4. Results

Part 1: Comparison of the in vivo fever reaction of the rabbit with the in vitro cytokine production measured by ELISA system

In order to determine whether the in vivo and in vitro reaction of the rabbit was comparable, a series of experiments was done using the same LPS (the international WHO endotoxin standard derived from *E. coli* O113) as in the in vivo experiment performed at the NIBSC, where rabbits had been challenged intravenously with different concentrations of *E. coli* and the fever reaction as well as the in vivo cytokine production had been measured (please refer to the introduction, Fig. 1). Additionally, a recent set of experiments at the Paul-Ehrlich Institute had been performed, where an in vivo fever threshold of altogether 171 rabbits between 0.5 and 2.0 EU/ml (5 and 20 EU/kg) had been determined. For this purpose, the whole blood of seven rabbits was challenged with different concentrations of *E. coli* LPS, and detection limits were determined by one-way ANOVA followed by Dunnett´s post test (all stimulations compared to unstimulated control). In these and all following figures, the graph´s x-axes show the LPS-concentrations expressed in EU (Endotoxin Units)/ml, 1 EU/ml corresponding to 100 picogram/ml of the WHO endotoxin standard, or alternatively in picogram (pg)/ml. The y-axes show the blood concentrations of the respective cytokine/chemokine in pg/ml, as measured by ELISA. Cytokine concentrations were calculated by pipetting known concentrations of recombinant cytokine into the ELISA assay and calculating the concentrations formed in the whole blood according to those. The variability is expressed as the standard deviation, SD. If the data follow a bell-shaped Gaussian distribution, then 68% of the values lie within one SD of the mean (on either side) and 95% of the values lie within two SD of the mean. The SD was calculated by Graph Pad Prism Software.

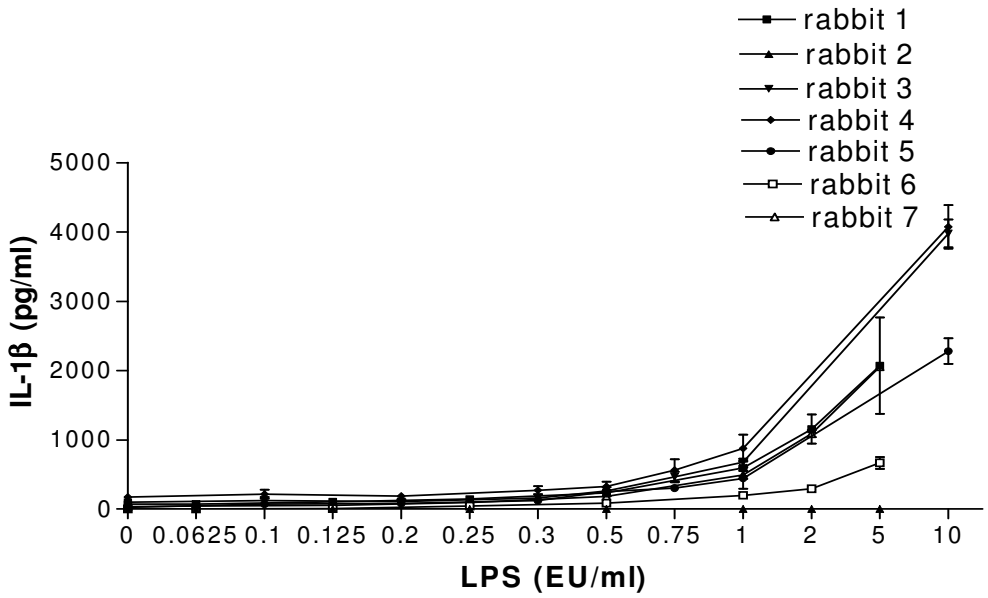

Fig. 2: In vitro IL-1β response of 7 rabbits towards the WHO reference LPS from *E. coli* O113

Detection limits							
rabbit	1	2	3	4	5	6	7
EU/ml	2.0	> 5	0.75	1.0	1.0	0.75	0.5

From the same supernatants, an IL-8 ELISA was performed (Fig. 3)

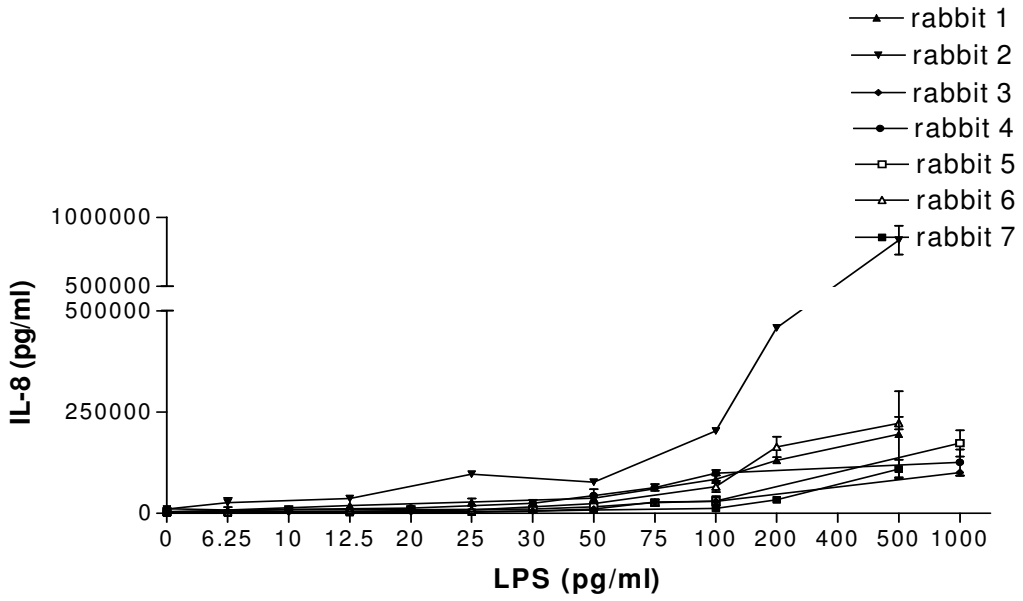

Fig. 3: In vitro IL-8 response of the same rabbits as in Fig. 2 towards LPS from *E. coli* O113

Detection limits							
rabbit	1	2	3	4	5	6	7
EU/ml	5.0	5.0	0.75	0.5	10	1.0	2.0

When the experiments were conducted, the IL-6 and TNF-α antibodies were not yet available. It was however possible to remeasure the supernatants of 2 rabbits later (Fig. 4 and 5).

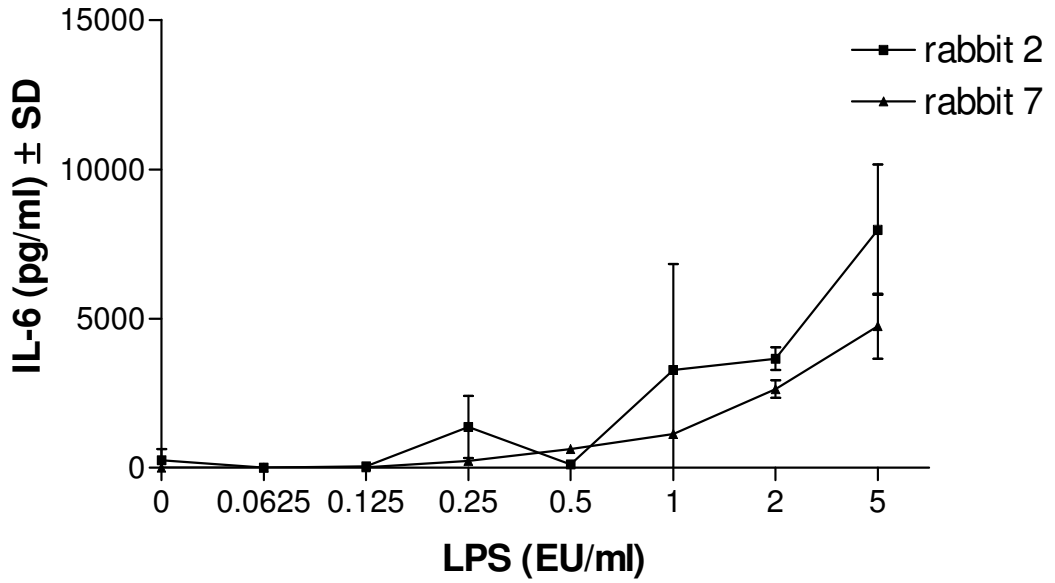

Fig. 4: In vitro IL-6 response of rabbit 2 and 7 from the Fig. 2 and 3 towards LPS from *E. coli* O113

Detection limits		
rabbit	2	7
EU/ml	5.0	1.0

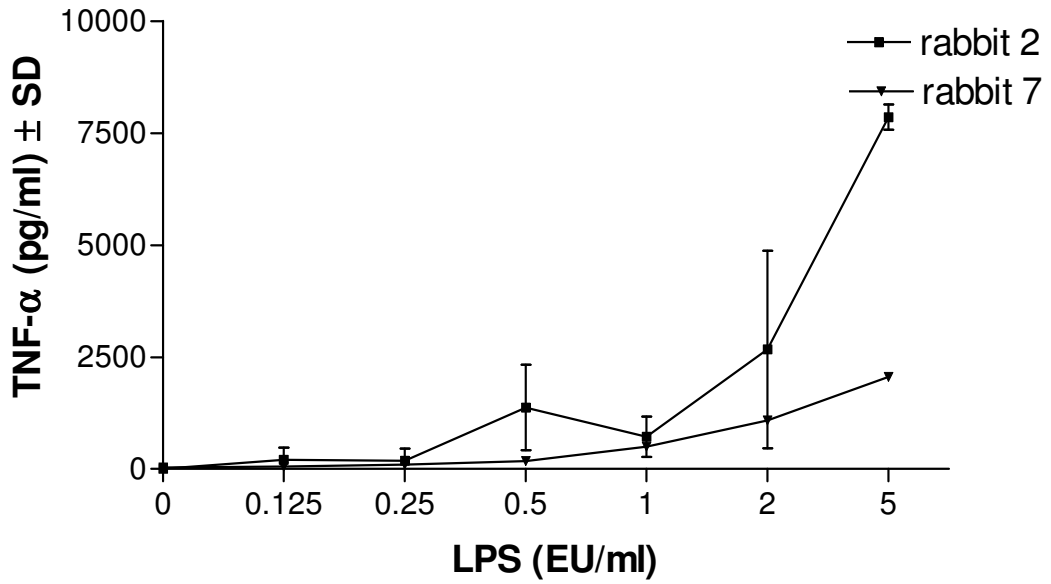

Fig. 5: In vitro TNF-α response of rabbit 2 and 7 from Fig. 2 and 3 towards LPS from *E. coli* O113

Detection limits		
rabbit	2	7
EU/ml	5.0	0.5

According to Mott, the average mature rabbit has 40-50 ml blood/kg bodyweight (Mott, 1967). In this case, 40 EU/kg applied in vivo correspond almost exactly to 1 EU/ml in vitro, which could be calculated as the first concentration inducing a significant cytokine release (Fig. 2). IL-8 seems to be a good and sensitive parameter as well. It is noticeable that the amounts of IL-8 produced exceed the amount of IL-1β by the factor 50. This of course requires a high predilution of the samples in the ELISA system in order to keep the optical density within the recombinant standard curve. The variance when measuring these samples is inevitably higher than when the supernatants can be measured nondiluted (coefficient of variation in the case of IL-1β usually below 15%, in the case of IL-8 sometimes > 35%), which of course affects the statistical evaluation. Rabbit 7 is a good example; the mean of the saline control is 4.66 ng/ml IL-8, at 0.3 EU/ml it was already at a mean of 10.5 ng IL-8/ml and at 0.5 EU/ml at 15.8 ng/ml. Still, the rise is not significant, since the standard deviation is 1.278 and 3.29, respectively. Therefore, the IL-8 readout is very sensitive, but the high dilution makes an evaluation difficult.

Another noticeable result was the cytokine response of rabbit 2. Failing completely to produce significant amounts of IL-1β when challenged with *E. coli* O113 LPS, it still made the highest amounts of IL-8 and normal amounts of IL-6 and TNF-α. It would have been interesting to have made a parallel in vivo challenge. Still, the phenomenon occurs from time to time and seems, from our observations, to restrict itself to *E. coli* O113 LPS and LTA (Schindler et al., 2003).

Having demonstrated that the in vivo and in vitro rabbit test show similar results, a wider variety of types of LPS was employed, comparing the in vitro rabbit blood test to the in vitro human blood test in order to see whether differences in the reactivity of both species could be found.

Part 2: Comparison of the in vitro reaction of humans and rabbits towards a variety of stimuli.

As a first step, the endotoxin of *E. coli* O113: H10 previously used in the in vivo and in vitro rabbit assay was employed in the whole blood of three human donors and IL-1β, IL-6, TNF-α and IL-8 were measured. It interested us whether the sensitivity of the humans towards the international WHO endotoxin standard would be similar to the in vivo and in vitro reaction of the rabbits in the former experiments and whether the four human readouts were comparable and useful.

4.1. *E. coli* O113: H10

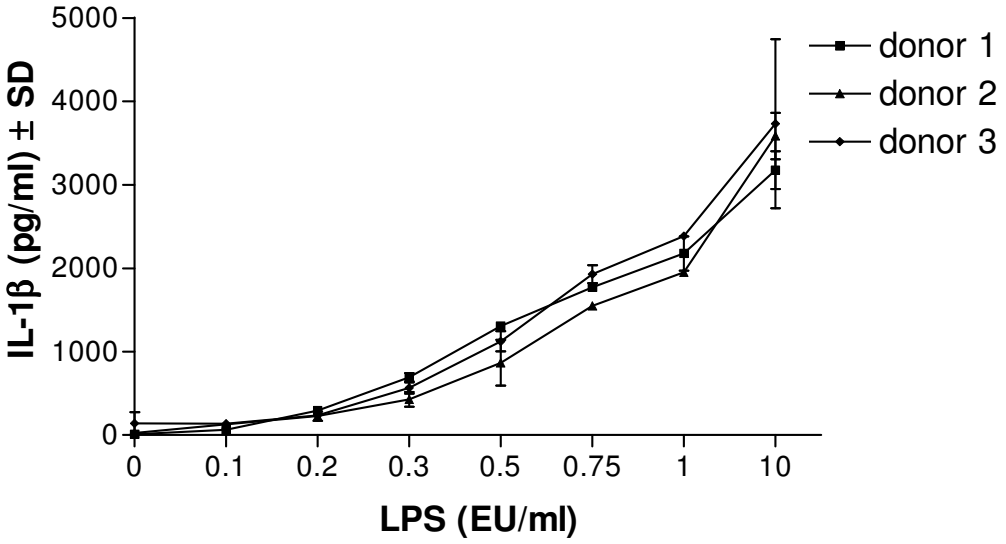

Fig. 6: In vitro IL-1β response of three human donors towards LPS from *E. coli* O113:H10

Detection limits			
donor	1	2	3
EU/ml	0.3	0.5	0.75

The IL-1β response of all donors is extremely uniform, much more than that of the rabbits in Fig. 2, which differ in their maximum response between 500 and 4000 pg/ml IL-1β. The detection limits correspond almost exactly to those of the rabbits. Variability is very low and the sensitivity of the whole blood assay followed by IL-1β measurement very good.

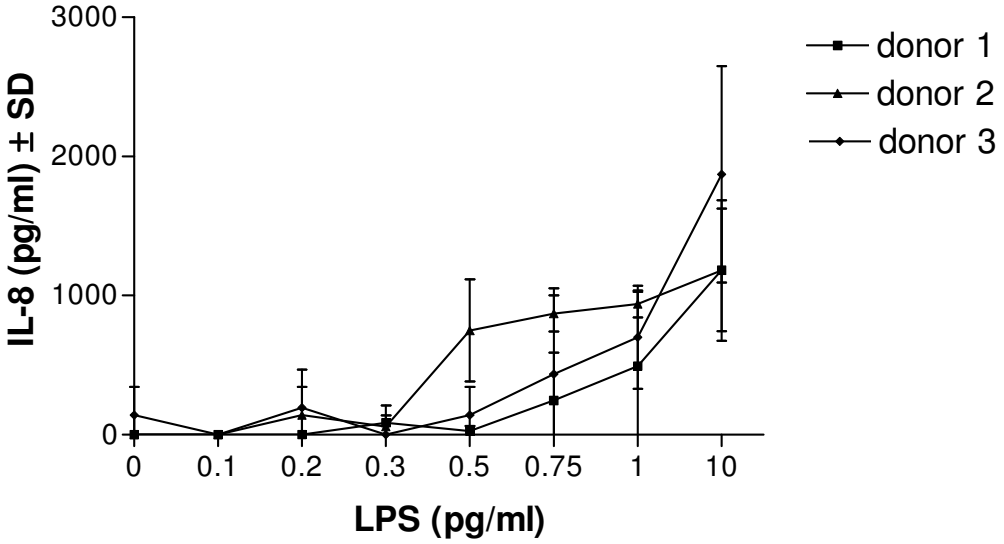

Fig. 7: In vitro IL-8 response of three human donors towards LPS from *E. coli* O113: H10

Detection limits			
donor	1	2	3
EU/ml	10	0.75	10

The IL-8 readout has a variance problem and therefore, the evaluation is hampered. Comparatively little amounts of IL-8 (2 ng/ml) are produced at all in this experiment, when compared to the rabbit IL-8 assay (0.2-1 µg/ml).

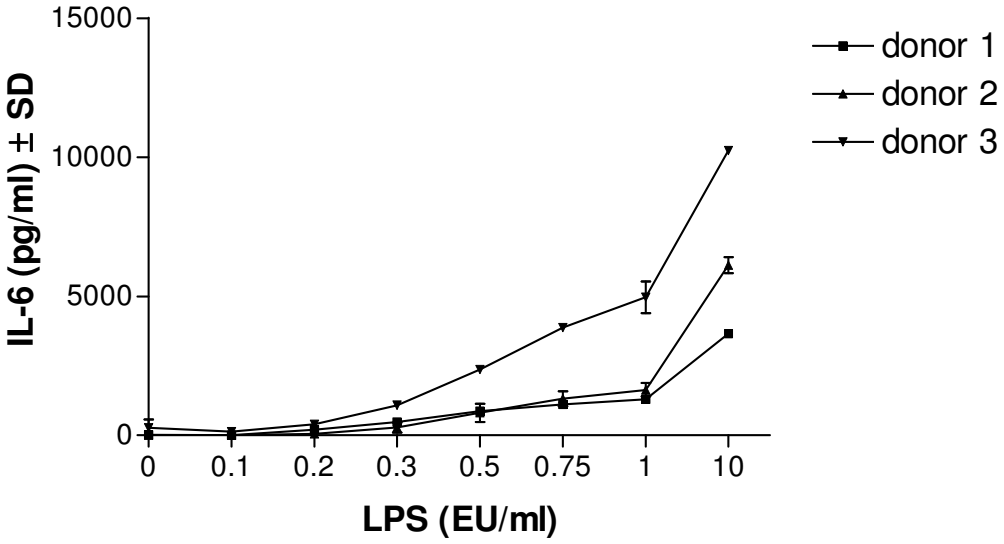

Fig. 8: In vitro IL-6 response of three human donors towards LPS from *E. coli* O113:H10

Detection limits			
donor	1	2	3
EU/ml	0.2	0.5	0.5

The IL-6 assay, then again, performs extremely well. All values hardly show any variation at all (as low as 12 pg/ml). The results are very similar to the human IL-1β assay, they seem even a little more sensitive. Still, the reaction of the individuals is less uniform than the IL-1β response. The amounts of IL-6 are very similar to those produced by the rabbits in Fig. 4.

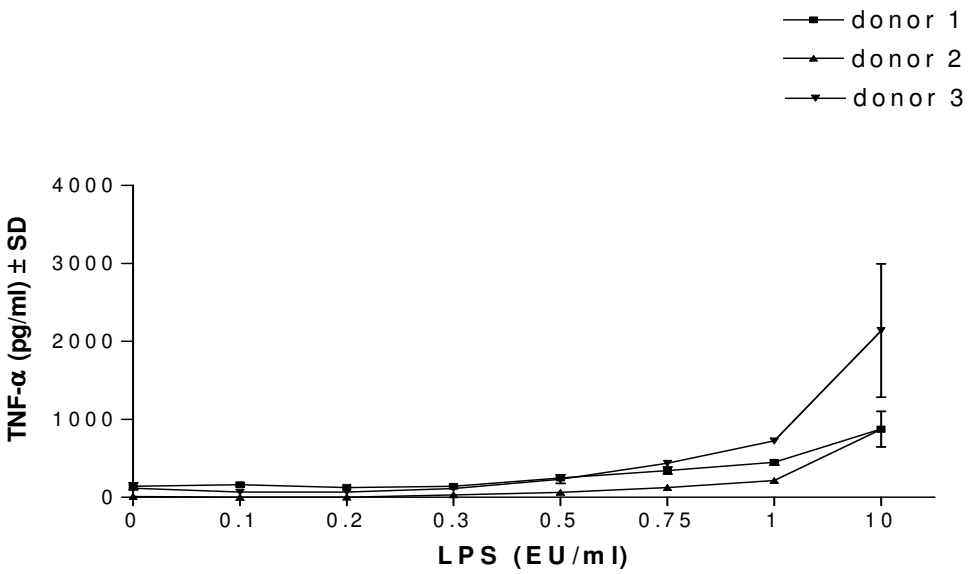

Fig. 9: In vitro TNF-α response of three human donors towards LPS from *E. coli* O113:H10

Detection limits			
donor	1	2	3
EU/ml	0.5	10	10

The TNF-α assay, then again, has a low variance and a low sensitivity. The amounts of TNF-α that can be retrieved from all three donors are relatively low when compared to the other assays.

Next, a wide variety of mostly Gram-negative stimuli was employed. The whole blood incubations for both species were performed in parallel, using the same materials and the same LPS dilutions whenever possible in order to ensure maximum comparability. It interested us whether by using the in vitro approach, differences in the sensitivity between humans and rabbits could be found which would maybe allow a retrospective judgment of the in vivo rabbit pyrogen test that had so far been the gold standard. The endotoxins used were three further types of LPS from *E. coli*, 2 types of LPS from *Salmonella*, one from *Shigella* and *Serratia*, respectively, and one Gram-positive stimulus, LTA from *Bacillus subtilis*.

4.2. *E. coli* O111: B4

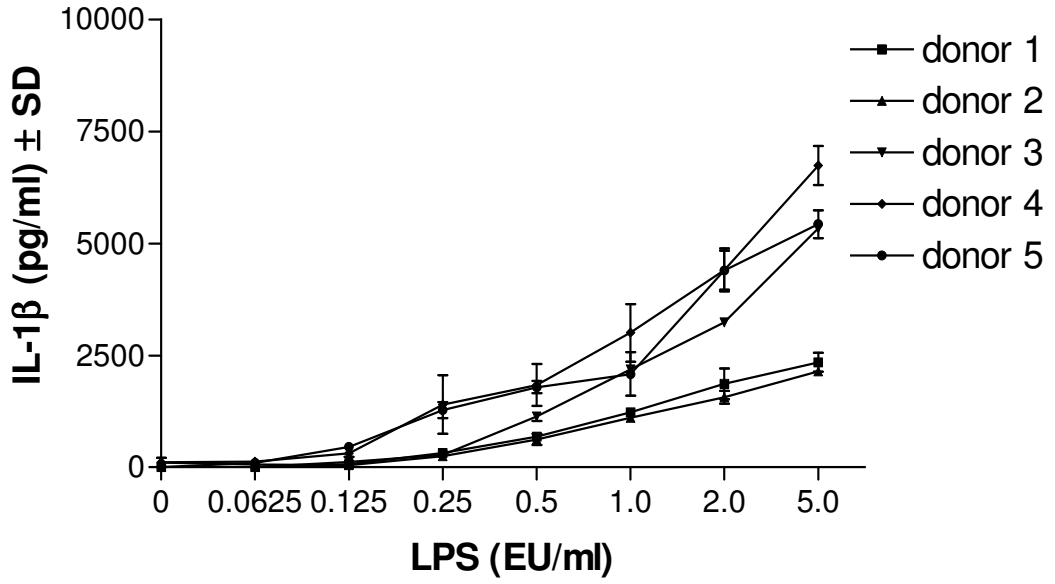

Fig. 10: In vitro IL-1β response of human whole blood incubated with LPS from *E. coli* O111: B4

Detection limits					
donor	1	2	3	4	5
EU/ml	0.5	0.5	0.25	0.5	0.25

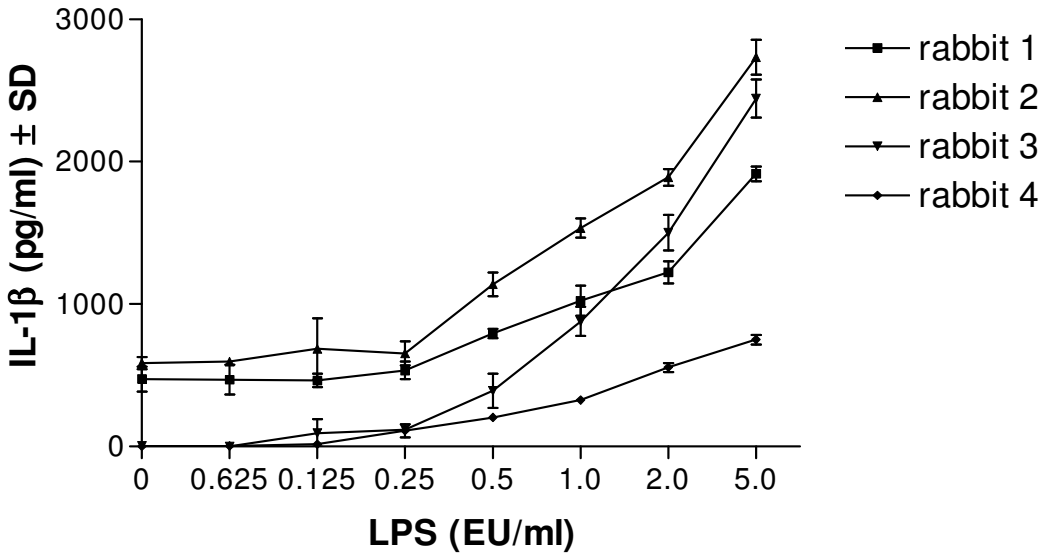

Fig. 11: In vitro IL-1β response of rabbit whole blood towards LPS from *E. coli* O111: B4

Detection limits				
rabbit	1	2	3	4
EU/ml	0.5	0.5	0.5	0.5

Again, the IL-1β assay shows a very good performance for both species. The detection limits of humans and rabbits are again very similar (Fig. 10 and 11). The human donors are less uniform this time in their IL-1β response (Fig. 6) but this does not affect their sensitivity. Next, the same supernatants were measured in the respective IL-8 ELISA (Fig. 12 and 13).

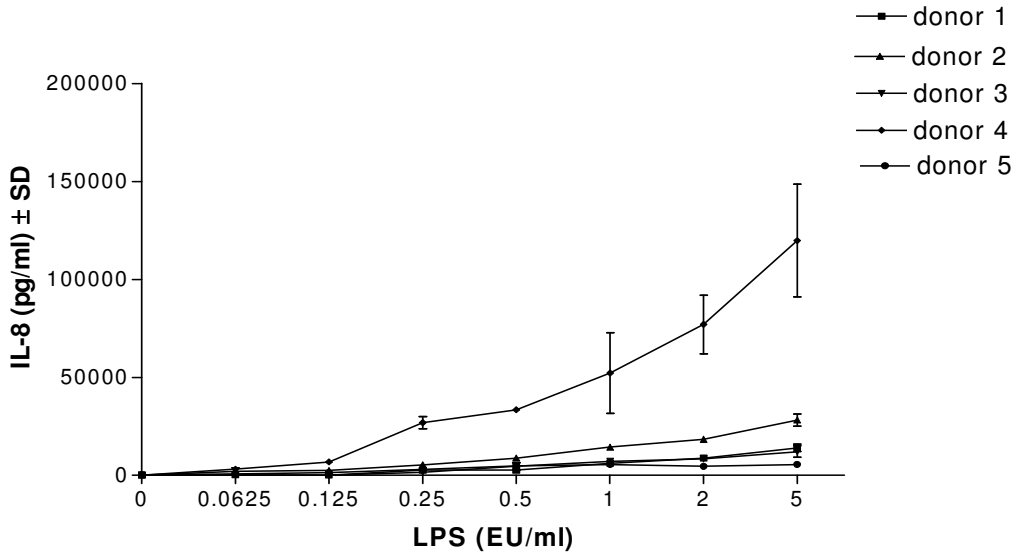

Fig. 12: In vitro IL-8 response of human whole blood towards LPS from *E. coli* O111:B4

Detection limits					
donor	1	2	3	4	5
EU/ml	1.0	0.25	0.5	1.0	0.25

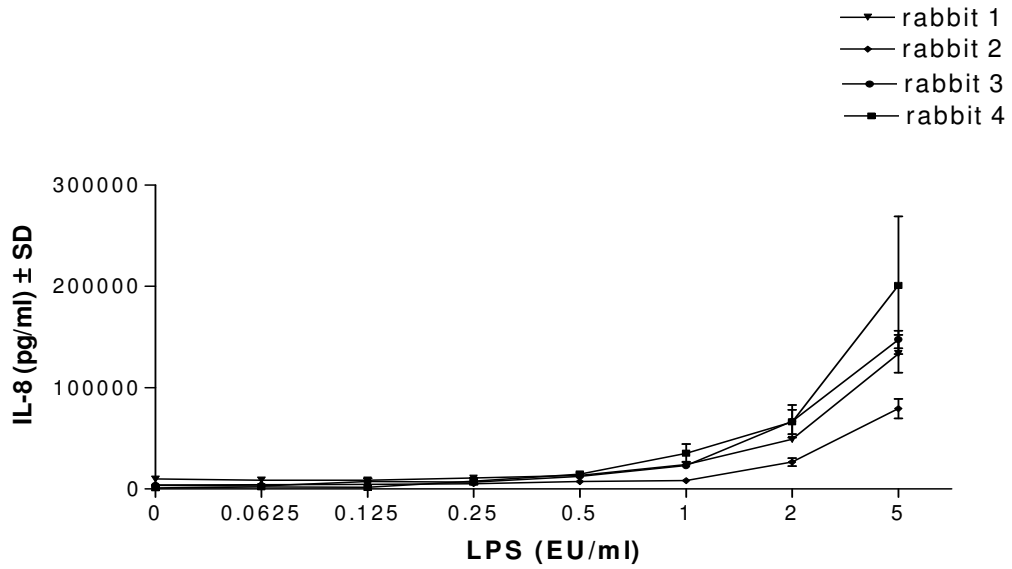

Fig. 13: In vitro IL-8 response of rabbit whole blood towards LPS from *E. coli* O111: B4

Detection limits				
rabbit	1	2	3	4
EU/ml	2.0	2.0	2.0	2.0

The amount of IL-8 is huge- 100 ng/ml and more, this time in both species. The detection limit is not as good as for IL-1β for the rabbit. For the humans it is an extremely sensitive parameter in this case. The performance of the human IL-8 assay could obviously be improved when compared to Fig. 7. Donor 4 differs remarkably from the others concerning his response (ca. 100 ng/ml maximum); compared to that, e.g. donor 5 doesn´t seem to produce any IL-8, but he responds at 0.25 EU/ml and makes 5 ng/ml IL-8 at the maximum challenge. The supernatants of both species were then measured for IL-6 (Fig. 14 and 15).

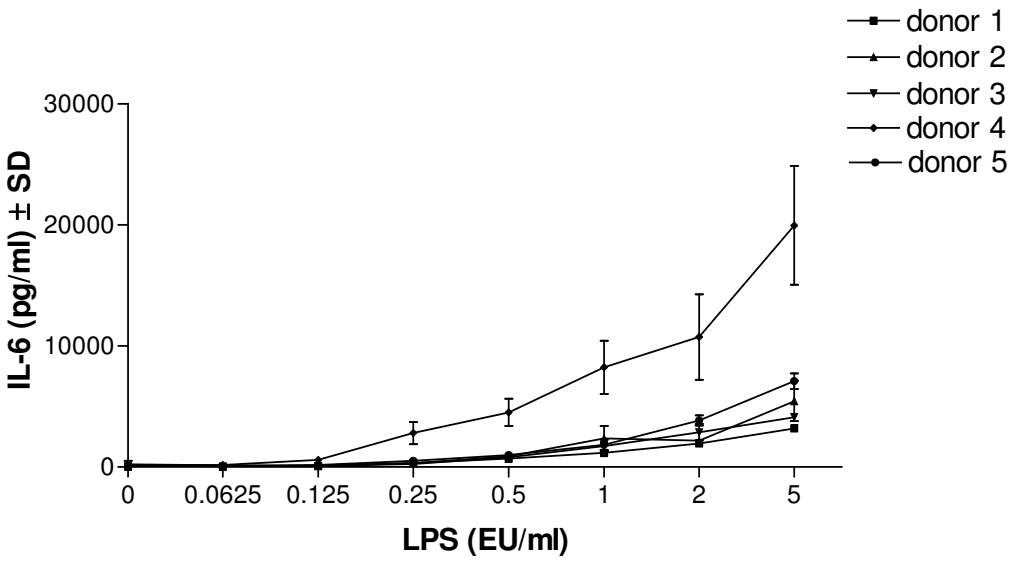

Fig. 14: In vitro IL-6 response of human whole blood towards LPS from *E. coli* O111:B4

Detection limits					
donor	1	2	3	4	5
EU/ml	0.25	0.5	1.0	1.0	1.0

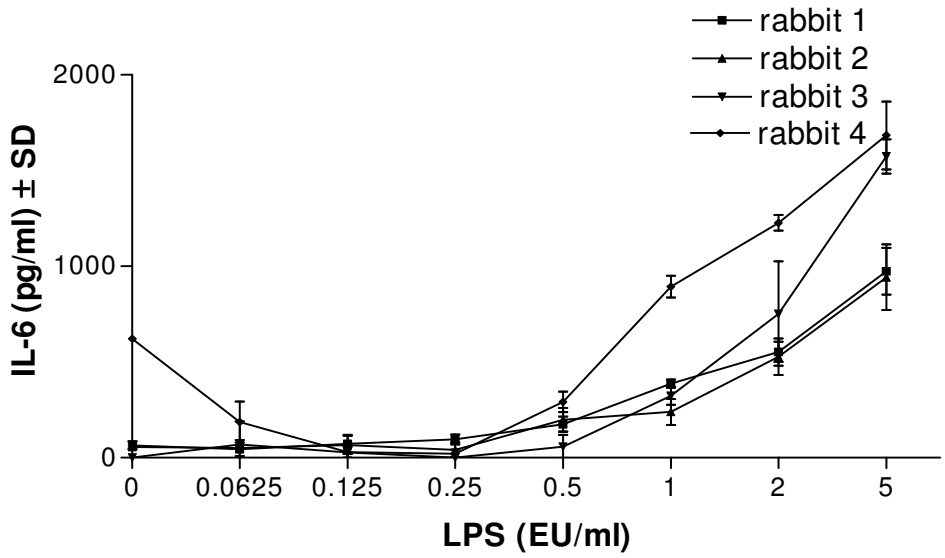

Fig. 15: In vitro IL-6 response of rabbit whole blood towards LPS from *E. coli* O111:B4

Detection limits				
rabbit	1	2	3	4
EU/ml	1.0	2.0	1.0	1.0

The humans produce more than IL-1β than IL-6, the rabbits make equal amounts. Human donor 4 again exceeds by far the response of the others (Fig. 14). The sensitivity is slightly lower than the one of the IL-1β assay in this experiment. The last endpoint measured in both species was then again TNF-α.

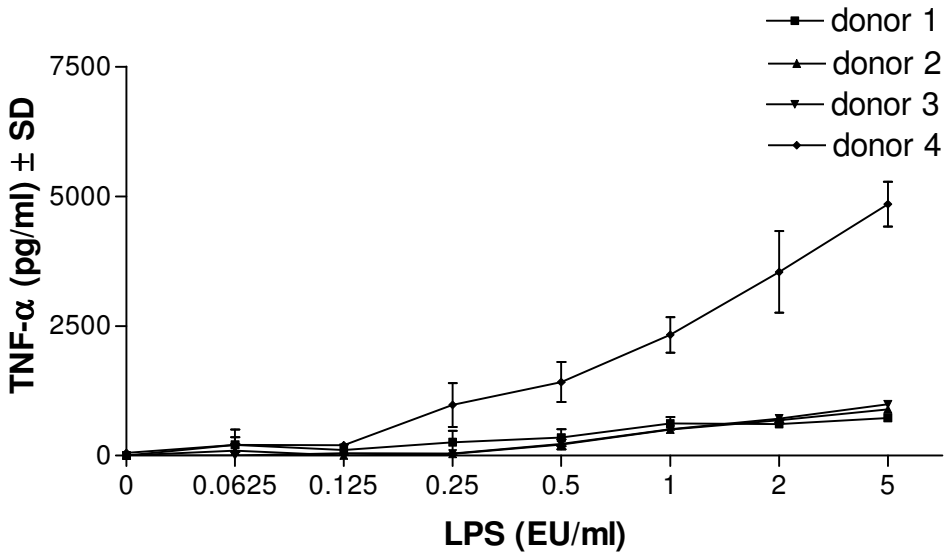

Fig. 16: In vitro TNF-α response of human whole blood towards LPS from *E. coli* O111:B4

Detection limits				
donor	1	2	3	4
EU/ml	1.0	1.0	0.5	0.5

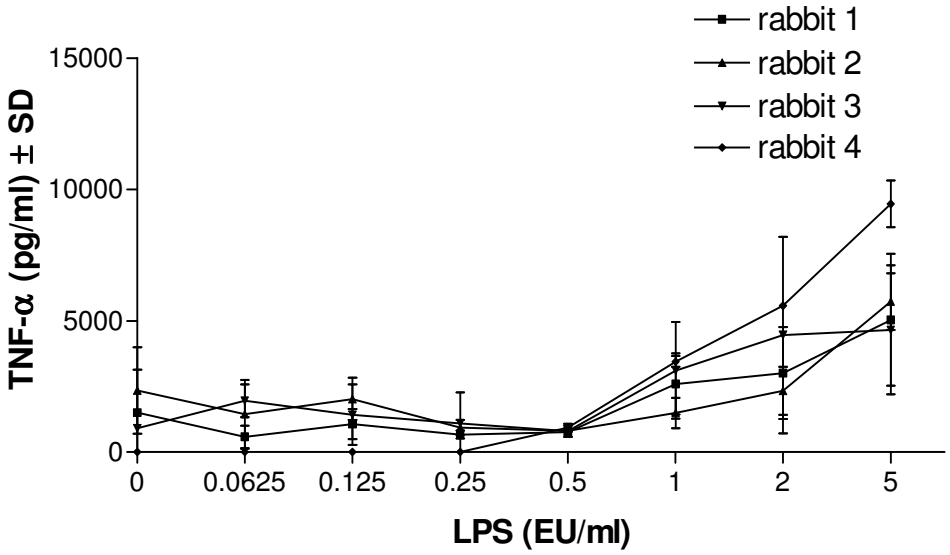

Fig. 17: In vitro TNF-α response of rabbit whole blood towards LPS from *E. coli* O111: B4

Detection limits				
rabbit	1	2	3	4
EU/ml	5.0	5.0	> 5	0.5

TNF-α seems to be reasonably sensitive in humans, but doesn´t perform too well in the rabbits. There is a certain basic level to be seen in almost all 3 rabbits at the negative control, hampering sensitivity in this case. The human donor 4 is a so-called high producer, making approximately five times the amounts of cytokines than the others. This difference is not so pronounced in the IL-1β assay (Fig. 10).

4.3. *E. coli* O128

A third LPS from *E. coli* was used, which had shown less pyrogenic activity than the other two in previous experiments in order to determine whether this difference was recognized in both species. Since no calibration to the standard endotoxin *E. coli* O113 had been done with this and the following stimuli, all further pyrogens are not expressed in EU/ml, but in pg (ng/µg, respectively)/ml. The following table shows the detection limit of each human or rabbit donor in pg/ml as determined by one-way ANOVA (Table 1).

cytokine	IL-1β	IL-8	IL-6	TNF-α
Humans				
Donor 1	100 pg/ml	200 pg/ml	100 pg/ml	400 pg/ml
Donor 2	200 pg/ml	100 pg/ml	100 pg/ml	200 pg/ml
Donor 3	400 pg/ml	200 pg/ml	200 pg/ml	400 pg/ml
Rabbits				
Rabbit 1	200 pg/ml	200 pg/ml	400 pg/ml	>400 pg/ml
Rabbit 2	200 pg/ml	200 pg/ml	400 pg/ml	>400 pg/ml
Rabbit 3	200 pg/ml	200 pg/ml	400 pg/ml	>400 pg/ml
Rabbit 4	400 pg/ml	100 pg/ml	100 pg/ml	400 pg/ml

Table 1: Limits of detection for LPS from *E. coli* O128 measured as cytokine response of humans and rabbits; LPS dilutions (pg/ml): 0/6.25/12.5/25/50/100/200/400

The LPS from *E. coli* O128 is indeed less active in both species. Again, the rabbit IL-6 and rabbit TNF-α as well as the human TNF-α assay are not as sensitive as the others. Since the data for the LPS from *E. coli* had not shown major differences in the human and the rabbit assays, LPS from *Salmonella* species were used next. The LPS from *S. typhosa* had previously shown a high pyrogenic potency, comparable to that of the *E. coli* WHO standard, whereas the LPS from *S. typhimurium* shows pyrogenic activity only at concentrations in the nanogram area. We addressed the question whether differences in the responses could maybe be found with another type of LPS.

4.4. *Salmonella typhosa* (Table 2)

cytokine	IL-1β	IL-8	IL-6	TNF-α
Humans				
Donor 1	30 pg/ml	50 pg/ml	30 pg/ml	100 pg/ml
Donor 2	50 pg/ml	50 pg/ml	50 pg/ml	50 pg/ml
Donor 3	50 pg/ml	50 pg/ml	50 pg/ml	50 pg/ml
Rabbits				
Rabbit 1	200 pg/ml	20 pg/ml	>200 pg/ml	>200 pg/ml
Rabbit 2	30 pg/ml	200 pg/ml	>200 pg/ml	>200 pg/ml
Rabbit 3	50 pg/ml	75 pg/ml	200 pg/ml	200 pg/ml
Rabbit 4	50 pg/ml	50 pg/ml	200 pg/ml	100 pg/ml
Rabbit 5	50 pg/ml	50 pg/ml	50 pg/ml	100 pg/ml

Table 2: Limits of detection for LPS from *Salmonella typhosa* measured as cytokine response of humans and rabbits; LPS dilutions (pg/ml):
humans: 0/10/20/30/50/75/100/200
rabbits 1-3: 0/10/20/30/50/75/100/200
rabbit 4 and 5 : 0/6.25/12.5/25/50/100/200/400

Again, the human assays perform extremely uniformly and show a high sensitivity. The rabbit IL-1β ELISA is the most sensitive and the one with the highest comparability to

the human system. IL-6 and TNF-α are not very sensitive, whereas the IL-8 does well. It was confirmed that the LPS from *S. typhosa* is a pyrogen that requires only minimum concentrations to evoke a reaction.

4.5. *Salmonella typhimurium* (Table 3)

cytokine	IL-1β	IL-8	IL-6	TNF-α
Humans				
Donor 1	250 pg/ml	1000 pg/ml	250 pg/ml	500 pg/ml
Donor 2	250 pg/ml	250 pg/ml	125 pg/ml	1000 pg/ml
Donor 3	250 pg/ml	500 pg/ml	500 pg/ml	1000 pg/ml
Rabbits				
Rabbit 1	1000 pg/ml	1000 pg/ml	5000 pg/ml	1000 pg/ml
Rabbit 2	1000 pg/ml	1000 pg/ml	5000 pg/ml	1000 pg/ml
Rabbit 3	500 pg/ml	1000 pg/ml	1000 pg/ml	1000 pg/ml

Table 3: Limits of detection for LPS from *Salmonella typhimurium* measured as cytokine response of humans and rabbits; LPS dilutions (pg/ml):
0/31.25/62.5/125/250/500/1000/5000

With the LPS from *S. typhimurium*, for the first time differences in the species-specific response could be observed. Even for the so far completely comparable IL-1β assay, the rabbit shows a 4 fold less sensitive response than do the humans (250 pg/ml for the humans, 1000 pg/ml for the rabbits). The human ELISAs show a differing sensitivity, with IL-1β and IL-6 as the most sensitive ones and TNF-α as the least, reminding of the results obtained with the LPS from *E. coli* O113 (Fig. 9). All 4 assays for the rabbit have a very low sensitivity.

Two more types of LPS with a high pyrogenic activity were used for comparison of the human and the rabbit species, that is LPS from *Shigella flexneri* and LPS from *Serratia*

marcescens. Both had evoked a cytokine response in the human at concentrations around 30 to 50 pg/ml in previous experiments.

4.6. *Shigella flexneri 1A* (Table 4)

cytokine	IL-1β	IL-8	IL-6	TNF-α
Humans				
Donor 1	50 pg/ml	50 pg/ml	75 pg/ml	100 pg/ml
Donor 2	75 pg/ml	75 pg/ml	100 pg/ml	>200 pg/ml
Donor 3	50 pg/ml	30 pg/ml	200 pg/ml	75 pg/ml
Rabbits				
Rabbit 1	400 pg/ml	400 pg/ml	>400 pg/ml	>400 pg/ml
Rabbit 2	50 pg/ml	400 pg/ml	>400 pg/ml	>400 pg/ml
Rabbit 3	100 pg/ml	400 pg/ml	400 pg/ml	>400 pg/ml

Table 4: Limits of detection for LPS from *Shigella* measured as cytokine response of humans and rabbits; LPS dilutions (pg/ml):
Humans : 0/10/20/30/50/75/100/200
Rabbits : 0/6.25/12.5/25/50/100/200/400

4.7. *Serratia marcescens* (Table 5)

cytokine	IL-1β	IL-8	IL-6	TNF-α
Humans				
Donor 1	75 pg/ml	30 pg/ml	50 pg/ml	50 pg/ml
Donor 2	75 pg/ml	50 pg/ml	50 pg/ml	75 pg/ml
Donor 3	30 pg/ml	30 pg/ml	30 pg/ml	50 pg/ml
Donor 4	75 pg/ml	75 pg/ml	50 pg/ml	100 pg/ml
Donor 5	30 pg/ml	30 pg/ml	50 pg/ml	200 pg/ml
Donor 6	50 pg/ml	100 pg/ml	75 pg/ml	200 pg/ml

Rabbits				
Rabbit 1	75 pg/ml	100 pg/ml	75 pg/ml	100 pg/ml
Rabbit 2	20 pg/ml	100 pg/ml	200 pg/ml	>200 pg/ml
Rabbit 3	50 pg/ml	100 pg/ml	200 pg/ml	>200 pg/ml
Rabbit 4	50 pg/ml	50 pg/ml	75 pg/ml	>200 pg/ml

Table 5: Limits of detection for LPS from *Serratia* measured as cytokine response of humans and rabbits; LPS-dilutions (pg/ml): 0/6.25/10/12.5/25/30/50/75/100/200/400

Rabbit Number 1 shows almost no response to the LPS from *Shigella*, which holds true for all the cytokines measured. The rabbits 2 and 3 react to this particular LPS with the production of IL-1β, but hardly show any other cytokine response. The LPS shows excellent activity in the human assays. The activity of the LPS of *Serratia*, on the other hand, is very comparable, especially again for the IL-1β assay. TNF-α has a relatively low sensitivity in both species.

Since, with the exception of *Salmonella typhimurium* and *Shigella*, no major differences in the reactivity to various types of LPS could be found, a so-called non-endotoxin stimulus derived from the Gram-positive *Bacillus subtilis* was employed and the reactivity was determined by ELISA (Fig. 18-25).

4.8. *Bacillus subtilis*

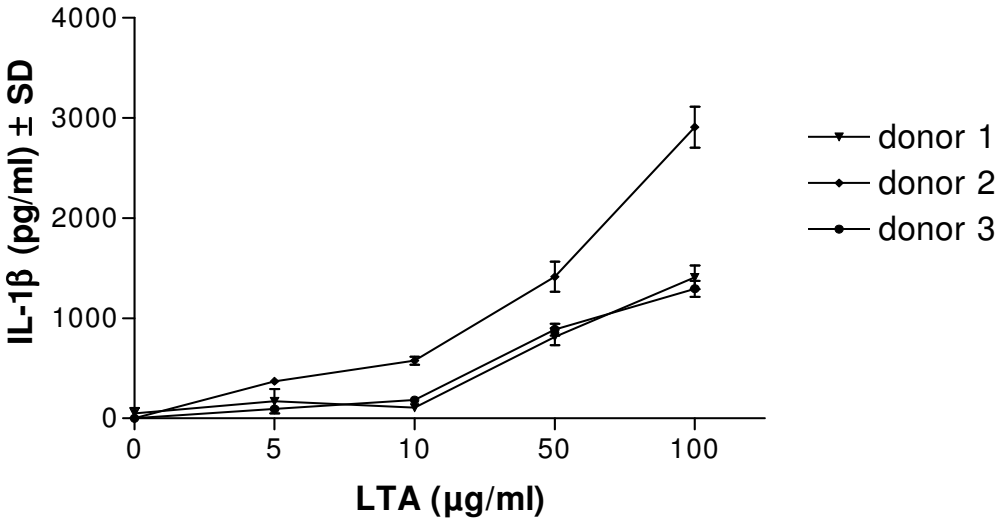

Fig. 18: In vitro IL-1β response of human whole blood towards LTA from *B. subtilis*

Detection limits			
donor	1	2	3
µg/ml	50	10	10

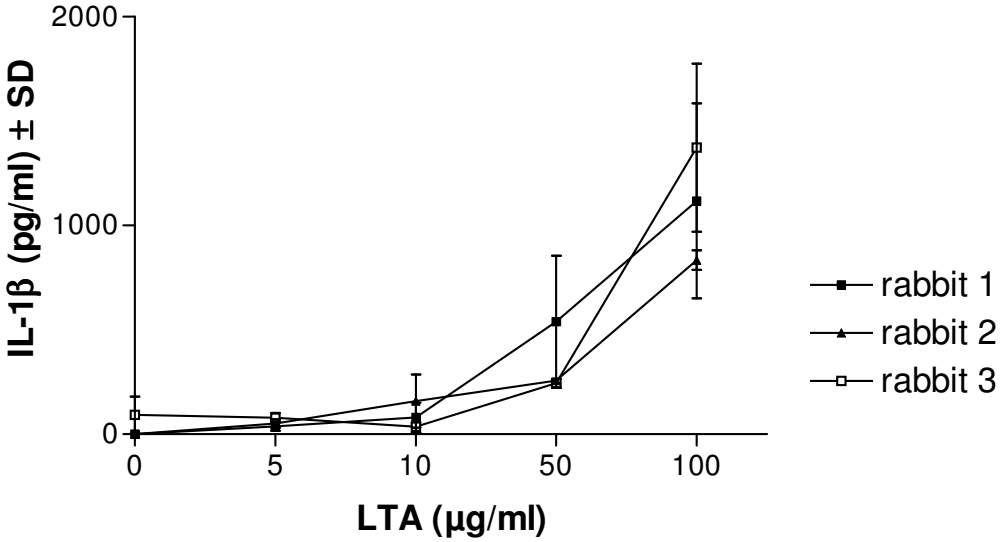

Fig. 19: in vitro IL-1β response of rabbit whole blood towards LTA from *B. subtilis*

Detection limits			
rabbit	1	2	3
µg/ml	100	50	100

The rabbits shows a reduced activity towards *B. subtilis*, when compared to the humans, even with the so far most reliable IL-1β readout. Rabbit 1 shows an extremely high variability in response which hampers the evaluation.

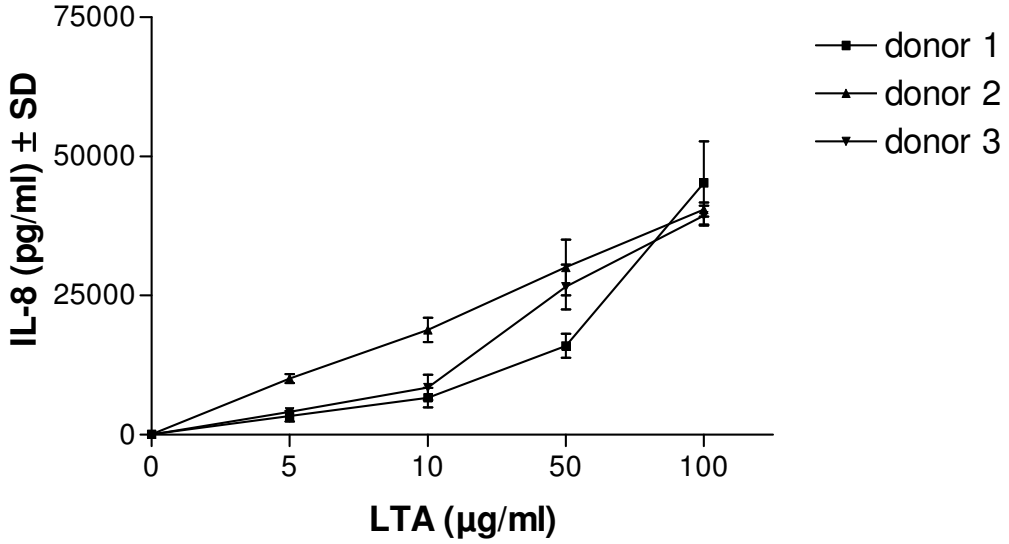

Fig. 20: In vitro IL-8 response of human whole blood towards LTA from *B. subtilis*

Detection limits			
donor	1	2	3
µg/ml	50	5	10

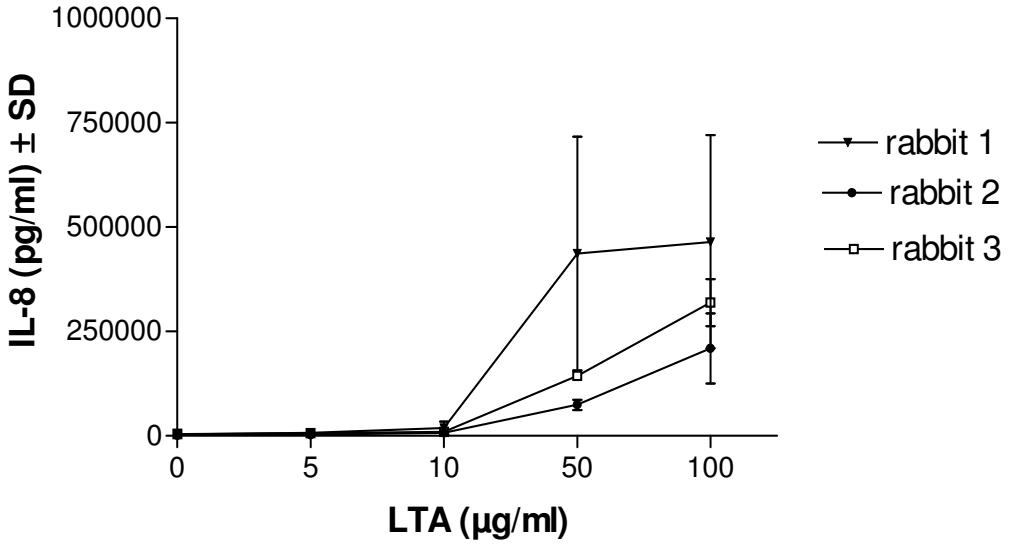

Fig. 21: In vitro IL-8 response of rabbit whole blood towards LTA from *B. subtilis*

Detection limits			
rabbit	1	2	3
µg/ml	100	100	50

The IL-8 response of the humans is also very sensitive. The rabbit IL-8 has a variance that is again too high to calculate a reasonable detection limit. The amounts of IL-8 exceed those of IL-1β by the factor 12 (humans) and 500 (rabbits).

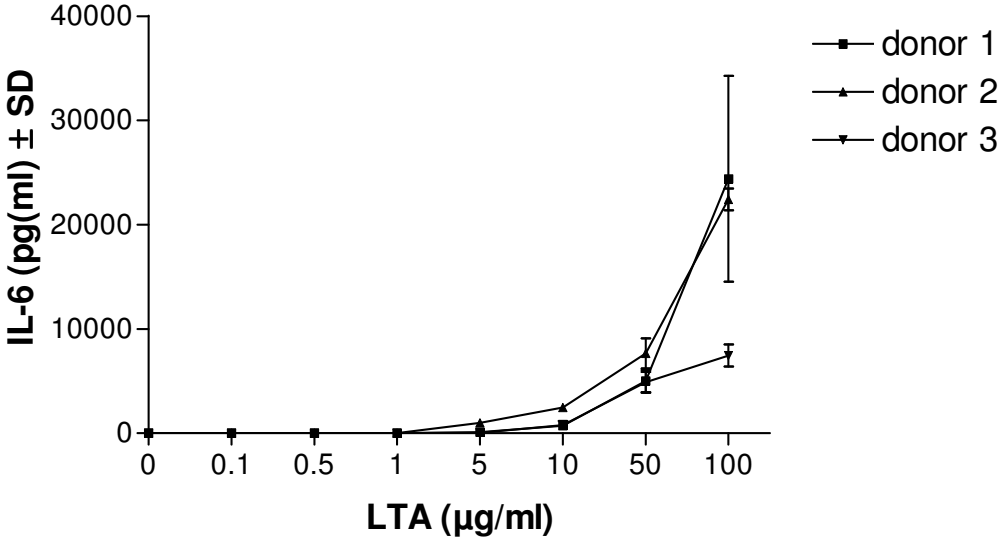

Fig. 22: In vitro IL-6 response of human whole blood towards LTA from *B. subtilis*

Detection limits			
donor	1	2	3
µg/ml	100	10	50

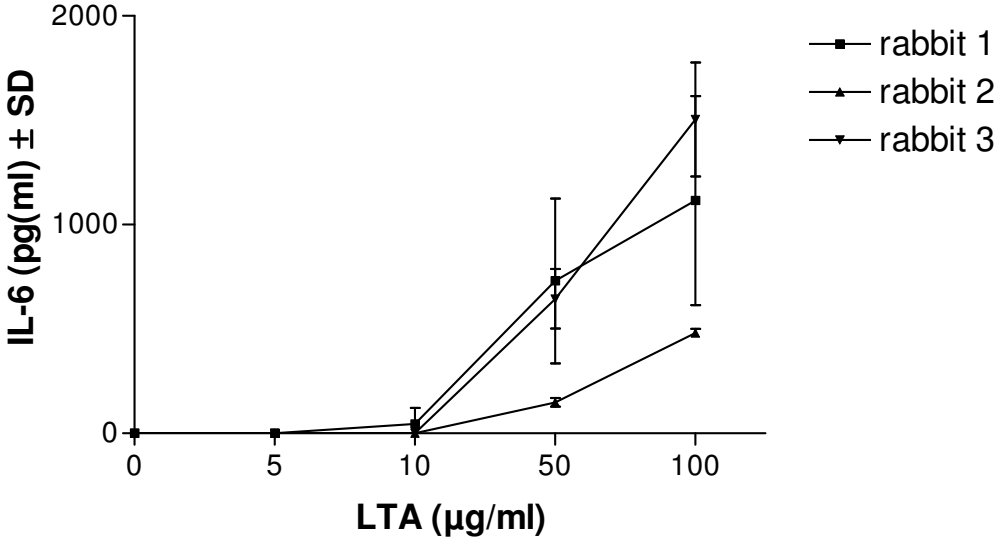

Fig. 23: In vitro IL-6 response of rabbit whole blood towards LTA from *B. subtilis*

Detection limits			
rabbit	1	2	3
µg/ml	50	50	50

The IL-6 assay of the rabbit is surprisingly sensitive in this case and shows a high sensitivity. The amounts of rabbit IL-6 are relatively low. The results differ strongly in between the different endpoints despite the high concentrations of stimulus used. As a final step, TNF-α was measured in both species.

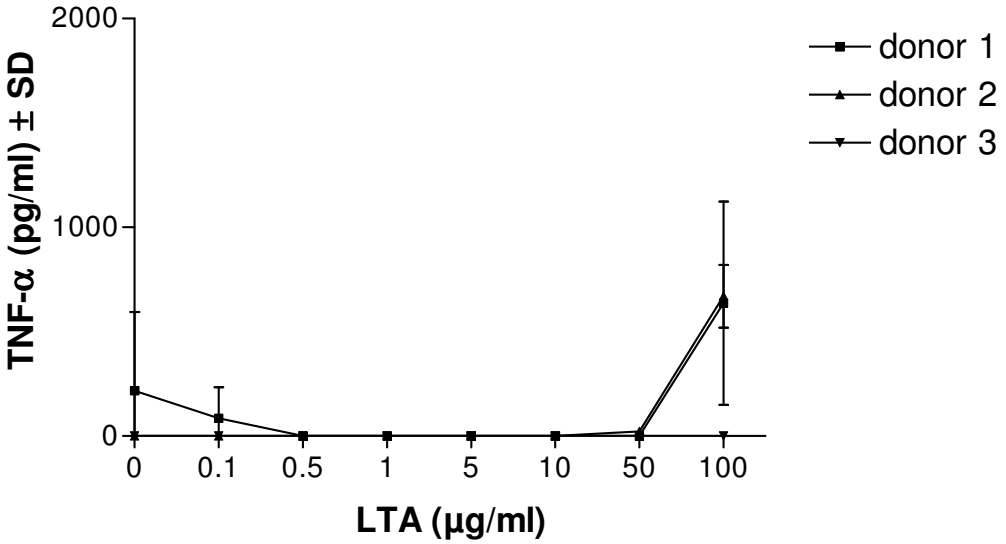

Fig. 24: In vitro TNF-α response of human whole blood towards LTA from *B. subtilis*

Detection limits			
donor	1	2	3
µg/ml	> 100	100	> 100

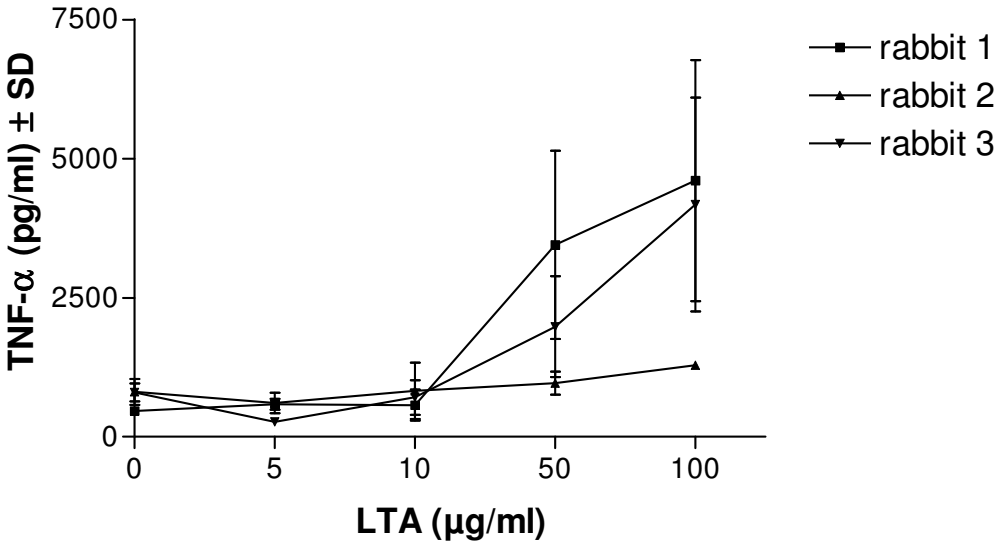

Fig. 25: In vitro TNF-α response of rabbit whole blood towards LTA from *B. subtilis*

Detection limits			
rabbit	1	2	3
µg/ml	50	> 100	100

The TNF-α assays show a very low sensitivity towards this stimulus in both cases. The best readout for this Gram-positive stimulus is in the case of the humans the IL-8 assay with the lowest detection limit and the highest response (Fig. 20) and the least sensitive by far the TNF-α readout. For the rabbits, IL-6 performs best (Fig. 23). All other readouts show a low sensitivity and high variability.

Part 3: Less potent Gram-negative stimuli in the rabbit IL-1β and rabbit IL-8

The results of the relatively little active LPS from *S. typhimurium* induced us to use two more Gram-negative stimuli that show little pyrogenic activity in the human whole blood in order to determine whether major differences occur systematically with such pyrogens. Additionally, the limits of detection for certain Gram-negative stimuli can differ very strongly between the LAL and the mammalian system (up to the factor 1000) (Hartung et al., 2000). It was therefore determined whether the reaction of the rabbit was similar to the human system if LPS was used that required concentrations of nanograms or even micrograms. For this, the relative potency of the following stimuli was determined by pretesting them in the human donor (data not shown) and perform a small dose-response curve for the rabbit in order to see whether the reaction of the rabbits would be in the same concentration range as in the humans. In this case, only IL-1β and IL-8 were measured.

4.9. *Bordetella pertussis*

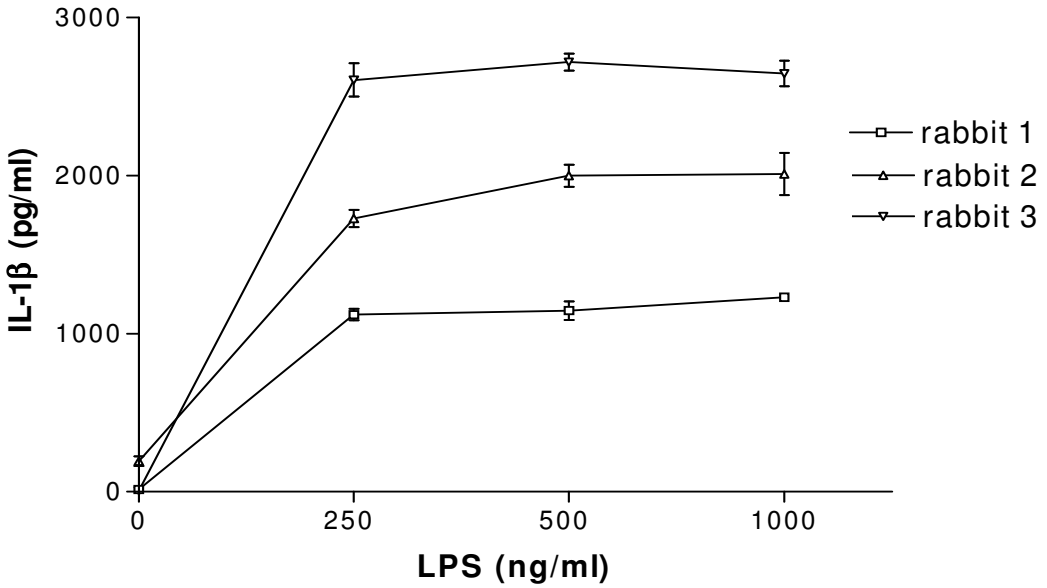

Fig. 26: In vitro IL-1β response of rabbit whole blood towards LPS from *Bordetella pertussis*

Detection limits			
rabbit	1	2	3
ng/ml	< 250	< 250	< 250

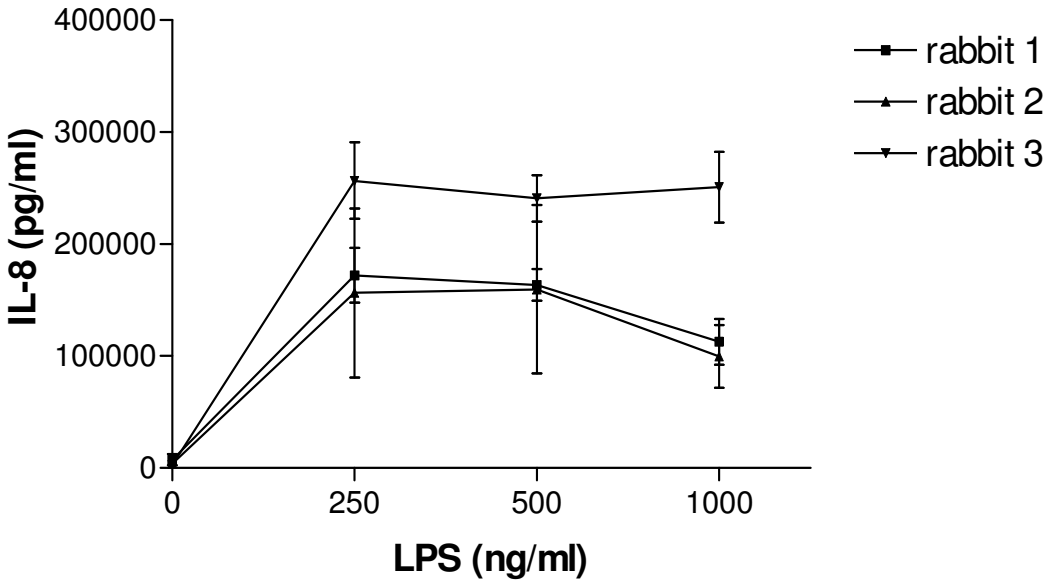

Fig. 27: In vitro IL-8 response of rabbit whole blood towards LPS from *Bordetella pertussis*

Detection limits			
rabbit	1	2	3
ng/ml	< 250	< 250	< 250

Both the IL-1β and the IL-8 assay show a strong positive response towards the LPS from *Bordetella pertussis* at the above concentrations. It is notable that both cytokine responses are not dose-dependent, that is, doubling the LPS concentration does not cause the cytokine response to increase, indicating that the chosen concentrations were too high. Therefore, only a sensitivity of < 250 ng/ml can be determined. The amount of IL-8 even decreases at the 1000 ng/ml concentration, with the IL-8 starting to dimerize.

4.10. *Pseudomonas aeruginosa*

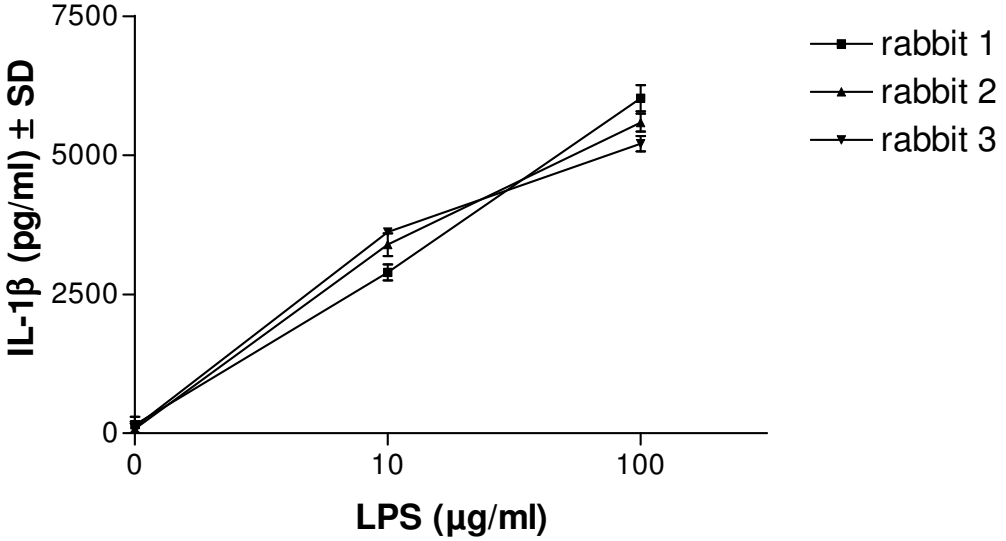

Fig. 28: In vitro IL-1β response of rabbit whole blood towards LPS from *Pseudomonas aeruginosa*

Detection limits			
rabbit	1	2	3
µg/ml	< 10	< 10	< 10

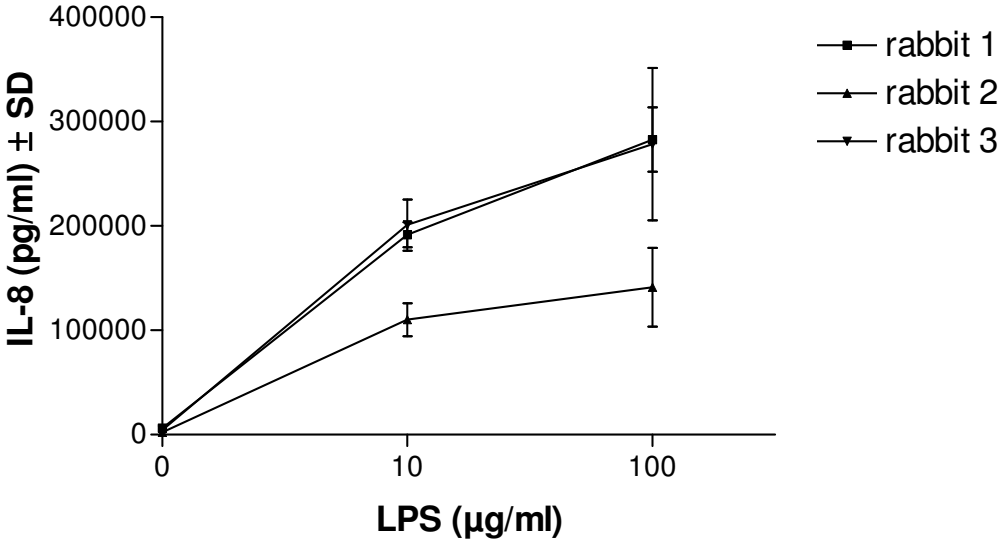

Fig. 29: In vitro IL-8 response of rabbit whole blood towards LPS from *Pseudomonas aeruginosa*

Detection limits			
rabbit	1	2	3
µg/ml	<10	<10	<10

With the LPS from *Pseudomonas aeruginosa* a good linearity in the cytokine response is visible. The lowest LPS concentration is not negative, so only a detection limit of < 10 µg/ml can be determined. Still, the response indicates that the chosen concentrations are in the right range. Both LPS are in the same concentration ranges as determined for the human. Again, IL-8 exceeds the amounts of IL-1β by the factor 100.

Part 4: Establishment of a bioassay (WEHI) in order to compare TNF-α production in human and canine whole blood

Another species that had been considered for in vivo pyrogen testing was the dog, having a more stable thermoregulation than the rabbit, but since it was reported to be less sensitive towards pyrogens (Co Tui, 1942), the rabbit was chosen as the gold standard. Since the WEHI procedures require a high number of dilutions, only a very small concentration-response curve could be done in order to see whether the human and the canine sensitivity would differ. As a first step, the ELISA and the WEHI system were compared: human whole blood was stimulated with the LPS of *Salmonella abortus equi* as in the previous experiments and the centrifuged supernatants were measured in parallel in the ELISA and in the WEHI bioassay (Fig. 30 and 31). It was to be determined whether the bioassay based on the apoptosis of murine cells upon contact with TNF-α would be as reliable as the already established human TNF-α ELISA. Since the bioassay measures only the amount of TNF-α that is biologically active, the readouts are about 20fold lower than in the ELISA system. In order to compare the methods, the ELISA readouts were divided by the ones of the WEHI and it was determined whether the thus calculated factor would be on the same level for all LPS concentrations (Fig. 32).

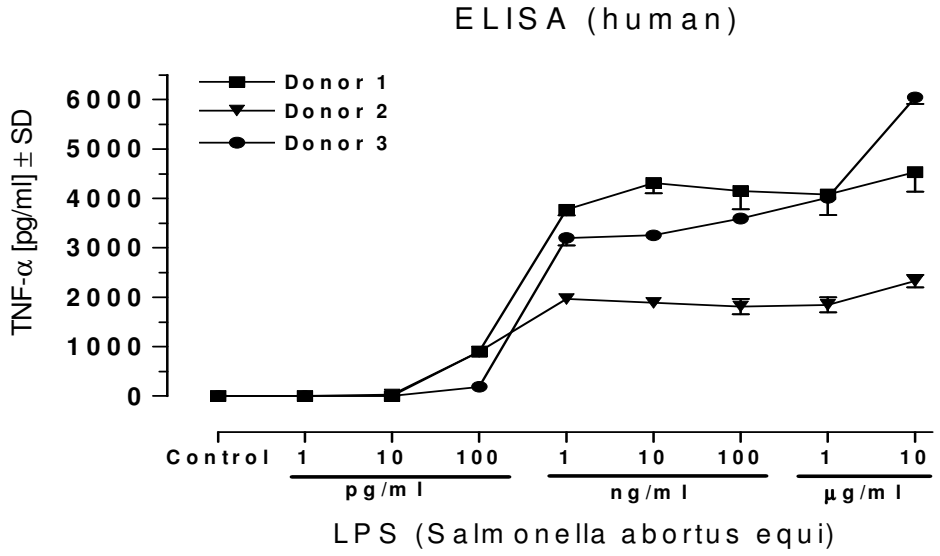

Fig. 30: Measurement of human TNF-α with an ELISA system

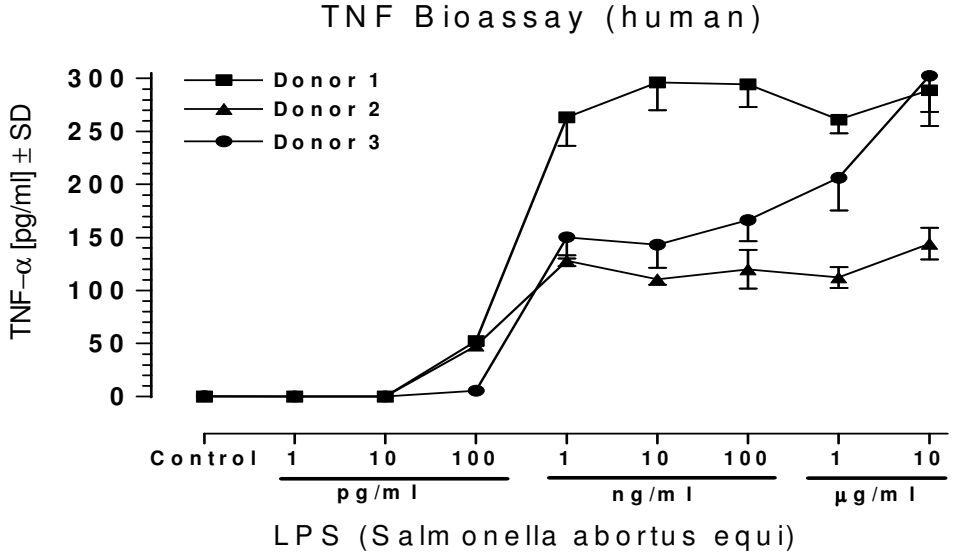

Fig. 31: Measurement of the same supernatants as in Fig. 30 in the TNF-α Bioassay (WEHI)

Even though the amounts of bioactive TNF-α found in the WEHI were much lower than those of the ELISA, the detection limits of all three donors as determined by one-way ANOVA were exactly the same: 1 ng/ml for donor 1 and 3 and 100 pg/ml for donor 2.

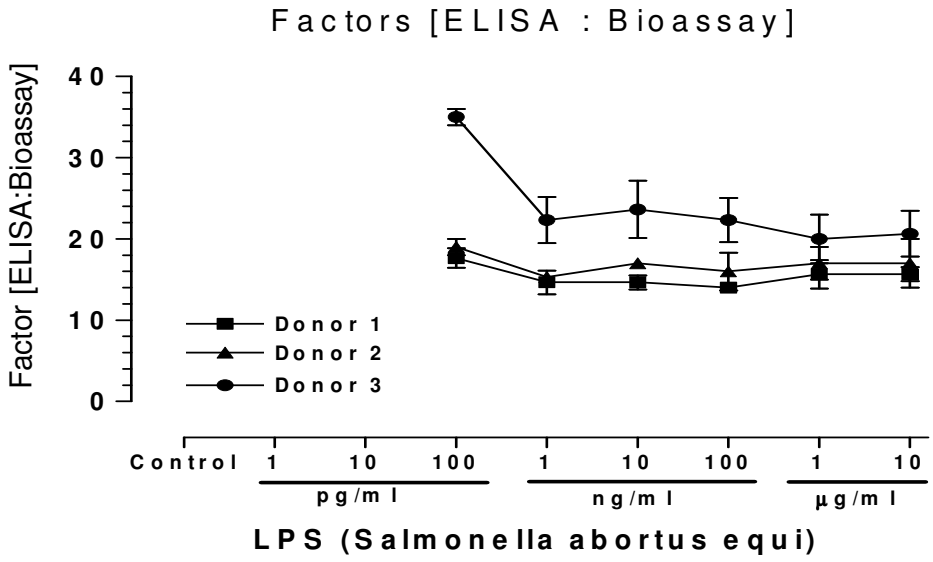

Fig. 32: Comparison of the results in the human ELISA system and the Bioassay; the ELISA readouts were divided by the WEHI readouts

The calculated factors for each value are very constant, indicating that the ELISA and the bioassay produce highly similar results. The next step was to measure canine supernatants in the WEHI assay in order to see whether the murine cells would show a cross-reaction towards bioactive canine TNF-α (Fig. 33). The same LPS from *Salmonella abortus equi* was used in the same concentrations and the canine blood incubation was handled exactly the same as the human (and the rabbit) assays before.

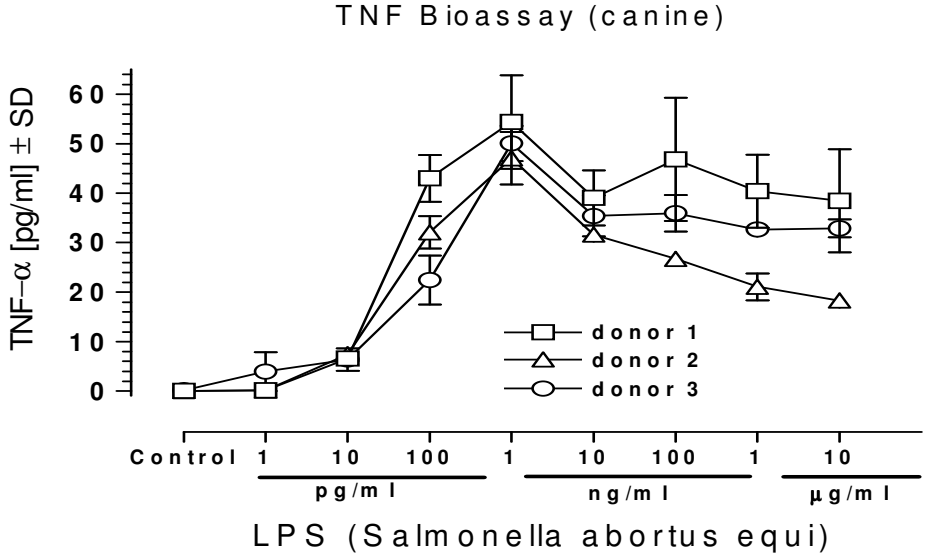

Fig. 33: Measurement of canine whole blood supernatants in the bioassay (WEHI)

The amounts of TNF-α detected are not high, indicating maybe that the cross-reaction of the murine cells is not complete and only a certain percentage of cells undergo apoptosis. Still, the sensitivities do not differ. All three dogs show a significant response at 100 pg/ml of endotoxin as determined by one-way ANOVA. It was therefore concluded that the reaction can be used for detecting canine TNF-α in a whole blood incubation.
Having established the canine bioassay, a further Gram-negative (the WHO standard *E. coli* O113) and a Gram-positive stimulus (LTA from *Staphylococcus aureus*) were used in order to see whether the WEHI assay would work reproducibly with canine blood when compared to the already established WEHI using human whole blood. Only a few concentrations could be tested, and a relatively low and a high one were chosen. No detection limit was determined in these cases, since the lowest concentration of LPS was not negative.

a) *E. coli* O113:H110

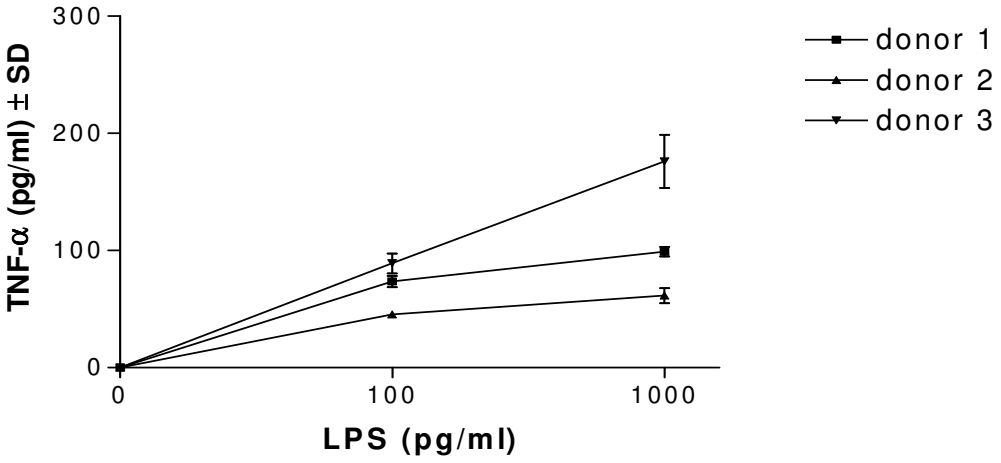

Fig. 34: Measurement of human whole blood supernatants after stimulation with *E. coli* O113: H10

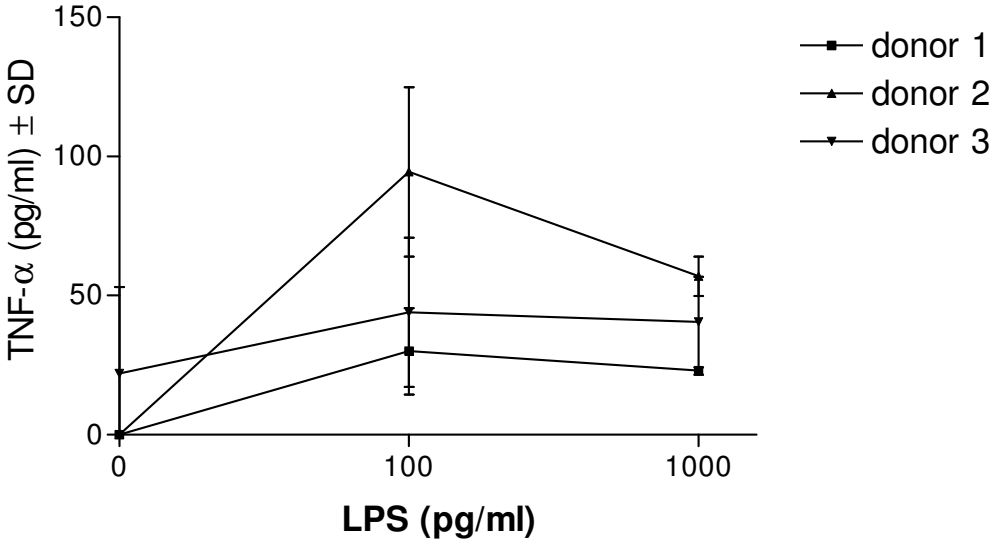

Fig. 35: Measurement of canine whole blood supernatants after stimulation with *E. coli* O113: H10

Again, the amounts of TNF-α detected in the canine system are lower than in the human assay and the variance is very high, with donor 3 exhibiting an elevated negative control, indicating that further optimization of the handling of the dog blood might be necessary. Still, both species show a reaction to the lower LPS concentration.

2) *Staphylococcus aureus*

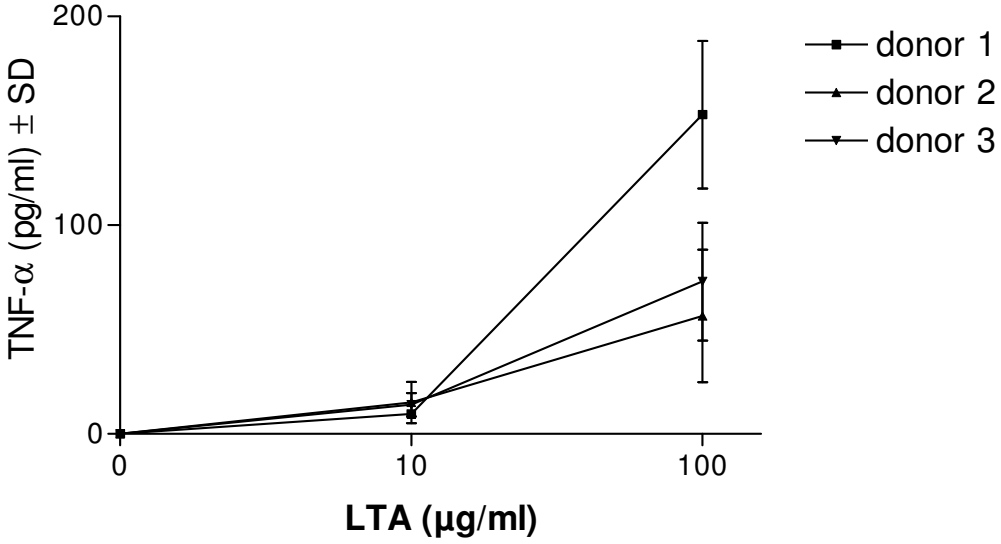

Fig. 36: Measurement of human whole blood supernatants after stimulation with LTA from *Staphylococcus aureus*

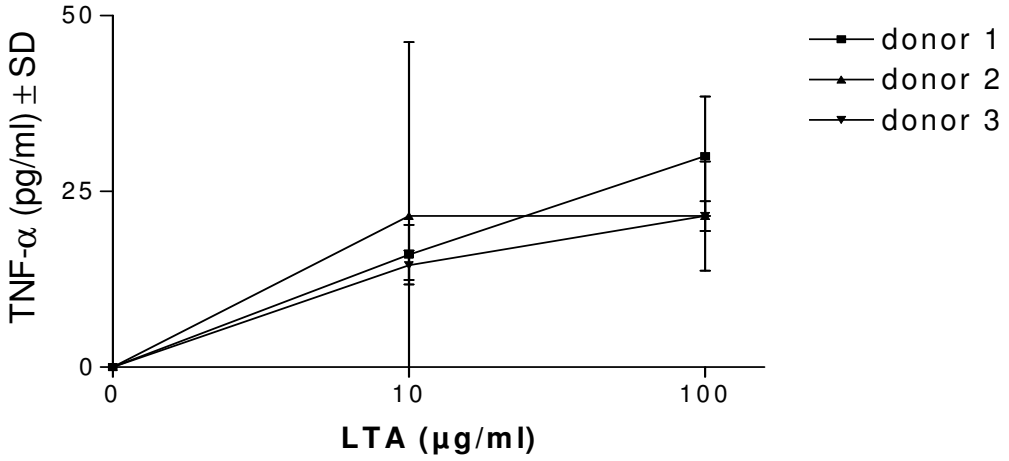

Fig. 37: Measurement of canine whole blood supernatants after stimulation with LTA from *Staphylococcus aureus*

Donor 2 shows a very high variance at 10 µg/ml (4 pg/ml vs. 39 pg/ml of TNF-α). Since only twofold values could be done, it is not possible to say which value (or if any of both values) is correct.

The other two donors show a positive reaction at this challenge, even if the amounts of cytokine detected are again very low.