

Ex vivo induction of cytokines by mould components in whole blood of atopic and non-atopic volunteers

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Abstract

We investigated the time-course release of IL-1 β and IL-8 protein as well as the steady state mRNA level of their genes in human whole blood after stimulation with LPS, β -1,3-D-glucan and mould extracts. We compared the response of 10 non-atopic and 10 atopic individuals. In parallel, cytokine protein release and the corresponding steady state mRNA level was determined by the standard ELISA and real-time on-line RT-PCR methods, respectively. Glucan induced the highest level of IL-1 β mRNA and protein release after 3 h. IL-8 was induced at 3 h after glucan, but not after LPS, induction, which indicates different induction pathways. At the time-points found to elicit the optimal cytokine response significantly higher basal cytokine mRNA levels and significantly lower LPS induced cytokine mRNA levels were observed in the non-atopic group. Generally, mould components induced cytokine mRNA steady state to lower levels in the atopics compared to the non-atopics. In contrast, no differences were found between the two groups in their capacity to induce cytokine protein release. These findings persisted after correction for the percentage of mononuclear cells. The data supported our hypothesis that “exhaustion” of the atopic immune system elicits lower basal cytokine mRNA levels and that analysis of cytokine gene expression has the potential to differentiate between non-atopic and atopic individuals.

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1. Introduction

Allergic and non-allergic asthma are respiratory conditions that occur after exposure to organic dust and allergens in the environment. Organic dust contains endotoxins like bacterial lipopolysaccharide (LPS) and β -1,3-D-glucan, a cell wall component of moulds. When an organism is stimulated with an endotoxin, many biologically active components are released from various cells [21]. Among these are the pro-inflammatory cytokine interleukin-1 β (IL-1 β) and the chemokine interleukin-8 (IL-8), which are integral components of the cytokine cascade release in the response to inflammatory stimuli [8].

The cytokines are defined as soluble mediators of cell-to-cell communication involved in inflammation and immunity. They mediate acute phase and inflammatory host responses that are thought to enhance host defense during infection and eliminate infectious agents like bacteria, viruses and fungi [14]. Cytokine production is influenced by factors such as smoking [27] and exercise [13], and excessive or inappropriate production of certain cytokines is involved in the pathology of acute and chronic inflammatory diseases [23,29] as well as autoimmune diseases [7] and some cancers [3]. The transcription of cytokine mRNA was demonstrated to be influenced by conditions such as typhoid fever [18].

IL-1 β is a major inflammatory mediator and is secreted mainly by monocytes but also by other cells like macrophages, neutrophils and endothelial cells [28]. IL-1 β is an early-response cytokine and the transcription of its mRNA after stimulation with LPS is observed within 15 min [8]. In whole blood as well as in human peripheral blood mononuclear cells (PBMC)

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stimulated with LPS, IL-1 β mRNA was reported to reach a maximum at 2–4 h, sustaining for 6–8 h before leveling off [18,22] whereas the protein release of IL-1 β , detectable after 2–4 h, reached a maximum after 6–8 h and the level sustained for 24 h [4,17,18,22].

IL-8 is produced by stimulated monocytes, macrophages, fibroblasts, endothelial cells, neutrophils and a number of other cells [2]. It is extremely long-lived and resistant to both high temperatures and enzymatic degradation [19]. This contrasts with the relatively short-lived kinetics of other cytokines such as TNF- α and IL-6 [5]. A rapidly induced IL-8 mRNA by IL-1 β and TNF α in monocytes, fibroblasts and endothelial cells with significant expression after 1 h reaching a maximum after 3–4 h and remaining elevated for more than 24 h was described by Baggiolini et al. [2]. LPS induced IL-8 mRNA transcription and protein release was also reported for whole blood and isolated macrophages [4,5,15]. For the IL-8 mRNA transcription a biphasic pattern was seen with the first peak at 1–4 h and the second peak at 16–24 h. The second increase was suggested to be primarily attributed to stimulation by TNF α [15], which like IL-1 β is an early-response cytokine induced by LPS stimulation. DeForge et al. [4] reported that the IL-8 protein release in whole blood upon stimulation with LPS was detected after 1 h, reaching a plateau level from 6 to 12 h and then the level increased again until 24 h. Hallsworth et al. [10] compared the protein release of the cytokines TNF- α , IL-1 β and IL-8 in un-stimulated and LPS-stimulated monocytes and macrophages from asthmatic and normal subjects and found for all three cytokines an elevated release in LPS-stimulated cells from asthmatic subjects.

Using the whole blood assay (WBA) we investigated whether there are differences in the response to LPS, β -1,3-D-glucan and different mould extracts among persons with and without atopy. The response was measured as IL-1 β and IL-8 protein release from the cells and as the steady state mRNA level of the respective genes. Also, the incubation time giving the optimal response to the inducers was determined.

2. Results

Using the whole blood assay (WBA) we analyzed the basal as well as the induced steady state mRNA level and the cellular release of the cytokines IL-1 β and IL-8. The induced level was expressed both as a ratio above the background level and as the induced level minus background, where the pure solvent of the inducers was added to the blood samples.

2.1. Basal level of cytokine steady state mRNA level

The basal steady state mRNA levels of IL-1 β and IL-8 elicited a different pattern for the two cytokines.

The pooled data of atopic and non-atopic individuals are shown in Fig. 1. The basal level of IL-1 β (Fig. 1A) was highest after 1 h of incubation and then rapidly leveled off, reaching zero after 20 h. The basal steady state mRNA level of IL-8 mRNA (Fig. 1B) showed a tendency to a biphasic pattern with a small significant peak after 1 h ($p < 0.003$) compared to the lower levels at 3 and 6 h, and at 20 and 23 h a second peak approximately seven times higher than the level at 1 h. The basal IL-8 mRNA levels were in a range 10 times higher than the basal IL-1 β mRNA levels.

2.2. Differences between non-atopics and atopics at the mRNA level

Cell differential-count showed no differences between atopics and non-atopics. Moreover, the basal steady state mRNA levels analyzed in the WBA only differed at few time-points between the atopics and the non-atopics (Table 1). For the basal steady state IL-1 β mRNA level at 1 and 3 h, the level for the non-atopics was significantly higher ($p = 0.001$) than for the atopics. For the basal IL-8 mRNA levels, the only difference between the two groups was found at 23 h where the non-atopic level was significantly higher ($p = 0.032$) than the atopic mRNA level.

For the induced ratio above background of IL-1 β and IL-8 mRNA, significant differences between atopics and non-atopics were found at some time-points upon treatment with LPS, β -1,3-D-glucan or various mould extracts (Table 1). For IL-1 β and IL-8, the steady state mRNA ratio induced by LPS was found to be significantly lower in non-atopics than in atopics after 1 h ($p < 0.001$) and 3 h ($p < 0.001$), respectively. Also, the *Rhodotorula rubra* (Rr) extract induced a significantly lower IL-8 mRNA ratio above background for non-atopics compared to atopics after 3 h ($p = 0.02$) whereas the opposite, a significantly higher ratio of IL-8 mRNA, was found in atopics after 1 h ($p = 0.028$) induction with glucan (Table 1). Finally, the IL-1 β mRNA ratio above background of non-atopics induced by *Penicillium chrysogenum* (Pc) after 6 h ($p = 0.01$) was significantly higher than the atopic ratio, while the non-atopic IL-8 mRNA ratio induced with Pc after 3 h ($p = 0.002$) was significantly lower than the atopic level.

When we studied the mRNA level minus background we found almost the same differences between atopics and non-atopics. The only extra information we gained was the higher induced IL-1 β level 3 h after *Cladosporium sphaerospermum* (Cs) and *Stachybotrys chartarum* (Sc) induction ($p = 0.02$). Also, a higher induced IL-8 mRNA level minus background was seen among non-atopics 1 h after Cs induction ($p = 0.001$) (Table 1). In addition, correction of the IL-1 β β 2M and IL-8 mRNA/ β 2M data to the percentage of mononuclear cells elicited similar results, except for Pc where the differences

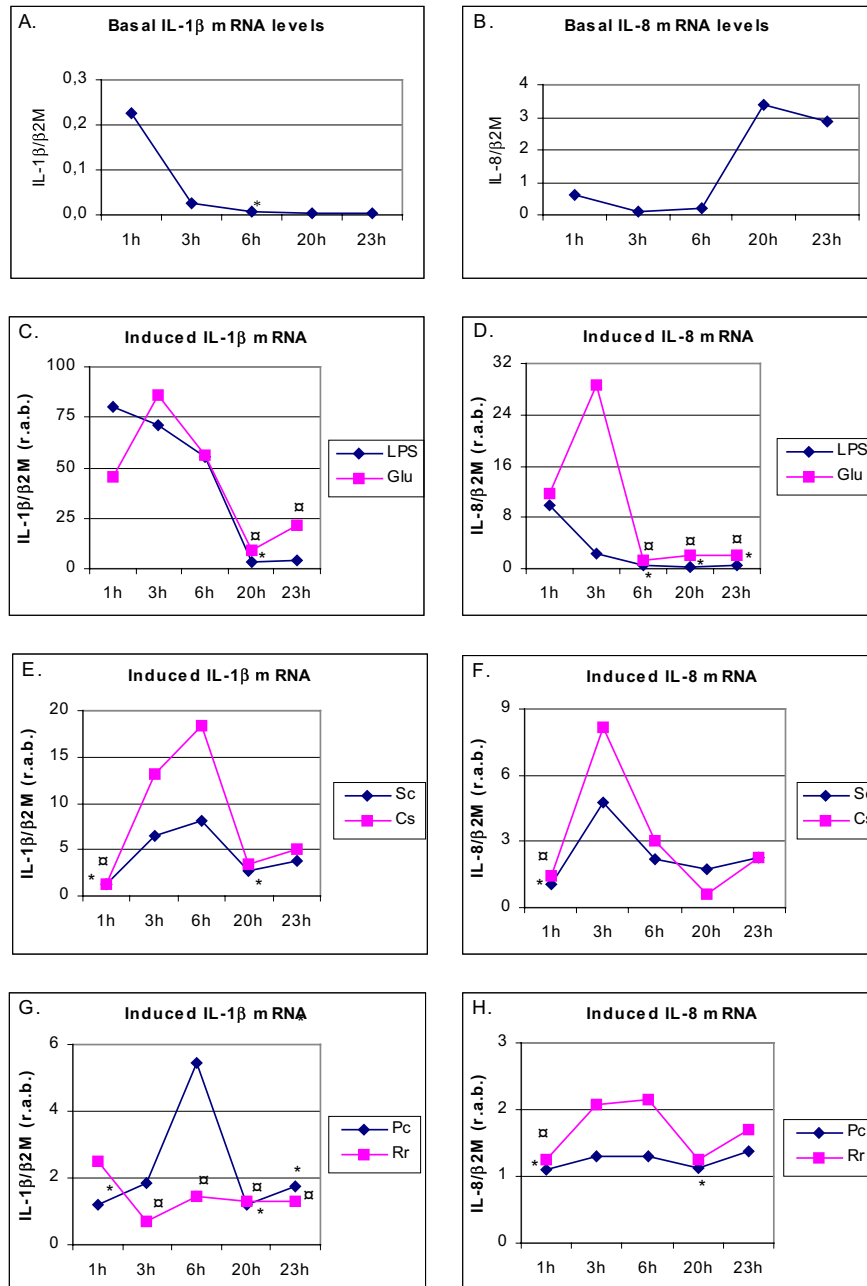


Fig. 1. Note the different vertical scales. The basal and induced IL-1 β and IL-8 mRNA levels in the whole blood assay. The ratio is determined from the pooled data of atopic and non-atopic individuals and is the mean of two to four independent measurements of up to 10 subjects in each group; r.a.b.: ratio above background; * and □ indicate levels not significant above background.

between atopics and non-atopics disappeared after correction and for Rr where no difference in IL-8 mRNA was found after 3 h.

2.3. Time-course study on induced cytokine steady state mRNA level

Due to the small study size, the data of the non-atopics and the atopics were pooled to determine the

optimal WBA incubation times to analyze the induced steady state mRNA level and release of the cytokines.

The induction of the steady state mRNA level with LPS and glucan, analyzed by WBA, reached a ratio 4- to 25-fold higher for IL-1 β and a ratio 1- to 25-fold higher for IL-8, compared to the induction with the various tested mould extracts (Fig. 1). Also, the mRNA levels after subtraction of basal mRNA level was much higher after LPS or glucan treatments than for mould extracts (Table 2). Contrary to the basal mRNA levels

Table 1

Significant differences of cytokine steady state mRNA and protein levels between atotics and non-atotics

	IL-1 β mRNA	IL-8 mRNA	IL-1 β protein	IL-8 protein
Basal	↑ 1 h ($p = 0.001$) ↑ 3 h ($p = 0.001$)	↑ 23 h ($p = 0.032$)	—	—
LPS	↓ 1 h ($p < 0.001$)	↓ 3 h ($p < 0.001$)	↑ 3 h ($p = 0.004$) ^a ↑ 20 h ($p = 0.048$) ^a ↑ 23 h ($p = 0.000$) ^a	↑ 3 h ($p < 0.001$) ^a
Rr	—	↓ 3 h ($p = 0.02$)	—	—
Glucan	—	↑ 1 h ($p = 0.028$)	—	—
Pc	↑ 6 h ($p = 0.01$)	↓ 3 h ($p = 0.002$)	—	—
Cs	↑ 3 h ($p = 0.02$) ^a	↑ 1 h ($p = 0.001$) ^a	—	—
Sc	↑ 3 h ($p = 0.02$) ^a	—	—	—

The significantly different basal and induced mRNA and protein levels of the cytokines IL-1 β and IL-8 are determined at the time-points giving the optimal response of the inducers. ↑: Significantly higher non-atopic level; ↓: Significantly lower non-atopic level; h: time-points; p values in brackets.

^a Only seen when mRNA or protein level minus background is used.

the induced IL-1 β steady state mRNA level was approximately 10- and 2-fold higher than the induced IL-8 steady state mRNA level calculated as ratio above background and level minus the background, respectively. For the level minus the background, however, the mould extract induced levels were higher for IL-8 after 20 and 24 h.

2.3.1. LPS and glucan induced steady state mRNA level

The LPS induced IL-1 β steady state mRNA ratio above background (Fig. 1C) showed the highest level at 1, 3 and 6 h, being not significantly different from each other with a decrease to background level after 20 h. The highest LPS induced ratio for IL-8 mRNA (Fig. 1D) was found after 1 h of incubation, which thereafter decreased, reaching the background level after 6 h. The

same response pattern was observed when the data are given as level minus background (Table 2).

Glucan induced the highest ratio above background of IL-1 β mRNA after 3 h (Fig. 1C) while the highest induced ratio for IL-8 mRNA (Fig. 1D) was observed at 1 and 3 h (not significantly different from each other), with a decrease to background level after 20 and 6 h, respectively. Similarly, for both cytokine mRNA the highest induced level minus background for glucan was observed at the first three time-course, 1, 3, and 6 h (Table 2). Correction of the data for mononuclear cells did not introduce any changes in the results.

2.3.2. Mould extract induced steady state mRNA level

The induced ratio above background of IL-1 β mRNA (Fig. 1E) by *C. sphaerospermum* (Cs) and *S. chartarum* (Sc) reached the highest steady state mRNA ratio at 3 and 6 h (not significantly different from each

Table 2

IL-1 β / β 2M and IL-8/ β 2M cytokine mRNA induction minus background level

	1 h		3 h		6 h		20 h		24 h		Significant differences ^a
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
<i>(a) IL-1β/β2M</i>											
LPS	10.75	10.62	1.75	1.60	0.54	0.24	0.02	0.02	0.01	0.02	1,2,3,4,5,6,7,8,9
Glucan	6.15	7.83	0.24	0.19	0.28	0.11	0.01	0.02	0.01	0.01	1,3,4,6,7,8,9
Sc	0.03	0.18	0.19	0.22	0.07	0.04	0.01	0.02	0.01	0.02	1,7,8,9
Cs	0.07	0.21	0.27	0.22	0.18	0.11	0.02	0.01	0.01	0.01	1,6,7,8,9
Pc	0.01	0.13	0.01	0.03	0.02	0.01	0.00	0.01	0.00	0.00	8,9
Rr	0.16	0.23	−0.01	0.01	0.00	0.03	0.00	0.00	0.00	0.01	1,2,4,6,7
<i>(b) IL-8/β2M</i>											
LPS	5.66	9.94	0.15	0.17	−0.15	0.14	−2.56	1.24	−1.61	1.58	2,3,4,5,6,7,8,9
Glucan	5.52	8.14	0.19	0.22	−0.02	0.12	−1.12	1.46	−0.86	0.86	3,4,5,7,9
Sc	−0.15	0.80	0.43	0.41	0.20	0.18	2.32	2.60	3.30	4.43	1,3,4,6,7,8,9
Cs	0.18	0.53	0.72	0.63	0.36	0.21	1.97	2.63	2.21	2.62	1,3,4
Pc	−0.06	0.38	0.03	0.07	−0.05	0.19	0.33	1.26	0.28	2.67	
Rr	0.24	0.80	0.12	0.07	0.15	0.15	0.53	0.95	1.02	2.19	4

^a 1–10: $p < 0.05$ t -test [(1) 1 h vs 3 h, (2) 1 h vs 6 h, (3) 1 h vs 20 h, (4) 1 h vs 24 h, (5) 3 h vs 6 h, (6) 3 h vs 20 h, (7) 3 h vs 24 h, (8) 6 h vs 20 h, (9) 6 h vs 24 h, (10) 20 h vs 24 h].

Table 3
IL-1 β and IL-8 protein release minus background level (pg/ml)

	3 h		20 h		23 h		Significant differences ^a
	Mean	SD	Mean	SD	Mean	SD	
<i>(a) IL-1β</i>							
LPS	1581	640	5986	1602	8234	2118	1,2,3
Glucan	2374	245	2346	965	2087	90	2
Sc	2	3	12	6	17	5	1,2
Cs	2	6	35	19	51	13	1,2
Pc	0	0	1	1	2	2	1,2
Rr	0	0	−1	0	−1	1	2
<i>(b) IL-8</i>							
LPS	75	41	262	99	688	554	1,2
Glucan	125	43	2448	1485	2733	1730	1,2
Sc	6	6	1021	723	1760	854	1,2
Cs	1	1	125	79	210	105	1,2
Pc	1	1	125	79	210	105	1,2
Rr	2	1	58	54	73	78	1,2

^a 1: $p < 0.05$ t -test 3 h vs 20 h. 2: $p < 0.05$ t -test 3 h vs 23 h. 3: $p < 0.05$ t -test 20 h vs 23 h.

other) while the highest ratio of IL-8 mRNA (Fig. 1F) was found after 3 h of incubation for both mould extracts. The same response tendency of these moulds was observed for level minus the background; however, for IL-8 a secondary significantly increased release was observed at 20 and 23 h (Table 2).

P. chrysogenum (Pc) induced the highest ratio above background of IL-1 β mRNA (Fig. 1G) after 6 h of incubation while the highest ratio of IL-8 mRNA was found at 3 and 6 h (not significantly different from each other) (Fig. 1H). The mould *R. rubra* (Rr) induced IL-1 β mRNA (Fig. 1G) to a ratio significantly above background after 1 h, which decreased to background levels after 3 h of incubation. The highest IL-8 mRNA ratios above background induced by the Rr extract (Fig. 1H) were found at 3 and 6 h (not significantly different from each other). Again a similar response pattern was observed for the release minus the background (Table 2) but as for the Sc or Cs moulds, the IL-8 level had a secondary increased release upon treatment with Pc or Rr at 20 and 23 h (Table 2).

2.4. Differences between non-atopics and atopics at the protein level

No significant differences in cytokine release from the cells were seen between atopics and non-atopics using the ratio above background either for the basal or for the induced ratio (Table 1). When we studied the cytokine level minus background, significantly higher induced IL-1 β release was seen among non-atopics after the following treatments: LPS, 3 h ($p = 0.004$) and 20 h ($p = 0.048$). For IL-8 the only significant difference was a higher induced level 3 h after LPS among non-atopics ($p < 0.001$) (Table 1). These results persisted after correction for the percentage of mononuclear cells.

Furthermore for the IL-1 β release, the difference between atopics and non-atopics became significant ($p = 0.000$) following LPS at 23 h also.

2.5. Basal level of cytokine protein release

The basal IL-1 β and IL-8 protein release from the cells was examined in parallel in the WBA with the cytokine mRNA determination. The pooled data of non-atopics and atopics are shown in Fig. 2. The basal, spontaneous release of both IL-1 β (Fig. 2A) and IL-8 (Fig. 2B) from the cells increased with time, reaching the highest determined level after 23 h.

As observed for the mRNA, the basal level of IL-8 protein release was approximately 20 times higher than the basal level of IL-1 β .

2.6. Time-course study on induced cytokine protein release

For the pooled data, LPS and glucan induced the IL-1 β protein release in the WBA to a ratio above background more than 100-fold higher than the induced ratio of the IL-8 protein. For the tested mould extracts the opposite effect was observed where the induced ratio of IL-8 protein release was 3- to 4-fold higher than the IL-1 β protein ratio. Similar induced ratios of the IL-8 protein were observed for LPS and Sc, and for glucan and Cs, whereas the ratio above background IL-1 β release caused by LPS and glucan was up to 200-fold higher than the ratio released after induction with the Cs and Sc extracts. The Pc and Rr extracts induced relatively low release of both cytokine proteins.

For both IL-1 β and IL-8 protein release, the ratios above background at the different time-points induced with LPS were not significantly different from each

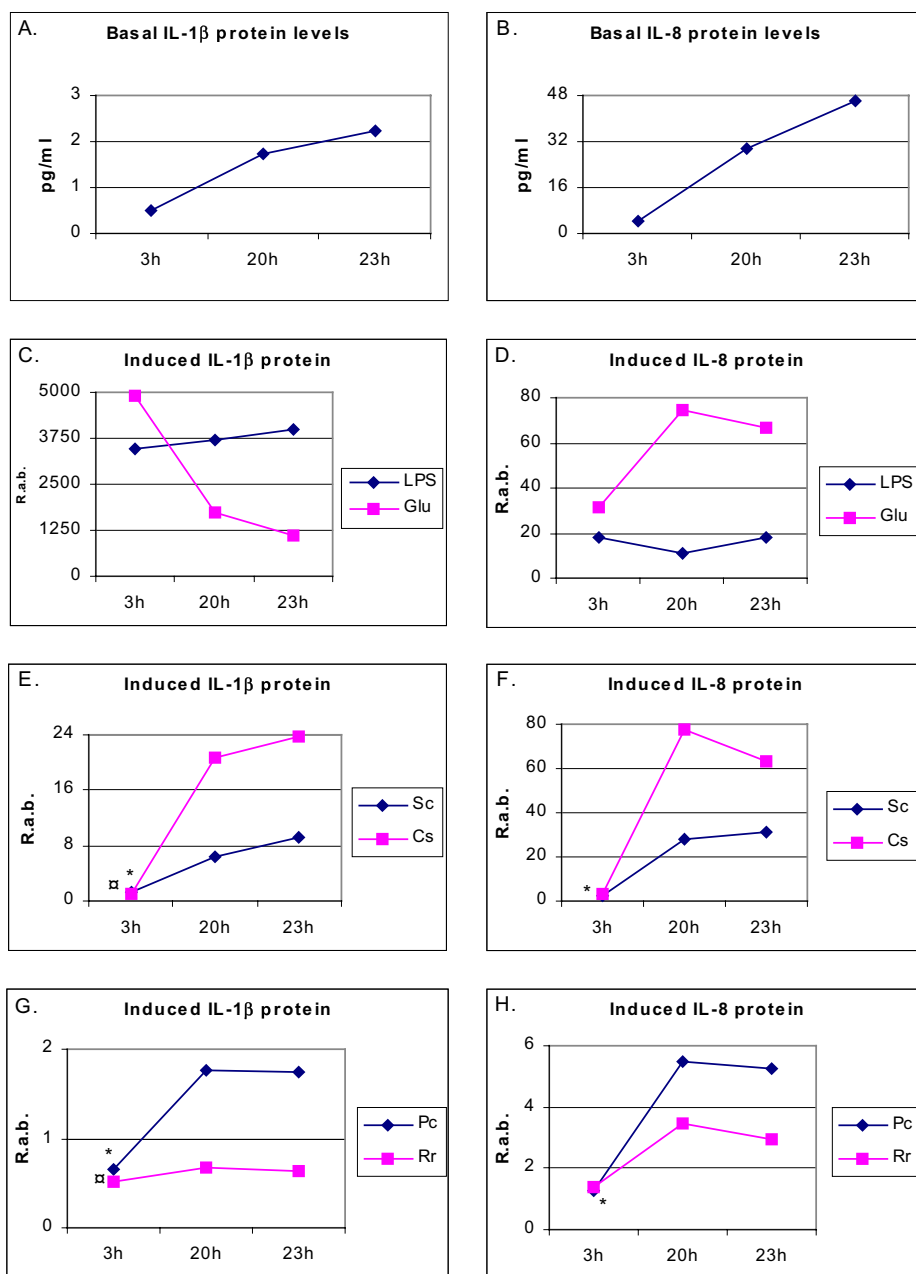


Fig. 2. Note the different vertical scales. The basal and induced IL-1 β and IL-8 protein release in the whole blood assay. The ratio is determined from the pooled data of atopic and non-atopic individuals and is the mean of two to four independent measurements of up to 10 subjects in each group. The background as well as the induced levels are determined in pg/ml. R.a.b.: ratio above background in pg/ml; * and α indicate levels not significant above background.

other (Fig. 2C and D) and neither was the glucan induced IL-8 protein ratio (Fig. 2D). However, the ratio of released IL-1 β protein induced with glucan changes with time, with the highest ratio at 3 h decreasing to the lowest level at 23 h (Fig. 2C).

The cytokine protein release induced by all the mould extracts followed the same pattern for both IL-1 β and IL-8 (Fig. 2E–H). All the moulds elicited a time dependent induced response, with the highest levels after 20 and 23 h of incubation.

Generally, the same response pattern was observed upon evaluation of the data by subtraction of the background level from the induced level (Fig. 2 and Table 3). For both cytokines all inducers, except IL-1 β induced by β -1,3-glucan and Rr, induced a time dependent significant increase in the level of cytokine release. The time-course elicited a decrease in IL-1 β release level after an initially high release at 3 h upon treatment with β -1,3-glucan, whereas Rr did not induce IL-1 β release during the time period (Table 3).

3. Discussion

Using the whole blood assay (WBA) we analyzed the time-course of the induced IL-1 β and IL-8 protein release from the cells and the steady state mRNA level of the corresponding genes. Then we compared the levels of an atopic and a non-atopic group to elucidate possible differences in the response between the two groups towards the different inducers.

3.1. Techniques

The WBA, which is an easy and reproducible method for assessment of cytokine production in human blood [6,31], has recently been established in our laboratory [24] and further optimized (Bonefeld-Jørgensen, EC, in preparation). In the WBA the cells are maintained in their physiological environment to avoid potential artifacts arising from isolation procedures of the cells. We have optimized several assay factors including the concentrations of the inducers as well as the optimal solvents for mould extracts (data not shown). Previously it has been shown that the viability of the cells is stable for at least 24 h [6,30]. Concerning the cell viability after exposure to LPS, we have earlier demonstrated that LPS is not cytotoxic to cell cultures in vitro [25]. For β -1,3-D-glucan, Hoffmann et al. [12] demonstrated that β -1,3-D-glucan exceeding 500 mg/ml, or at double the concentration compared to this study, did not have any cytotoxic effect on rat alveolar macrophages ex vivo. Thus, these studies concerning the effects of LPS and β -1,3-D-glucan on the cell viability ex vivo strongly indicate that the low concentration of mould extracts used in the present study is not cytotoxic to the blood cells.

For determination of the gene expression at the steady state level, we used real-time RT-PCR. On-line monitoring of the PCR reaction allows a faster and more accurate measurement of gene expression than conventional PCR quantification and is suggested to be “the method of choice for quantification of cytokine profiles” [9]. Errors resulting from post-PCR analysis are removed and since the quantification is based on the early exponential amplification phase, differences in efficiency of amplification have less effect on the results. We used the two-step RT-PCR to also avoid differences in the efficiency of the reverse steady state mRNA level since one-step RT-PCR was shown to be less efficient than two-step RT-PCR [16,32]. The use of fluorogenic probes allows us to measure low expressed cytokines in a reproducible way, since these probes are sensitive and can detect ≤ 10 copies per reaction [26].

Previous studies have shown that the blood sampling procedure [11], the collection tubes [20] and the source of LPS [1] have influences on the cytokine mRNA expression and protein release. Therefore, in order to determine the effect of the inducer it is necessary to

determine the time-course of the basal level in non-induced samples, and include this in the calculations. To elucidate a component's capacity to induce cytokine response as well as the level obtained by inducement, we have expressed our results both as a ratio above the background and as the induced level minus background. By doing this, it will be easier to compare results between laboratories where the blood sampling procedure, the collection tubes, the source of LPS etc. are different.

3.2. Non-atopics vs atopics

The comparison between the atopic and non-atopic volunteers was performed at time-points found to be optimal for determination of the effects of the inducers.

For both cytokine mRNAs the basal levels were found to be significantly higher among the non-atopics compared to the atopics. We hypothesize that this might be the result of an “exhaustion” of the immune system of the atopics as related to their basal inflammatory status.

Interestingly, the mRNA levels of the non-atopics generally were induced to a higher level compared to the mRNA level of the atopics. However, LPS induced both IL-1 β and IL-8 mRNA to a higher level in atopics compared to non-atopics and also the mould extracts of *P. chrysogenum* (Pc) and *R. rubra* (Rr) induced the IL-8 mRNA to a higher level in the atopics than in the non-atopics. In contrast, glucan and *C. sphaerospermum* (Cs) induced the IL-8 mRNA in non-atopics to a higher level than in atopics and also Pc, Cs and *S. chartarum* (Sc) all induced a higher IL-1 β mRNA level in non-atopics compared to atopics.

When we compared the mRNA ratio above background with mRNA level minus background, similar information was obtained. However, the latter method showed a significantly higher IL-1 β mRNA level 24 h after glucan and 3 h after Cs and Sc induction, and also the IL-8 mRNA level was higher after 1 h induction with Cs. Correction of the cytokine mRNA data for mononuclear cells showed similar results except for two mould species, Pc and Rr, where all differences or a single (IL-8 mRNA after 3 h) one became insignificant, respectively.

Thus, we found that the basal mRNA level and the LPS induced steady state mRNA level in atopic and non-atopic volunteers were similar for the two cytokine genes, whereas the response upon exposure to glucan and some mould extracts differed between the genes. Therefore, we hypothesize that different pathways are involved in the induction of IL-1 β and IL-8. Moreover, these data also convinced us that no LPS contaminations are present in either of the inducing components.

At the protein level we found no differences between the two groups, either at the basal level or at the induced release when we studied the ratio above background.

When we studied the protein level minus background we observed significantly higher induced IL-1 β and IL-8 levels 3 h after LPS induction among non-atopics. Hallsworth et al. [10] did not observe significant differences in the basal IL-1 β and IL-8 protein release from monocytes and macrophages from normal and asthmatic subjects. However, after LPS stimulation for 24 h, monocytes from asthmatic subjects produced 4-fold more IL-1 β than monocytes from normal subjects and also the IL-8 production was elevated four times in the macrophages from the asthmatic subjects. This is in contrast to our study of the level minus background where we found a doubling of the IL-1 β release among non-atopics 3 and 20 h after LPS treatment and a 4-fold increase of IL-8 release among non-atopics 3 h after LPS stimulation compared to atopics. These differences may not be attributed to the fact that purified monocytes and macrophages were used in the study undertaken by Hallsworth et al. [10] and we used whole blood, since Yaqoob et al. [31] and Nerad et al. [17] showed significant correlations between cytokine release measured in whole blood and purified monocytes, although the level of the cytokines was significantly lower in whole blood preparations. We believe, that the different results reflect differences between normal, healthy subjects, asthmatic subjects and atopic subjects, probably because asthmatics like the persons studied by Hallsworth et al. [10] had a greater ongoing inflammation compared to the non-symptomatic atopics from our panel. The differences could also be due to different cell populations in the peripheral blood of atopics as compared to non-atopics. Because of the very few monocytes determined in the blood samples, we related the data of cytokine release from cells to segmented and non-segmented white blood cells. We did not find any difference in the cell differential-count between atopics and non-atopics and as the cell count has not been shown be related to atopy, we believe that the results are related to differences in cell sensibility rather than different cell populations.

3.3. Cytokine RNA

We included a time study in our investigation since we had no knowledge of the time-course response of several of the inducers included in our investigation. Others have also reported time-course studies of LPS induction; however, to our knowledge no other investigations have studied the effects of glucan and mould extracts on cytokines in human whole blood.

Netea et al. [18] also used LPS-stimulated human whole blood to determine the steady state mRNA level of IL-1 β and found a time-course similar to our findings. They found an increased expression after 2 h LPS stimulation, with maximum values reached after 4–6 h before leveling off. As for calculation of our data, Netea

et al. [18] also expressed the results as a ratio vs the amount of the housekeeping gene β -2-microglobulin mRNA to correct for fluctuations in the leukocyte count. However, the background levels were not reported in this study [18].

Also, data for the LPS induced steady state mRNA level of IL-8 in human whole blood have been reported [4,5]. The amounts of IL-8 mRNA were normalized to actin but not expressed relative to the background level. In these studies a biphasic pattern was reported with the first increase at 4 h, a plateau from 6 to 12 h followed by the second increase, which was still escalating at 24 h. In our study we found that the basal steady state mRNA level of IL-8 followed a similar biphasic pattern, whereas the LPS induced IL-8 mRNA levels given either as a ratio above background or as the level minus background were decreasing with time, with the highest level at the 1 h time-point and being at background levels at 6, 20 and 23 h.

We found the basal IL-1 β mRNA level to be 10 times lower than the basal IL-8 mRNA level. Upon induction the IL-1 β mRNA levels were up to 10-fold higher than the IL-8 mRNA ratio above background. Also, the IL-1 β mRNA steady state level minus the background was higher than IL-8 mRNA after LPS or glucan treatment. This suggests that IL-1 β mRNA is more responsive to LPS and glucan than IL-8 mRNA and that different pathways may be involved in the cellular regulation. Generally, in the time-course study similar patterns of fold induced mRNA levels of IL-1 β and IL-8 by the different inducers were observed giving no indication of the involvement of IL-1 β in IL-8 steady state mRNA level. However, for the mould *R. rubra* (Rr) the early 1 h maximum induced ratio of IL-1 β mRNA and in comparison the delayed maximum induced IL-8 mRNA ratio at 3 h may indicate involvement of IL-1 β in IL-8 gene activation. This suggestion is supported by the response patterns of the mould extracts given as level minus the background as all the mould extracts caused secondary increase of IL-8 mRNA at the late time-course points.

Since only minor changes were observed in correction for mononuclear cells, our data support the fact that mononuclear cells play a central role in cytokine expression.

3.4. Cytokine protein

The release of IL-1 β protein upon LPS treated whole blood has been investigated by Nerad et al. [17], DeForge et al. [4] and Netea et al. [18]. In all three studies, IL-1 β protein was detectable after 2–4 h and the maximum level was reached after 6–8 h, sustaining until the 24-h time-point. In the study undertaken by Nerad et al. [17], cytokine production was normalized per 10⁶ mononuclear cells whereas DeForge et al. [4] and Netea

et al. [18] did not normalize the cytokine release. In neither of the studies the basal levels were integrated in the calculation of the results.

DeForge et al. [4] also determined the IL-8 protein release in LPS-stimulated whole blood and found a biphasic pattern similar to that seen for IL-8 steady state mRNA level. However, as for IL-1 β the released levels were not normalized or expressed relative to the non-induced control samples.

In our study the basal cytokine protein levels were increasing with time from the 3-h time-point up to the 20-h time-point for IL-1 β protein and up to the 23-h time-point for IL-8 protein. The fold release above background of both cytokines induced by LPS was unchanged by the time-course, whereas the LPS induced level after subtraction of the background for both cytokines increased with time. We suggest that this difference indicates that the cytokine level increases with time upon treatment with LPS but the cellular capacity to respond does not change over time.

We determined the protein levels at 3, 20 and 23 h. Probably if we had included one to two other time-points, e.g. 6 and 8 h, we might also have seen the highest level of IL-1 β after 6–8 h and not after 20 h of LPS induction as well as a biphasic pattern for IL-8 protein release as reported by DeForge et al. [4].

The basal IL-1 β protein levels were in a range 20 times lower than the basal IL-8 protein levels. However, after induction with LPS and glucan the IL-1 β protein release reached after 3 h a level between 20- and 200-fold higher than the IL-8 protein release, concerning level minus background and fold above background, respectively. The mould extracts, however, induced a three to five times higher release of IL-8 than the release of IL-1 β protein after 20 and 23 h. This may indicate that IL-8 protein release is more sensitive to the mould extracts than the IL-1 β protein, or an effect of the initial IL-1 β release on the subsequent IL-8 release as shown by other studies [4].

As observed for the cytokine mRNA data, the inducers in general elicited similar time-course patterns for the two cytokines. For glucan treatment, however, the maximum fold induced release of IL-1 β and IL-8 at 3 and 20 h, respectively, may indicate that the relatively delayed increase of IL-8, although not significant, may involve IL-1 β .

3.5. Conclusion

In summary our time study elicited the highest effects of LPS and glucan induction of IL-1 β and IL-8 steady state mRNA level after 1 and/or 3 h. For the mould extracts the highest induced mRNA levels were seen after 3 and/or 6 h. Concerning induced cytokine protein release from the cells, the highest levels were observed after 20 and/or 23 h, except for glucan, which induced

the highest fold IL-1 β protein release after 3 h. These data indicate that generally the induced cytokine activation upon allergen provocation is initiated at the gene level followed by an increased release from the cell after approximately 20 h. Interestingly, glucan induced the highest level of IL-1 β mRNA and protein release after 3 h. This may be explained by a simultaneous activation at the gene level and induced release of IL-1 β protein stored in the cell. It is also noteworthy that IL-8 (mRNA and protein) is induced at 3 h after glucan induction whereas no effect on IL-8 is seen after LPS induction, indicating an inductive pathway different from that for IL-1 β .

The similar time-course patterns for IL-1 β and IL-8 steady state mRNA level and protein release by different inducers do not indicate the involvement of IL-1 β in IL-8 steady state mRNA level and protein release. However, the steady state mRNA level of IL-1 β induced by the mould Rr may be involved in the IL-8 steady state mRNA level detected after induction with this mould, and the glucan induced protein release of IL-1 β from the cells may be involved in the cellular release of IL-8 protein, since they have a second peak at 23 h.

Firstly, we conclude upon comparison to other studies, that it is necessary to express the results both as induced ratio above background and as induced level minus background to be able to obtain the effect of the inducers alone and not the effects of other factors influencing the WBA.

Secondly, we found the optimal times to determine the effects of the inducers on IL-1 β and IL-8 steady state mRNA level and protein release. This is of great importance in response evaluations since the effect of the inducers may be lost if not determined at the right time.

The lower basal steady state mRNA level and higher LPS induced steady state mRNA levels of the atopics support our theory about “exhaustion” of the atopic immune system. This hypothesis is further supported by the higher release of cytokines among non-atopics, especially as seen after LPS induction.

Finally, we found several significant differences between the atopic and the non-atopic group at the cytokine steady state mRNA level but only for LPS induced level of protein release. This shows that the analysis of cytokines at the steady state mRNA level of induced cells has the potential to distinguish between non-atopic and atopic individuals.

4. Materials and methods

4.1. Materials

EDTA blood collection tubes were obtained from Meda, Herlev, DK. LPS from the *Escherichia coli*

serotype 055:B5 was purchased from Fluka and the GenElute Mammalian Total RNA Kit was purchased from Sigma–Aldrich, Vallensbaek Strand, DK. β -1,3-D-Glucan was from Curdlan, Art 032-09902, Wako Pure Chem. Industries Ltd., Amsterdam, The Netherlands. The moulds and the skin prick-test kit were obtained from ALK Abello, Frederiksberg, DK. The QIAamp RNA Blood Mini Kit and the Qiagen Plasmid Maxi Kit were obtained from Qiagen, Merck Eurolab A/S, Albertslund, DK. Also, Tween-20 was from Merck Eurolab. The Superscript Preamplification System for First Strand cDNA Synthesis using random hexamers, PCR buffer, $MgCl_2$, the *Taq* polymerase, the restriction enzymes used and RPMI were purchased from Life Technology, Tåstrup, DK. The ELISA kits were purchased from R&D Systems, London, UK. Spin-X columns were from Costar, Biotech Line A/S, Lyngø, DK. The pGEM-T Easy Vector was purchased from Promega, Bie & Berntsen, Rødovre, DK.

4.2. Preparation of the inducers

LPS was dissolved in RPMI and glucan in RPMI + 18% 0.05 M NaOH while the mould extracts were made by homogenizing the mould powder in a 0.05% Tween-20 solution in RPMI for 30–90 s until the powder was dissolved. The samples were stored at -20°C . The concentrations of the inducers were 0.1 mg/ml LPS; 2.5 mg/ml glucan; 1.0 mg/ml *C. sphaerospermum* (Cs), *S. chartarum* (Sc) and *P. chrysogenum* (Pc); and 10.0 mg/ml *R. rubra* (Rr).

4.3. Whole blood assay

After a skin prick-test with 10 common inhalant-allergens, 10 atopics and 10 non-atopics were chosen and peripheral blood was drawn and collected in 10 ml EDTA tubes. Within 4 h, using aseptic handling, aliquots of blood (900 μl) were placed in sterile 1.5 ml Eppendorf tubes and stimulated with 100 μl of inducer (1. LPS; 2. β -1,3-D-glucan; 3. Cs; 4. Sc; 5. Pc; 6. Rr; and solvent controls 7. RPMI; 8. Tween-20). The samples were gently vortexed for 5 s and incubated with open lids for 1, 3, 6, 20 and 23 h at 37°C , in 5% CO_2 and humidified air for RT-PCR and ELISA analyses. For each sample, single incubations were made for RT-PCR while double incubations were performed for ELISA.

4.4. ELISA

After induction, 500 μl of RPMI was added to each sample. The samples were briefly vortexed and centrifuged for 3 min at 10,000 rpm and the supernatant (900 μl) was transferred to new Eppendorf tubes. Then samples were flash frozen in ethanol and dry ice and stored

at -80°C . The ELISA assay was performed according to the manufacturer's instructions.

4.5. RT-PCR

Upon incubation, total RNA was purified with the QIAamp RNA Blood Mini Kit or the GenElute Mammalian Total RNA Kit following the instructions from the manufacturer. The RNA was stored at -80°C until reverse steady state mRNA level. Reverse steady state mRNA level was performed using 1 μg total RNA with the SuperScript Preamplification System for First Strand cDNA Synthesis using random hexamers. The resulting cDNA was frozen at -80°C until use. Real-time PCR was employed with the on-line detection of the PCR reaction based on fluorescence monitoring (Lightcycler, Roche, Hvidovre, DK). Hybridization probes were used to monitor the amount of specific target sequence. Both primers and probes were designed by TIB MolBiol, Berlin, Germany, and purchased at DNA Technology, Aarhus, Denmark. The sequences of the forward (5'–3') and reverse (5'–3') primers of the cytokines as well as the internal control β 2M are: TgTACgATCACTgAACTgCA and gAAgTCAGTTATATCCTggC for IL-1 β ; TCTTggCAGCCTTCCTgATT and TCCAgACAgAgCTCTCTTCCATC for IL-8; and CCAGcAgAgAATggAAAgTC and gACAAgTCTgAA-TgCTCCAC for β 2M. The sequences of the hybridization probes LC 640 (5'–3') and FL 530 (5'–3') used for quantification of the cytokines as well as the internal control are: ATTTgAgTCTgCCCCAgT-TCCCCA and TggTACATCAgCACCTCTCAAgCAG for IL-1 β ; gACATCTAAgTTCTTTAgCACTCCTTggCA and AACTgCACCTTCACACAgAgCTgC for IL-8; and TTCTTCAGTAAgTCAACTTCAATgTCggA and ATgA-AACCCAgAACACATAgCAATTCAg for β 2M. Firstly, the expression of the target genes coding for IL-1 β and IL-8 was normalized to the steady expression of the housekeeping gene β -2-microglobulin (β 2M) which was found to be unaffected by the different inducing agents. Secondly, these β 2M corrected data were further corrected for the percentage of mononuclear cells to test a possible different response of cell types. For the three genes, each PCR reaction was optimized with respect to the $MgCl_2$ concentration, the optimal concentration of primers and probes and the best PCR cycling. The optimal reactions were achieved at same amounts of $MgCl_2$, primers and probes, while separate PCR cycling programs were necessary.

The mastermix was prepared according to the number of samples. For each sample the following were added: 2.0 μl 1 $\times$ PCR buffer + 1.2 μl 3 mM $MgCl_2$ + 1.0 μl 0.5 μM primer mix + 1.5 μl 0.15 μM probe mix + 0.4 μl 0.2 mM dNTP mix + 0.5 μl 2.5 U/ μl *Taq* polymerase + 11.4 μl H_2O . The 18 μl mastermix was placed in a capillary in the pre-cooled adapter block and

2 µl of cDNA (plasmid standards, see below, samples or negative control) was added. The capillaries were closed and centrifuged for 5 s at 2000 rpm before they were placed in the Lightcycler rotor at the following positions: 1–7 standards; 8–30 samples; 31 and 32 negative control. The negative control used was water.

Denaturation of IL-1 β and β 2M was performed at 95 °C for 30 s while IL-8 was denatured for 60 s. The PCR amplification programs were as follows: IL-1 β , denaturation at 95 °C for 0 s, annealing at 56 °C for 10 s followed by fluorescence measurements and elongation at 72 °C for 17 s for a total of 33 cycles; IL-8, 95 °C for 1 s, 60 °C for 20 s and 72 °C for 10 s for a total of 30 cycles; β 2M, 95 °C for 0 s, 60 °C for 10 s and 72 °C for 5 s for a total of 30 cycles. Each run was followed by cooling at 40 °C for 30 s. The amplicon length was 433 bp for IL-1 β , 211 bp for IL-8 and 125 bp for β 2M.

4.6. Cloning of plasmid standards

Cytokine cDNA fragments were generated by RT-PCR with the gene specific primers given in Table 1. The PCR program was followed by 10 min at 72 °C to ensure the addition of adenosine at the 3'-ends of the amplified fragments. Each amplicon was purified on a Spin-X column and the PCR product was purified by butanol–phenol–chloroform extraction followed by ethanol precipitation. The fragments were ligated into the pGEM-T Easy Vector according to the protocol and transformed into competent *E. coli* cells (genotype K12; *lac-pro*, *supE*, *thi*, *hsdD5/F'* *traD36*, *proA* + *B*, *lacI*^q, *lac Z* Δ M15). The cloned plasmids were verified by restriction enzyme digestion.

The Qiagen Plasmid Maxi Kit was used for the large scale preparation and the concentration of the plasmids was determined by measuring absorbance at 260 and 280 nm using the Shimadzu UV-160 A spectrophotometer (Struers, Denmark) and the preparation was diluted to a concentration of 0.1 µg/µl and stored at 4 °C. The plasmids were linearized with the *ScaI* enzyme and stocks of 0.01 µg/µl plasmid were stored at –20 °C. Before use, this stock was diluted 10–10⁶ times for the standard curve.

4.7. Quantification

Each cDNA sample was diluted five times. From this dilution all three genes were quantified in sequential separate runs. For each gene at least two independent runs were performed.

The signal of the samples was quantified from the standard curve by the Lightcycler software (version 3.0) using the Fit Points method and proportional baseline adjustment.

For each sample the expressions of IL-1 β and IL-8 were determined relative to the housekeeping gene β 2M.

Because of inter-run variations and drifting basal levels, the results are expressed relative to the basal levels after normalization against the housekeeping gene. The basal non-induced levels were determined in samples incubated with the solvents RPMI or Tween-20 solution for each time-point.

4.8. Statistical analysis

Statistical analysis was performed in Microsoft Excel (Windows 98). The *t*-test was carried out with the two-tailed test with different variance and considered statistically significant with $p \leq 0.05$. The statistical calculations were performed at three levels, data minus background, ratio above background and after correction for percent mononuclear cells.

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