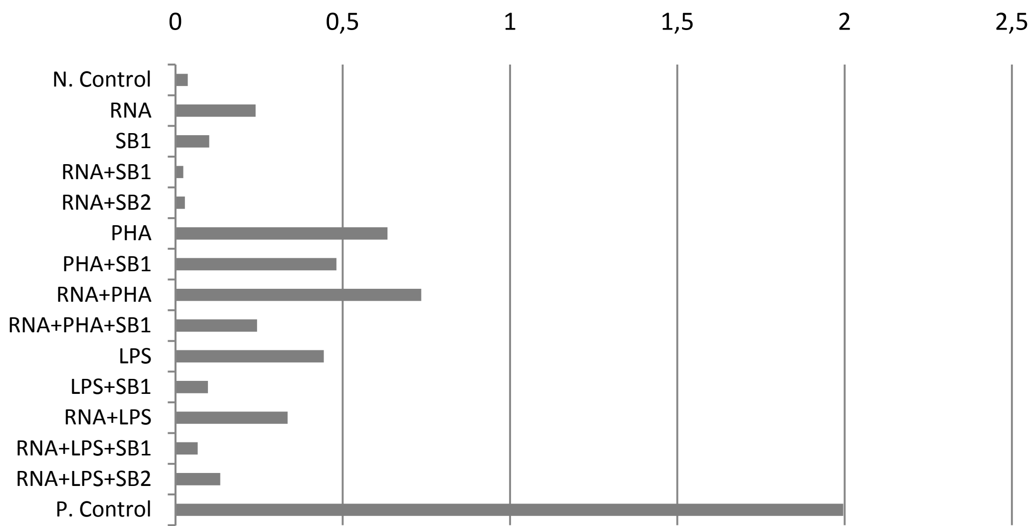


Figure 9. TNFα screening results (n=11) given as optical density at 450 nm, negative control have been subtracted from all values.

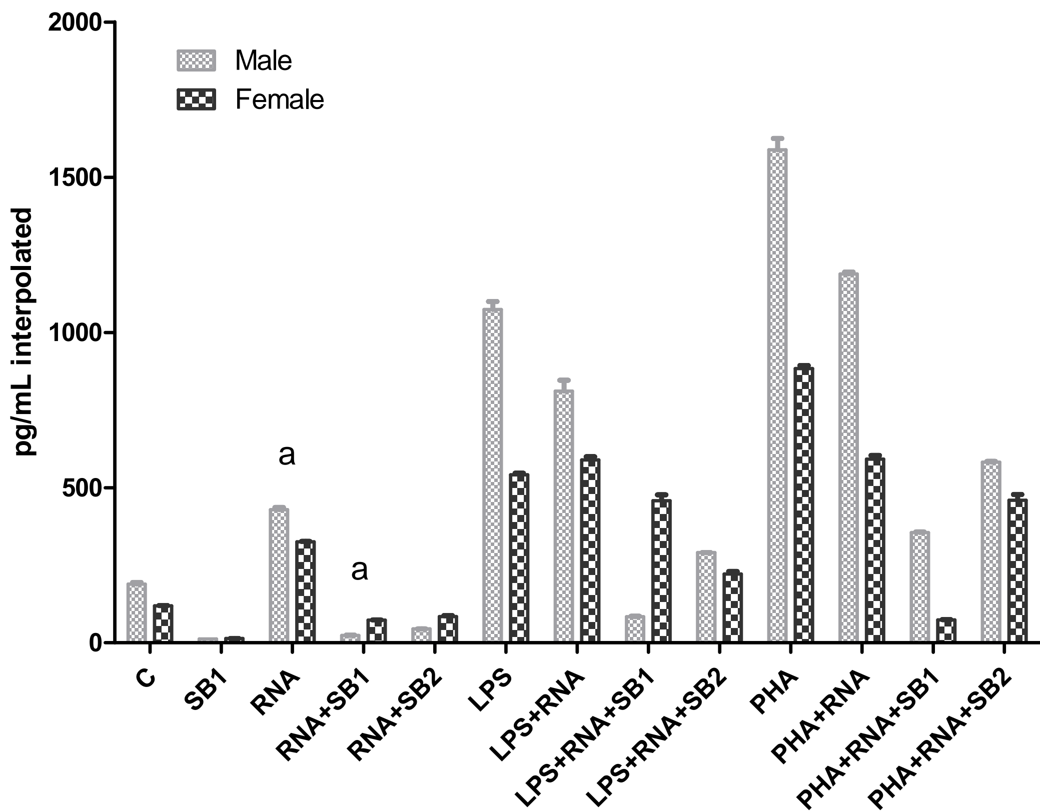


Figure 10. TNF-α concentrations (interpolated) for male (n=5) and female (n=6) subjects. Letters (a) indicate significant difference between treatments groups ($p<0.05$). Error bars are difference in two parallel analyses. High standard concentration was 1000 pg/mL.

RNA (500 μg/ml), LPS (1 μg/ml) and PHA (5 μg/ml) increased secretion of TNF-α compared to controls (fig 9, 10). PHA and PHA + RNA induced the highest IL-10 secretion of 883-1589 pg/mL and 592-1188 pg/mL respectively. RNA induced IL-10 in the range 325-429 pg/mL.

RNA did not increase the LPS induced TNF-α secretion, and there was a decrease in secretion with PHA+RNA. RNA but not PHA and LPS induced secretion of TNF-α was significantly decreased by SB202190. Spontaneous TNF-α secretion in the control group was in the range 119-189 pg/mL. The TNF-α secretion is almost consistently higher in male samples, especially with RNA, PHA and LPS stimulation.

PBMC secretion of GM-CSF

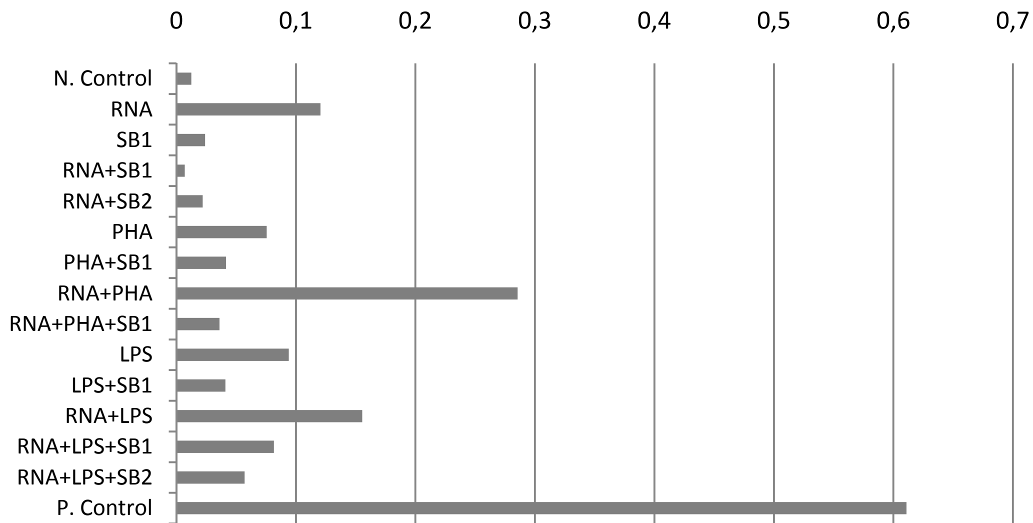


Figure 11. Optical density (450 nm) results for GM-CSF screening with ELISArray kit, negative control have been subtracted from all values, n=11.

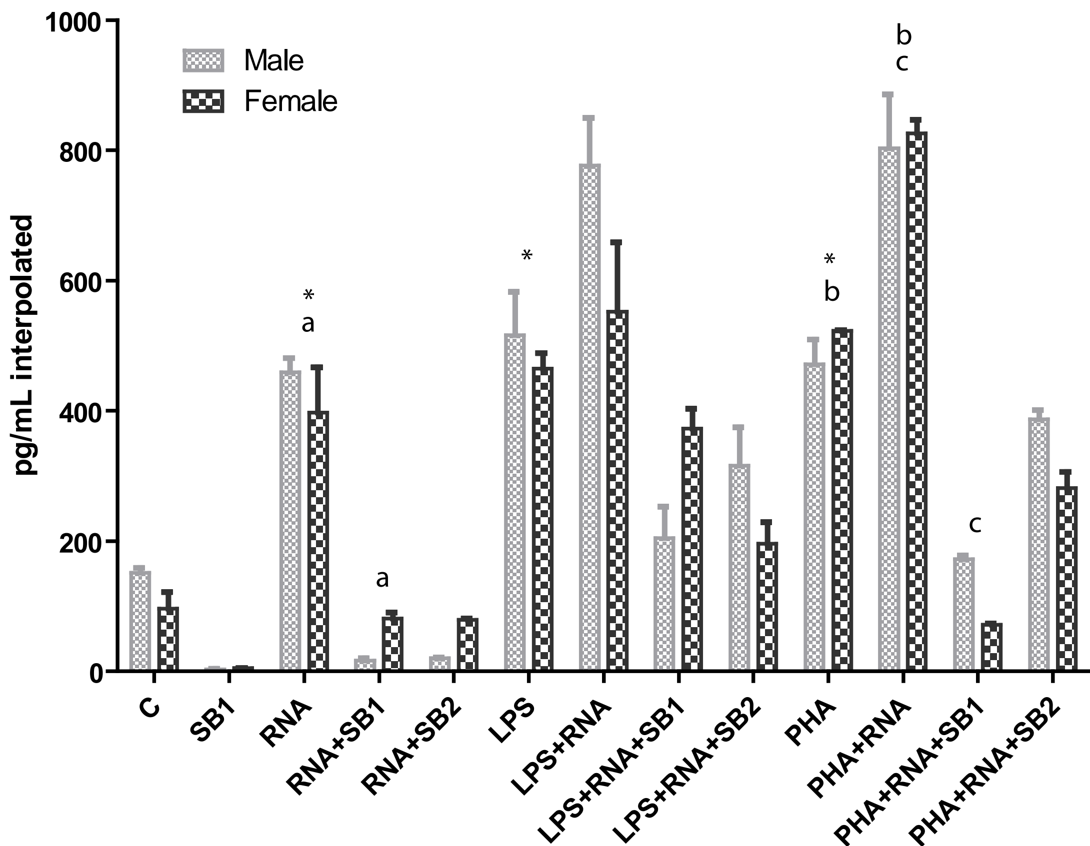


Figure 12. GM-CSF concentrations (interpolated) for male (n=5) and female (n=6) subjects. Asterixes (*) indicate significant difference from control ($p<0.05$), letters (a, b, c) indicate significant difference between treatment groups ($p<0.05$). Error bars are difference in two parallel analyses. High standard concentration was 500 pg/mL.

RNA (500 μg/ml), LPS (1 μg/ml) and PHA (5 μg/ml) increased secretion of GM-CSF compared to controls (fig 11, 12). PHA+RNA and LPS+RNA induced the highest GM-CSF secretion of 759-827 pg/mL and 550-741 pg/mL respectively. RNA induced GM-CSF in the range of 396-459 pg/mL. RNA significantly increased PHA induced GM-CSF secretion. The RNA, PHA and LPS induced secretion of GM-CSF was significantly decreased by SB202190. Spontaneous GM-CSF secretion in the control group was in the range of 97-151 pg/mL. There was a tendency for higher GM-CSF secretion in the male samples.

DISCUSSION

The purpose of my thesis was to study the effect on immune modulation of cytokines by dietary RNA in humans. RNA and nucleotides are thought to have beneficial effects on human health, by supporting the immune system during infections. As we wanted to challenge immune cells with LPS and PHA, the only feasible way to do this was by culturing of peripheral blood mononuclear cells. *In vivo* experiments with LPS or PHA challenge is not allowed due to ethical considerations. Usage of human materials for research must be approved by an ethical committee (REK-vest), criteria for approval are among others, informed consent by subjects and that the research gives relevant/useful information and knowledge. The process of writing an application for approval can be advantageous because it requires the project to be well planned and have defined goals.

DISCUSSION OF METHODS

There are many benefits of using PBMC cultures; it is easily obtained and isolated with established methods and it is widely used for investigating the interplay between various immune cells. When investigating mechanisms of immune cells, it is easier to control variables *in vitro* than *in vivo*, where many unknown and uncontrollable variables are at play, also to test various toxic substances would be unethical in other ways than cell culture experiments. The use of Giemsa staining and an inverse microscope are useful for confirming the presence of leukocytes and to control for infection and take pictures of the cultured cells.

Sources of error include infections of the cultures due to inadequate sterile technique, inaccurate pipetting when stimulants are added, and the chance of substances added to the wrong wells. The counting of cells with Bürker chamber can be a source of error since this is a subjective method, the use of an exact standardized procedure is important, and ideally the isolated cells should have been counted by two persons.

The method used for detecting cytokines was enzyme-linked immunosorbent assay (ELISA), more specific a solid phase heterogeneous sandwich immunoassay, with colorimetric based reading. Premade optimized kits, with all necessary reactants included, makes this a quick, easy and convenient though somewhat expensive option. Alternatively a chemiluminescent ELISA could have been used, as this has a broader dynamic range than standard colorimetric ELISAs. Other alternatives could have been ELISpot or PCR. ELISPOT is based on capturing cells in an antibody matrix, and measures the number of secreting cells and gives an indirect impression of the amount of secreted cytokines. An important difference of this method from ELISA, in that it measures only one specific cell type. This method has a very high sensitivity, this can be an advantage for some cytokines that are hard to measure, e.g. IL-4 (Díaz and Mateu 2005), and also, only a low number of cells are required.

PCR is also a very sensitive method and as ELISpot, it requires only a small amount of cells. However, this is a more laborious method, and also requires more adjustments to get optimal results. And the results do not necessarily reflect the amount of cytokines secreted (Ekerfelt, Ernerudh et al. 2002).

Our approach was to use a multi array kit for a screening, and then to further quantify selected cytokines using cytokine-specific kits, where quantification is done by extrapolating from a standard curve made from known concentrations. The screening kits are a very convenient and quick way to get an overview of the cytokines present in a sample. However, the concentration of the different cytokines in a sample may vary, some may be below the detection limit while others are above the positive samples, thus requiring different dilutions for the various cytokines. This makes a quick screening inaccurate for some cytokines. Also, this method may not be sensitive enough or give an incorrect impression of some of the cytokines that are very hard to detect such as IL-2 and IL-4 (Ekerfelt, Ernerudh et al. 2002). The kits also had some problems, and according to the manufacturer the IL-6 antibodies become unstable after some time, although the kits we used were not past the expiry date, they were apparently too old for an accurate detection of at least this cytokine.

Statistics

The major limitation in this study was the cost of the ELISA kits used. As we wanted to analyze several cytokines, we chose to pool supernatants from several individuals for each ELISA kit, this limited the possibility for finding statistical significance, but nevertheless the results show clear tendencies for the different treatments. Still I chose to test difference in means between treatments with the pooled results from female and male groups by using t-test. These results cannot be interpreted as significant differences between various treatments of the eleven subjects I sampled, but is rather a test for difference assuming interpersonal variation is low or no larger than the variation between the pooled gender groups. I find that this method is justified by the goal of the study; to find how RNA affected cytokine secretion relative to the comprehensively studied stimulants we used. Studies have showed large individual variations in cytokine secretion from human PBMC (Louise 1998, Grimble 2002), due to this potentially large variance, statistically significant results might not have been achieved with individual analysis anyway, considering the small sample size we used (n=11). Although the possibly large inter-subject variations in the PBMCs response to stimuli, we used a relatively homogenous group regarding age, health and socioeconomic status, and as the individual differences will be averaged by the sample pooling, we assumed that this would give a reasonable representation of average cytokine secretion for healthy individuals. It is known that sex hormones affect the immune system, so to eliminate the influence of gender on the variance, samples were pooled in two groups, females (n=6) and males (n=5). We assumed that if differences could be observed in this small sample of healthy individuals, these differences would be significant in later studies with immunodeficient patient groups.

The results show that for most of the cytokines and stimuli there is relatively little difference in secretion between females and males. This may be taken as an indication that there are no major deviations in the cytokine secretion for the sampled individuals.

DISCUSSION OF RESULTS

In this study we found that RNA from baker's yeast, alone, induces cytokine secretion in PBMC isolated from healthy individuals. From the multi array kit, RNA, LPS and PHA induced IL-1α, IL-1β, IL-6, IL-8, IL-10, TNF-α and GM-CSF. In contrast, IL-2, IL-4, IL-12 and IL 17α were not induced in detectable amounts. RNA alone did not induce any IFN-γ secretion. Especially PHA, but also LPS, induced significantly amounts of IFN-γ. Previously, Holen et al. (2005) showed that TNF-α, IL-10 and IFN-γ secretion were increased in healthy PBMCs co-cultured with RNA and virus antigen. Interestingly the p38 MAPK inhibitor alone induced significantly amounts of IL-1β and TNF-α, the same is observed in salmon head kidney cell cultures (Holen, personal communication). The p38 MAPK may be endogenously regulated, so that inhibition leads to upregulation/activation and activation leads to downregulation, in an attempt to control signaling. Interestingly, RNA but not PHA and LPS, seemed to induce IL-8 secretion according to the multi array kit. The inhibitor did not influence this secretion, indicating that IL-8 secretion is not initiated via the p38 MAPK signaling cascade. IL-8 is a chemokine secreted by phagocytes and many other cell types in response to inflammatory stimuli from viruses, bacteria and pro-inflammatory cytokines among others. It plays a role as a chemoattractant guiding leukocytes to the source of infection or cellular stress. RNA may function as a cell-stress or pathogen danger signal causing the induction of IL-8.

Although RNA alone stimulated IL-1β, TNF-α and IL-10 secretion, RNA did not significantly increase the high LPS or PHA induced secretion of these cytokines. The inhibitor significantly reduced cytokine secretion, indicating the involvement of p38. Overall there was a higher IL-1β induction in the female cell culture, both similar and opposite findings related to gender differences have been reported (van Eijk, Dorresteijn et al. 2008). There was generally a higher TNF-α secretion in the male cell cultures. IL-10 inhibition was approximately equal in PHA and LPS treatments, to levels lower than the control. Both NF-kB and p38 pathways have been associated with IL-10 secretion, so the observed reduction could be caused directly by inhibiting p38 or by the reduced secretion of pro-inflammatory cytokines e.g. TNF-α (Asadullah, Sterry et al. 2003; Baker, Wang et al. 2009). SB inhibited TNF-α secretion slightly more when co-cultured with

LPS compared to when co-cultured with PHA. This could be explained by the findings that TNF-α release from T- cell stimulation is only partly dependent on p38 (Schafer, Wang et al. 1999).

IL-1β and TNF-α are typical pro-inflammatory cytokines and key mediators in innate immune response, both affecting several different cell types (Dinarello 1996; Schottelius, Moldawer et al. 2004). Bacteria, viruses, stress factors, polynucleotides, PHA and inflammatory cytokines are some of the inducers of IL-1β and TNF-α. Numerous cell types secrete IL-1β and TNF-α, including non-immune cells, but monocytes and macrophages are a primary source during inflammatory response (Schottelius, Moldawer et al. 2004). Both cytokines are highly inflammatory, and tightly regulated, inappropriate secretion may cause tissue destruction and organ injury. The stimulatory effect of RNA is possibly caused by a priming of the cells involved in the innate immune system causing them to secrete pro-inflammatory cytokines, perhaps similar to how DAMPs derived from destruction of host cell and tissue can activate innate immune cells and inflammatory response (Kono and Rock 2008).

When RNA was co-cultured with LPS or PHA it induced an increase in PBMC secretion of GM-CSF. The quantity of GM-CSF induced by RNA was comparable to that induced by PHA and LPS. GM-CSF is typically thought of as a growth factor for maturation of myeloid cells, but has also been shown to function as a pro-inflammatory cytokine with direct effects on multiple types of mature cells. Secretion is induced by inflammatory signals, such as IL-1, TNF-α or LPS, in several cell types, including macrophages, endothelial cells, smooth muscle cells and others (Hamilton 2008). The effects on macrophages, neutrophils and eosinophils include increased survival, proliferation, differentiation and activation/priming. Of the mature cell types, macrophages are one of the main targets, from which populations with antigen presenting and dendritic cell-like capabilities are generated. With a second stimulus such as LPS, GM-CSF typically primes macrophages and monocytes to become activated, show an increased response to stimuli and secrete the pro-inflammatory cytokines TNF, IL-6, IL-12 and IL-23 (Hamilton 2008).

These different effects of GM-CSF could explain its increased secretion when co-cultured with LPS and PHA; when LPS or PHA are present, more macrophages/monocytes are primed to secrete pro-inflammatory cytokines as IL-1β and TNF-α which again increase the secretion of GM-CSF. This general activation of innate immune response by GM-CSF may be the reason it is induced in relatively high quantities; RNA may function by triggering a general danger signal inducing secretion followed by activation of other cell types for a more specific response. The inhibitor reduced secretion of this cytokine, but mostly the RNA induced secretion, suggesting that p38 is less involved in LPS and PHA induced secretion of GM-CSF.

Such non-specific activations or priming of cells to a pro-inflammatory state could be useful in enhancing infection resistance or enhancing vaccines as with the use of CpG oligodeoxynucleotides (Klinman 2004).

The pyrinidyl imidazole compound SB 202190 selectively prevents activation downstream of p38 MAPK, and we found that it inhibited secretion by RNA in particular but also by LPS and PHA. This indicates that RNA, at least partly, signals through p38 MAPK pathway. The inhibitory effect of SB on cytokine secretion varied with the strength of stimuli the cells were exposed to; the stimuli that induced high secretion, such as PHA and LPS, and these in combination with RNA, were also the least inhibited. The concentration at which the inhibitor was used (5 μM) should completely block p38 (Schafer, Wang et al. 1999) This indicates that PHA and LPS also signals through other pathways than p38 MAPK

In most of the cell cultures added SB 202190 alone; the cytokine secretion was almost completely inhibited indicating that in our cultures SB did not activate the secretion of the measured selected cytokines. Both p38 MAPK and extra cellular signal-regulated protein kinase (ERK) pathways are activated by LPS or T-cell receptor stimulation activation (Morley 1997, Carter et al. 1999). Thus, the secretion observed in inhibitor treated samples is likely the result of activation of other pathways than p38, or that the

p38 MAPK is endogenously up-regulated as mentioned before. Interestingly, it has been reported that the inhibition of p38 by SB202190 activates c-Jun N-terminal kinase (JNK) and that this effect apparently not is caused by compensation of reduced p38 (Muniyappa and Das 2008). If this is correct it obscures attempts to dissect p38 pathways, as results from the use of these inhibitors could be attributed to either inhibition of p38 or activation of JNK. However, any activation of JNK by the inhibitor should also result in cytokine secretion, not observed in particularly large amounts in the cytokines tested.

Even though RNA stimulated secretion was the treatment most inhibited by SB, RNA and SB co-treatment still induced some cytokine secretion, compared to SB alone. This indicates that the cytokine inducing effect of RNA is partly dependent on activation of the p38 MAPK pathway. Additionally, the fact that SB didn't completely inhibit RNA induced cytokine secretion, as when it was used alone, is an indication that the effect of RNA on cytokine secretion is a receptor mediated event, as opposed to a need for exogenous nucleotides for salvage synthesis.

If the effect of RNA was caused by fueling the salvage pathway, I would suspect the cytokine secretion in RNA treatment to be inhibited to the same degree as when SB was used alone. The means by which RNA activates p38 is uncertain, some TLRs activate p38, but the signal transduction pathways of these receptors are complex and still not fully understood. Yeast such as *Saccharomyces cerevisiae* are an important source of nucleotides. But the lack of processing procedures or detailed information about the product makes it difficult to determine if the nucleotides are present in the form of mononucleotides, polynucleotides or short fragments, or whether it contains methylated CpG motifs, which may be important for finding which receptors are involved with RNA stimulation.

Previous studies have found the stimulating effect of nucleotides or nucleic acids mostly with T-cell dependent antigen (Jyonouchi 1994; Nagafuchi 2007). Jyonouchi (1994) showed that when murine spleen cells primed with T-cell dependent antigen where stimulated with RNA, the number of antibody producing cells increased. Antibody producing cells stimulated with T-cell independent antigen did not increase with RNA. When B-cells were stimulated with LPS no effect of RNA was found. The stimulating effect is attributed to polyribonucleotides (RNA), when treated with ribonuclease the effect was nullified, the same when removing small oligonucleotides or chemically modifying or degrading the RNA. In contrast, Holen et al. (2005) found a stimulatory effect of both nucleotides and RNA from baker's yeast on human PBMC.

Jyonouchi (1994) also reports that mice fed a nucleotide free diet had a decreased specific antibody response to intra peritoneal priming with T-cell dependent antigens. The response to T-cell independent antigen and LPS was not affected by nucleotide free diet. The immune response was restored after intra peritoneal administration of mononucleotide-nucleoside mixture. The same mononucleotides had no effect in vitro and no effect in normal fed mice. The difference between *in vitro* and *in vivo* study results may be explained by a local and brief activation by polynucleotides at the site of tissue injury or inflammation, i.e. danger hypothesis.

In a recent review Nagafuchi (2007) reports that mice fed nucleotide supplemented diet had lower serum IgE levels and lower IgG1:IgG2a ratio. Also, spleenic lymphocytes from the mice fed nucleotide supplemented diet had increased IFN-γ and lower IL-4 production in response to ovalbumin (T-cell dependent) stimulation. These results suggest that dietary nucleotides may, through enhancing IL-12 production by macrophages, up-regulate the antigen specific Th1 response and reduce the IgE response.

As in animal studies, polyribonucleotides (RNA) have been found to increase immunoglobulins in response to T-cell dependent stimuli in both human PBMC and human umbilical cord blood mononuclear cells *in vitro* (Jyonouchi 1994). There was an increase in IgG and IgM in PBMC and an increase of IgM from cord blood mononuclear cells. Brief RNA incubation of T-cells resulted in a nonspecific activation, which was suppressed when T-cells were allowed to interact with non-T-cells, similar to the results from animal studies.

The findings of the present study add to the reputed role of RNA in modulating both innate and adaptive immunity. RNA itself approximately doubled cytokine secretion compared to control of all the investigated cytokines except of IFN-γ, probably as a result of priming the innate first defence immune cells. The pro-inflammatory cytokine secretion effect was most prominent with the T-cell dependent stimuli, and the effect is probably through interacting with T-cells as suggested by others (Jyonouchi, Sun et al. 2001).

The lack of IL-12 response in my experiment is incongruent with previous results, also the irregular IFN-γ response do not fit the suggested Th1 activation by nucleotides. This may be attributed to the fact that I used RNA as opposed to nucleotides, and may indicate a difference in activation pathway between nucleotides/nucleosides and oligonucleotides.

The importance of nucleotides in infant nutrition is already well established (Schaller, Buck et al. 2007). The means by which nucleotides and nucleic acids modulate immune responses is still not fully understood. Although this was an *in vitro* study, the effect of nucleotides and nucleic acids on immune cells *in vitro* and in feeding studies have similarities, even though nucleotides and nucleic acids are extensively metabolized in the intestine (Jyonouchi, Sun et al. 2001).

Perhaps three models of immune modulation by nucleotides and nucleic acids can be proposed; 1) by changing the environment of nucleic acids and nucleotides intestinal DCs are exposed to thus altering their ability to maintain immune homeostasis. Nucleotides and nucleic acids are ubiquitous in human nutrition, and may have plaid a role equally important to the co-evolved relationship between commensal bacteria and the intestinal immune system. Upsetting this balance between immune tolerance and inflammation may activate immune cells in intestinal lymphoid tissue, maybe also affecting peripheral immune cells. 2) There is evidence that nucleotides play an important role in supplying enterocytes, which have a limited ability for *de novo* synthesis, nucleotide supplementation have inhibited intestinal bacterial translocation in protein deficient mice and reduced diarrheal incidence in infants (Adjei and Yamamoto 1995; Schaller, Buck et al. 2007). Nucleotides may thus be required for proper intestinal barrier function. 3) By acting as endogenous or exogenous danger signals, activating TLRs and immune cells nonspecifically by priming the innate immune system, or specifically depending on co-stimulatory signals, as observed with *in vitro* studies.

Further studies using PBMC from humans with autoimmune diseases or allergies could be of interest to further the knowledge of the immunostimulating properties of nucleotides and nucleic acids, and their role in Th1 – Th2 balance. There might be a role for dietary RNA in ameliorating allergic conditions. Clinical studies with oligonucleotides as vaccine adjuvants have been conducted (Klinman 2004). Enteral and parenteral nutrition might benefit from nucleotides; also the inclusion of nucleotides could be beneficial in treating malnutrition related immune deficiencies in developing countries where sanitation and medical facilities may be inaccessible.

To sum up, we have found that RNA induces cytokine secretion from peripheral blood mononuclear cells from healthy humans. The effect of RNA seems most likely to be a priming of immune cells by activating them in a general manner. The mechanism behind this stimulatory effect is unknown, but is likely receptor mediated, and partly involving the p38 mitogen activated protein kinase signaling cascade. These results suggest that dietary RNA have an immunostimulatory effect which can be used to fight infections, stimulate immune function in compromised individuals, and potentially be used as vaccine adjuvant.

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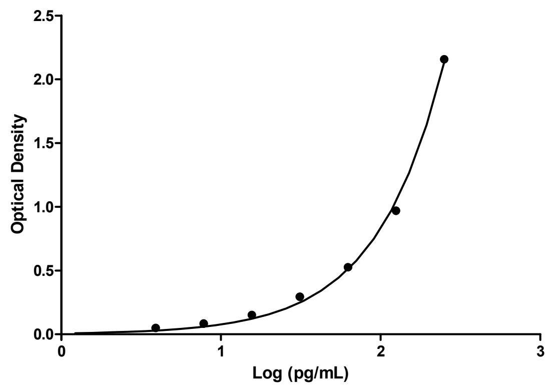
ABBREVIATIONS

BSA	Bovine serum albumin
GM-CSF	Granulocyte monocyte colony stimulating factor
IFN-γ	Interferone-γ
IL-1β	Interleukine-1β
IL-10	Interleukine-10
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinase
PCR	Polymerase chain reaction
PHA	Phytohemagglutinine
SB	SB202190
SB1	SB202190 in 5 μM concentration
SB2	SB202190 in 1 μM concentration
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-α

STANDARD CURVES FOR QUANTIKINE® KITS



Figur 13. Standard curve for IL-1β, concentration range 0-250 pg/mL.

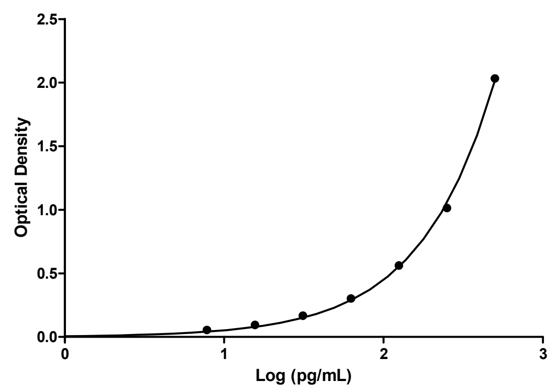


Figure 14. Standard curve for IL-10, concentration range 0-500 pg/mL.

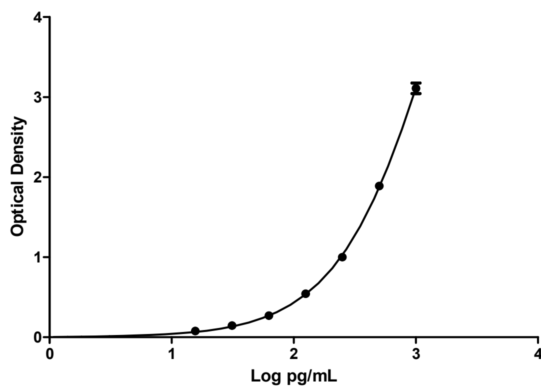


Figure 15. Standard curve for TNFα, concentration range 0-1000 pg/mL.

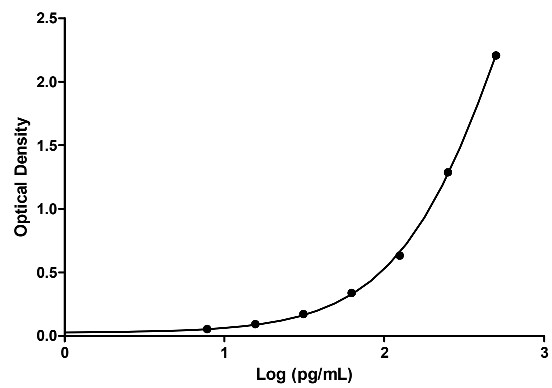


Figure 16. Standard curve for GM-CSF, concentration range 0-500 pg/mL.

T-TEST OF SIGNIFICANT DIFFERENCE IN MEANS BETWEEN FEMALE AND MALE GROUP RESULTS

Table 4. T-test for difference in mean between treatment groups.

IL-1b

	Significant different mean	p
C vs RNA:	no	0.084
C vs LPS	yes	0.008
C vs PHA	no	0.060
LPS vs LPS+RNA	no	0.960
PHA vs PHA+RNA	no	0.660
RNA vs RNA+SB1	yes	0.046
LPS+RNA vs LPS+RNA+SB1	yes	0.004
PHA+RNA vs PHA+RNA+SB1	yes	0.037

IL-10

	Significant different mean	p
C vs RNA:	yes	0.016
C vs LPS	yes	0.025
C vs PHA	yes	0.001
LPS vs LPS+RNA	no	0.890
PHA vs PHA+RNA	no	0.330
RNA vs RNA+SB1	yes	0.006
LPS+RNA vs LPS+RNA+SB1	yes	0.014
PHA+RNA vs PHA+RNA+SB1	yes	0.023

TNF-α

	Significant different mean	p
C vs RNA:	no	0.071
C vs LPS	no	0.093
C vs PHA	no	0.135
LPS vs LPS+RNA	no	0.738
PHA vs PHA+RNA	no	0.532
RNA vs RNA+SB1	yes	0.029
LPS+RNA vs LPS+RNA+SB1	no	0.054
PHA+RNA vs PHA+RNA+SB1	no	0.177

GM-CSF

	Significant different mean	p
C vs RNA:	yes	0.018
C vs LPS	yes	0.010
C vs PHA	yes	0.010
LPS vs LPS+RNA	no	0.267
PHA vs PHA+RNA	yes	0.008
RNA vs RNA+SB1	yes	0.014
LPS+RNA vs LPS+RNA+SB1	no	0.115
PHA+RNA vs PHA+RNA+SB1	yes	0.008

Table 5. Raw data from multiarray kits, values are optical density at 450 nm.

	IL-1A	IL-1B	IL-2	IL-4	IL-6	IL-8
Neg. control	0,223	0,126	0,056	0,044		
Neg. control	0,049	0,076	0,075	0,047	0,045	0,081
Neg. control	0,046	0,082	0,078	0,045	0,061	0,066
Control	0,128	0,112	0,059	0,046	3,497	1,936
RNA	0,302	0,187	0,060	0,045		
RNA	0,251	0,291	0,071	0,042	3,793	4,264
RNA+SB1	0,067	0,086	0,053	0,044		
RNA+SB1	0,055	0,085	0,071	0,050	0,952	4,101
RNA+SB2	0,084	0,087	0,052	0,047		
SB1	0,081	0,088	0,052	0,053	3,040	1,538
LPS	0,369	0,312	0,056	0,050	4,216	1,934
PHA	0,339	0,293	0,066	0,048	4,109	2,033
LPS+SB1	0,084	0,105	0,061	0,045	3,956	1,803
PHA+SB1	0,096	0,094	0,280	0,056	2,913	1,841
RNA+LPS	0,479	0,297	0,052	0,047		
RNA+LPS	0,422	0,478	0,064	0,045	3,947	3,424
RNA+LPS+SB1	0,119	0,114	0,058	0,054		
RNA+LPS+SB1	0,138	0,185	0,070	0,044	3,683	3,910
RNA+LPS+SB2	0,129	0,125	0,055	0,057		
RNA+PHA	0,349	0,462	0,086	0,056	4,227	4,495
RNA+PHA+SB1	0,060	0,135	0,142	0,050	2,776	4,204
Pos. control	2,112	1,231	0,852	1,340		
Pos. control	1,880	2,054	0,936	0,079	0,377	2,516
Pos. control	2,564	1,431	0,993	1,509	2,332	0,099

	IL-10	IL-12	IL17-	INF-γ	TNF-α	GM-CSF
Neg. control		0,042	0,057	0,053	0,059	0,045
Neg. control	0,049	0,044	0,047	0,047	0,075	0,045
Neg. control	0,047	0,052	0,051	0,052	0,065	0,057
Control	0,276	0,055	0,054	0,077	0,102	0,069
RNA		0,062	0,048	0,050	0,225	0,112
RNA	0,505	0,053	0,045	0,047	0,388	0,219
RNA+SB1		0,053	0,051	0,060	0,093	0,052
RNA+SB1	0,069	0,044	0,049	0,047	0,087	0,052
RNA+SB2		0,052	0,048	0,052	0,087	0,067
SB1	0,222	0,058	0,071	1,185	0,166	0,080
LPS	0,821	0,075	0,070	0,091	0,509	0,150
PHA	0,807	0,075	0,137	3,292	0,700	0,132
LPS+SB1	0,170	0,069	0,054	0,060	0,161	0,097
PHA+SB1	0,136	0,062	0,065	1,801	0,546	0,097
RNA+LPS		0,055	0,047	0,060	0,346	0,130
RNA+LPS	0,716	0,052	0,050	0,055	0,458	0,271
RNA+LPS+SB1		0,053	0,052	0,053	0,122	0,084
RNA+LPS+SB1	0,155	0,050	0,054	0,059	0,144	0,169
RNA+LPS+SB2		0,064	0,050	0,070	0,193	0,102
RNA+PHA	0,898	0,054	0,155	1,990	0,810	0,331
RNA+PHA+SB1	0,116	0,047	0,058	0,639	0,319	0,081
Pos. control		1,896	1,151	1,404	1,552	0,553
Pos. control	1,663	1,598	0,724	1,404	2,445	0,844
Pos. control	1,603	2,473	1,103	1,768	2,185	0,582

Table 6. Raw data from Quantikine kits, optical densities at 450 nm and 570 nm.

IL-1β

Standard	**450 nm**		**570 nm**	
500	2,302	2,204	0,043	0,049
250	1,348	1,303	0,040	0,037
125	0,656	0,680	0,034	0,037
62,5	0,356	0,387	0,034	0,033
31,2	0,208	0,218	0,043	0,041
15,6	0,123	0,130	0,033	0,036
7,8	0,087	0,085	0,032	0,031
0	0,046	0,046	0,033	0,033
Males				
Control	0,816	0,875	0,047	0,046
RNA	2,070	2,183	0,049	0,053
SB1	0,062	0,062	0,045	0,048
RNA+SB1	0,132	0,111	0,033	0,041
RNA+SB2	0,142	0,129	0,034	0,033
PHA	2,265	2,086	0,058	0,058
LPS	2,456	2,148	0,049	0,040
PHA+RNA	3,202	2,934	0,045	0,041
LPS+RNA	3,137	2,894	0,051	0,047
PHA+RNA+SB1	0,933	0,972	0,049	0,049
PHA+RNA+SB2	1,901	1,827	0,043	0,043
LPS+RNA+SB1	1,257	0,937	0,040	0,041
LPS+RNA+SB2	1,754	1,409	0,043	0,038
DMSO1	0,059	0,058	0,042	0,041
DMSO2	0,067	0,054	0,040	0,031
Females				
Control	0,651	0,466	0,039	0,043
RNA	2,085	1,721	0,050	0,047
SB1	0,060	0,059	0,038	0,038
RNA+SB1	0,513	0,436	0,041	0,042
RNA+SB2	0,466	0,456	0,035	0,036
PHA	2,332	2,330	0,045	0,047
LPS	2,195	2,081	0,042	0,041
PHA+RNA	3,162	3,090	0,049	0,042
LPS+RNA	2,645	2,190	0,052	0,050
PHA+RNA+SB1	0,433	0,419	0,041	0,044
PHA+RNA+SB2	1,519	1,377	0,044	0,049
LPS+RNA+SB1	1,894	1,730	0,045	0,046
LPS+RNA+SB2	0,943	1,174	0,042	0,044

IL-10

Standard	**450 nm**		**570 nm**	
500	4,863	4,797	0,151	0,082
250	2,104	2,056	0,047	0,046
125	1,105	1,008	0,043	0,040
62,5	0,604	0,602	0,041	0,041
31,2	0,342	0,341	0,039	0,037
15,6	0,205	0,210	0,037	0,041
7,8	0,132	0,123	0,033	0,032
0	0,097	0,086	0,039	0,034
Males				
Control	0,802	0,824	0,043	0,045
RNA	1,707	1,690	0,044	0,046
SB1	0,049	0,051	0,048	0,046
RNA+SB1	0,190	0,183	0,039	0,037
RNA+SB2	0,180	0,182	0,038	0,038
PHA	2,877	2,928	0,050	0,051
LPS	2,973	2,958	0,050	0,051
PHA+RNA	3,911	3,986	0,054	0,054
LPS+RNA	2,374	2,449	0,051	0,050
PHA+RNA+SB1	0,367	0,372	0,041	0,041
PHA+RNA+SB2	0,656	0,680	0,039	0,041
LPS+RNA+SB1	0,264	0,277	0,037	0,040
LPS+RNA+SB2	0,568	0,590	0,043	0,038
DMSO1	0,064	0,071	0,043	0,040
DMSO2	0,055	0,095	0,037	0,047
Females				
Control	0,946	0,986	0,047	0,050
RNA	1,832	1,928	0,050	0,045
SB1	0,105	0,126	0,037	0,042
RNA+SB1	0,322	0,354	0,039	0,043
RNA+SB2	0,367	0,318	0,042	0,043
PHA	3,009	2,728	0,050	0,049
LPS	2,577	2,253	0,049	0,048
PHA+RNA	3,005	3,049	0,065	0,050
LPS+RNA	2,815	2,910	0,051	0,052
PHA+RNA+SB1	0,185	0,187	0,042	0,045
PHA+RNA+SB2	0,663	0,623	0,044	0,047
LPS+RNA+SB1	0,548	0,554	0,043	0,043
LPS+RNA+SB2	0,583	0,565	0,042	0,047

TNF-α

Standard	450 nm		570 nm	
1000	3,228	3,096	0,052	0,053
500	1,925	1,948	0,052	0,042
250	1,050	1,031	0,040	0,041
125	0,598	0,568	0,040	0,040
62,5	0,311	0,307	0,039	0,040
31,2	0,188	0,183	0,040	0,039
15,6	0,119	0,119	0,042	0,040
0	0,046	0,040	0,041	0,04
Males				
Control	0,819	0,860	0,0425	0,045
RNA	1,667	1,718	0,042	0,047
SB1	0,094	0,093	0,042	0,042
RNA+SB1	0,136	0,141	0,036	0,036
RNA+SB2	0,228	0,232	0,037	0,038
PHA	4,107	4,207	0,056	0,057
LPS	3,259	3,361	0,048	0,052
PHA+RNA	3,514	3,538	0,054	0,054
LPS+RNA	2,668	2,829	0,0525	0,047
PHA+RNA+SB1	1,442	1,454	0,045	0,041
PHA+RNA+SB2	2,167	2,150	0,048	0,046
LPS+RNA+SB1	0,391	0,401	0,036	0,032
LPS+RNA+SB2	1,224	1,223	0,044	0,046
DMSO1	0,122	0,126	0,036	0,039
DMSO2	0,124	0,118	0,039	0,033
Females				
Control	0,550	0,555	0,042	0,041
RNA	1,342	1,350	0,046	0,042
SB1	0,098	0,095	0,04	0,038
RNA+SB1	0,346	0,357	0,036	0,039
RNA+SB2	0,421	0,394	0,0425	0,039
PHA	2,937	2,884	0,049	0,044
LPS	2,055	2,030	0,045	0,053
PHA+RNA	2,212	2,150	0,04	0,043
LPS+RNA	2,206	2,123	0,053	0,048
PHA+RNA+SB1	0,366	0,361	0,047	0,05
PHA+RNA+SB2	1,846	1,732	0,042	0,043
LPS+RNA+SB1	0,943	0,997	0,0465	0,049
LPS+RNA+SB2	1,154	1,125	0,048	0,045

GM-CSF

Standard	450 nm		570 nm	
500	2,302	2,204	0,043	0,049
250	1,348	1,303	0,040	0,037
125	0,656	0,680	0,034	0,037
62,5	0,356	0,387	0,034	0,033
31,2	0,208	0,218	0,043	0,041
15,6	0,123	0,130	0,033	0,036
7,8	0,087	0,085	0,032	0,031
0	0,046	0,046	0,033	0,033
Males				
Control	0,816	0,875	0,047	0,046
RNA	2,070	2,183	0,049	0,053
SB1	0,062	0,062	0,045	0,048
RNA+SB1	0,132	0,111	0,033	0,041
RNA+SB2	0,142	0,129	0,034	0,033
PHA	2,265	2,086	0,058	0,058
LPS	2,456	2,148	0,049	0,040
PHA+RNA	3,202	2,934	0,045	0,041
LPS+RNA	3,137	2,894	0,051	0,047
PHA+RNA+SB1	0,933	0,972	0,049	0,049
PHA+RNA+SB2	1,901	1,827	0,043	0,043
LPS+RNA+SB1	1,257	0,937	0,040	0,041
LPS+RNA+SB2	1,754	1,409	0,043	0,038
DMSO1	0,059	0,058	0,042	0,041
DMSO2	0,067	0,054	0,040	0,031
Females				
Control	0,651	0,466	0,039	0,043
RNA	2,085	1,721	0,050	0,047
SB1	0,060	0,059	0,038	0,038
RNA+SB1	0,513	0,436	0,041	0,042
RNA+SB2	0,466	0,456	0,035	0,036
PHA	2,332	2,330	0,045	0,047
LPS	2,195	2,081	0,042	0,041
PHA+RNA	3,162	3,090	0,049	0,042
LPS+RNA	2,645	2,190	0,052	0,050
PHA+RNA+SB1	0,433	0,419	0,041	0,044
PHA+RNA+SB2	1,519	1,377	0,044	0,049
LPS+RNA+SB1	1,894	1,730	0,045	0,046
LPS+RNA+SB2	0,943	1,174	0,042	0,044

Table7. Number of cells collected; cells pr ml, total number of cells pr sample and number of cells pr well in cultures.

Person	cells/ml	cells tot.	cells pr. well
1			
2	1.1×10^6	2.4×10^7	6.6×10^5
3	8.0×10^5	1.7×10^7	4.7×10^5
4	1.4×10^6	3.7×10^7	1.0×10^6
5	1.8×10^6	2.7×10^7	7.5×10^5
6	1.9×10^6	4.1×10^7	1.1×10^6
7	2.2×10^6	4.9×10^7	1.4×10^6
8	1.8×10^6	3.9×10^7	1.1×10^6
9	2.1×10^6	3.6×10^7	9.9×10^5
10	1.0×10^6	3.6×10^7	9.9×10^5
11	5.2×10^5	1.3×10^7	3.8×10^5
Avg	$\mathbf{1.5 \times 10^6}$	$\mathbf{3.2 \times 10^7}$	$\mathbf{8.8 \times 10^5}$
SD	5.8×10^5	1.1×10^7	3.1×10^5

INFORMED CONCENT FORM & APPROVAL LETTER FROM REK-VEST

Forespørsel om deltakelse i forskningsprosjektet

”Nukleotider og effekter på humane immunceller (PBMC) in vitro”

Bakgrunn og hensikt

Dette er et spørsmål til deg om å delta i en forskningsstudie for å undersøke effekten av nukleotider (finnes i matvarer og morsmelk) på cytokin (signalmolekyl) produksjon hos humane immun celler som blir dyrket i cellekultur-brett. Tidligere forskning har vist at nukleotider kan endre signalmolekyl-produksjonen hos immun celler. Noe som blant annet kan gi forandringer i immunforsvaret mot bakterier og virus. Vi ønsker at friske frivillige personer vil delta i masterstudien. NIFES og Institutt for indremedisin er ansvarlig.

Hva innebærer studien? Det blir tatt blodprøver av deg 1 gang. Blodet blir sentrifugert og immunceller blir isolert fra de sentrifugerte prøvene. Cellene blir sådd ut i cellekulturbrett og tilsatt nukleotider. Cellene skiller ut signalmolekyler (cytokiner) i mediet og dette mediet blir frosset ned i påvente av at prøvene skal analyseres for cytokiner. Analysene skjer i ELISA-brett hvor brønnene er koblet med antistoffer mot ulike cytokiner. Prøvematerialet tilføres brønnene og en fargereaksjon måles for å finne mengde cytokin sammenlignet med en

kontroll-prøve. Ditt navn, alder og kjønn blir registrert. Navnet kodes, antall år og kjønn følger prøvene i masterstudien. Det er umulig for andre å koble navnet ditt til resultatene.

Mulige fordeler og ulemper

Det kreves 1.gang blodtaking i et par rør. Prøven taes av en bioingeniør slik at et eventuelt ubehag ved stikk skal bli minst mulig. Oppleves dette som belastende kan personen avslutte deltagelsen umiddelbart.

Hva skjer med prøvene og informasjonen om deg? Immunceller isolert fra din blodprøve blir sådd ut i cellekulturer samme dag blodprøven blir tatt. Cellekultur -mediet blir samlet etter 3 døgn og frosset ned på -20 grader. Cellene blir destruert.
Supernatantene blir oppbevart til de er analysert for cytokiner ved hjelp av kommersielle ELISA-kit. Deretter blir de kastet/destruert. Kun alder og kjønn blir knyttet til prøvene og blir beskrevet i master studien. En kode knytter navnet ditt til prøvene. Denne opplysningen blir også slettet ved forsøkenes slutt (Sommer 2009).

Prøvene tatt av deg og informasjonen som registreres om deg skal kun brukes slik som beskrevet i hensikten med studien. Alle opplysningene og prøvene vil bli behandlet uten navn og fødselsnummer eller andre direkte gjenkjennende opplysninger. En kode knytter deg til dine opplysninger og prøver gjennom en navneliste.
Det er kun autorisert personell knyttet til prosjektet som har adgang til navnelisten og som kan finne tilbake til deg. Det er kun Prosjektleder E. Holen og Masterstudent Ole Petter Nuland som har koden for navn knyttet til prøvene.
Det vil ikke være mulig å identifisere deg i resultatene av studien når disse publiseres.

Frivillig deltakelse
Det er frivillig å delta i studien. Du kan når som helst og uten å oppgi noen grunn trekke ditt samtykke til å delta i studien. Dette vil ikke få konsekvenser for din videre behandling. Dersom du ønsker å delta, undertegner du samtykkeerklæringen på siste side. Om du nå sier ja til å delta, kan du senere trekke tilbake ditt samtykke uten at det påvirker din øvrige behandling. Dersom du senere ønsker å trekke deg eller har spørsmål til studien, kan du kontakte Elisabeth Holen mail: eho@nifes.no tlf nr 95241632

Ytterligere informasjon om studien finnes i kapittel *A – utdypende forklaring av hva studien innebærer.*
Ytterligere informasjon om biobank, personvern og forsikring finnes i kapittel B – *Personvern, biobank, økonomi og forsikring.*

Samtykkeerklæring følger etter kapittel B.

Kapittel A- utdypende forklaring av hva studien innebærer

I undersøkelsen vil kun friske frivillige personer delta.

Bakgrunnsinformasjon om studien : Nukleotider finnes i mat og i morsmelk. Det er kjent at nukleotider i morsmelk er bra for barnets tarmceller og immunforsvar. Derfor tilsettes morsmelkerstaninger nukelotider. I denne undersøkelsen ønsker vi å finne ut om nukleotider kan påvirke immunceller fra friske personer. Prosjektleder har tidligere vist i en pulikasjon at nukleotider kan forandre utskillelse av signalmolekyler fra immunceller som er utsatt for et virus antigen.
I denne studien ønsker vi å undersøke en eventuell utskillelse av mange signalmolekyler, noe som er mulig ved å bruke ELISA-kit hvor en kan analysere opp til 12 signalmolekyler samtidig.
Vi ønsker å få en indikasjon på om nukleotider kan påvirke immunforsvaret til friske personer.
Resultater fra denne undersøkelsen kan få betydning for en eventuell ny undersøkelse som også vil omfatte utvalgte pasientgrupper.

Hvis du vil delta vil du gi blod 1 gang. Ingen annen kontakt er senere nødvendig om deltakeren ikke ønsker informasjon om resultatene.
Blodprøvene kan taes etter avtale mellom master studenten/prosjektleder og forsøkspersonen
Blodprøvetakingen medfører intet ubehag annet enn et stikk.

Kapittel B - Personvern, biobank, økonomi og forsikring

Personvern
Opplysninger som registreres om deg er alder og kjønn. Navn kobles mot en kode til prøvene er ferdig analysert. Navn blir deretter slettet.

NIFES ved administrerende direktør er databehandlingsansvarlig.

Biobank
Det er opprettet og godkjent en forskningsbiobank ved NIFES, godkjent for midlertidig lagring av humane prøver.

Cellesupernatanter opprinnelig isolert fra blod prøvene som blir tatt og informasjonen utledet av dette materialet vil bli lagret i en forskningsbiobank ved NIFES. Hvis du sier ja til å delta i studien, gir du også samtykke til at det biologiske materialet og analyseresultater inngår i biobanken. Livar Frøyland er ansvarshavende for forskningsbiobanken. Biobanken med dine prøver planlegges å vare til sommer 2009. Etter dette vil materiale og opplysninger bli destruert og slettet etter interne retningslinjer.

Utlevering av materiale og opplysninger til andre
Intet materiale eller opplysninger vil bli gitt til andre en masterstudenten og prosjektleder, som ikke vil gi disse videre til andre personer i eller utenfor Norge. Alt materiale og opplysninger vil bli destruert ved studiens slutt.

Rett til innsyn og sletting av opplysninger om deg og sletting av prøver
Hvis du sier ja til å delta i studien, har du rett til å få innsyn i hvilke opplysninger som er registrert om deg. Du har videre rett til å få korrigert eventuelle feil i de opplysningene vi har registrert. Dersom du trekker deg fra studien, kan du kreve å få slettet innsamlede prøver og opplysninger, med mindre opplysningene allerede er inngått i analyser eller brukt i vitenskapelige publikasjoner.

Økonomi og NIFES rolle
Dette er masterstudie, ingen kommersielle interesser er involvert

Studien og biobanken er finansiert gjennom forskningsmidler fra NIFES. Dette er en masterstudie hvor ingen kommersielle interesser er involvert.

Forsikring
Ingen forsikring er tegnet for de frivillige deltagerne

Informasjon om utfallet av studien
Resultatet av studien vil offentliggjøres i en masteroppgave ved UIB. Frivillige personer kan få innsyn i egne resultater.

Samtykke til deltakelse i studien

Jeg er villig til å delta i studien

(Signert av prosjektdeltaker, dato)

Stedfortredende samtykke når berettiget, enten i tillegg til personen selv eller istedenfor

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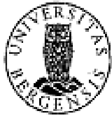
(Signert av nærstående, dato)

Jeg bekrefter å ha gitt informasjon om studien

(Signert, rolle i studien, dato)



UNIVERSITETET I BERGEN

Regional komité for medisinsk og helsefaglig forskningsetikk, Vest-Norge (REK Vest)

Elisabeth Holen
Nasjonalt institutt for ernærings- og sjømatforskning (NIFES)
PB 2029 Nordnes
5817 Bergen

Deres ref	Vår ref	Dato
	2008/14812-CAG	15.01.2009

Ad. prosjekt: Nukleotider og effekter på humane immunceller (PBMC) in vitro (268.08).

Det vises til din søknad om godkjenning av forskningsprosjekt, datert 20.11.08 og tilbakemelding gitt 15.01.09.

Komiteen behandlet prosjektsøknaden i møte den 16.12.09.
REK-Vest v/leder behandlet tilbakemeldingen.

Det søkes om å benytte den generelle forskningsbiobanken kalt "Oppbevaring av alt humant biologisk materiale fra ulike forskningsprosjekter i en felles forskningsbiobank ved NIFES", (REK nr 04/6121).
REK Vest legger til grunn at ansvarshavende av den generelle forskningbiobanken godkjenner at biobanken benyttes i dette prosjektet. REK Vest har ingen innvendinger mot at den generelle biobanken benyttes.

REK Vest noterer at man etter REK Vests anbefalinger kun inkluderer friske forsøkspersoner i prosjektet.

En har følgende merknader til informasjonsskrivet til deltakerne:

- Det må fremgå at samtykket kan tilbakekalles og at deltaker har rett til å kreve det biologiske materialet destruert og innsamlede opplysninger slettet eller utlevert. Adgangen til å tilbakekalle samtykket eller kreve destruksjon, sletting eller utlevering gjelder ikke dersom opplysningene allerede er inngått i vitenskapelige arbeider, er anonymisert eller har inngått i et annet biologisk produkt, jf. Biobankloven § 14.
- Informasjonsskrivet til deltakerne må forenkles. Uttrykk som "nukleotider" "ELISA-brett", "brønner", "supernatant" må forklares eller utelates. For at deltakelse skal bygge på et reelt informert samtykke, må prosjektleder sikre at informasjon er språklig tilgjengelig for forsøksdeltaker. Forespørselen må utarbeides slik at bakgrunn for henvendelsen er tydelig, kortfattet og poengtert beskrevet på et lettfattelig norsk. Deltakeren skal gis tilstrekkelig informasjon, men ikke nødvendigvis alle detaljer om hva prosjektet går ut på.

Postadresse
Postboks 7804
5020 Bergen

rek-vest@uib.no
www.etikkom.no/REK
Org no. 874 789 542

Regional komité for medisinsk og helsefaglig forskningsetikk, Vest-Norge
Telefon 55 97 84 97 / 98 / 99

Besøksadresse
Haukeland Universitetssykehus

En har ingen videre merknader til prosjektet.

Vedtak:
Prosjektet godkjennes på vilkår av at ovennevnte merknader tas til følge.

Komiteen ber om å få tilsendt sluttrapport evt. trykt publikasjon for studien.

Vennlig hilsen

Jon Lekven
leder

Camilla Gjerstad
førstekonsulent

Kopi: Livar Frøyland, ansvarshavende av generell biobank.

De regionale komiteene for medisinsk og helsefaglig forskningsetikk foretar sin forskningsetiske vurdering med hjemmel i Forskningsetikklovens § 4. Saker vedrørende forskningsbiobanker behandles i samsvar med Biobankloven. Saksbehandlingen følger Forvaltningsloven.

Komiteenes vedtak etter Forskningsetikklovens § 4 kan påklages (jfr. forvaltningsloven § 28) til Den nasjonale forskningsetiske komité for medisin og helsefag. Klagen skal sendes REK-Vest (jfr. forvaltningsloven § 32). Klagefristen er tre uker fra den dagen du mottar dette brevet (jfr. forvaltningsloven § 29).