Cytokine responses to lipopolysaccharide in vivo and ex vivo : Genetic polymorphisms and inter-individual variation

Schippers, E.F.

CHAPTER 6

IL-10 and toll-like receptor-4 polymorphisms and in vivo and ex vivo response to endotoxin

E.F. Schippers[1], C. van 't Veer[2], S. van Voorden[1], C.A.E. Martina[1], T.W.J. Huizinga[3], S. le Cessie[4], J.T. van Dissel[1]

Departments of [1]Infectious Diseases, [3]Rheumatology and [4]Medical Statistics, Leiden University Medical Center, Leiden, the Netherlands
[2]Department of General Surgery, University of Maastricht, Maastricht, the Netherlands

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IL-10 and toll-like receptor-4 polymorphisms and the in vivo and ex vivo response to endotoxin☆

Emile F. Schippers[a,*], Cornelis van 't Veer[b], Sjaak van Voorden[a], Cerithsa A.E. Martina[a], Tom W.J. Huizinga[c], Saskia le Cessie[d], Jaap T. van Dissel[a]

[a]*Department of Infectious Diseases, C5-P42, Leiden University Medical Center, PO Box 9600, 2300 RC Leiden, The Netherlands*
[b]*Department of General Surgery, University of Maastricht, Maastricht, The Netherlands*
[c]*Department of Rheumatology, Leiden University Medical Center, Leiden, The Netherlands*
[d]*Department of Medical Statistics, Leiden University Medical Center, Leiden, The Netherlands*

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Abstract

To determine to what extent lipopolysaccharide-induced IL-10 production capacity is determined by polymorphisms in toll-like receptor-4 (TLR4) and the IL-10 promoter region, we measured in vivo IL-10 and TNF-α production in patients undergoing elective cardiopulmonary bypass surgery, a major surgical trauma associated with ischemia-reperfusion injury that triggers an endotoxemia and profound inflammatory response in most patients. Ex vivo the IL-10 and TNF-α production was measured in a whole blood stimulation assay, using 3 LPS concentrations. Positive correlations were found between TNF-α and IL-10 production ex vivo, upon stimulation with each of the LPS concentrations. Also, the estimated TNF-α and IL-10 EC_{50}, and TNF-α_{max} and IL-10_{max} were positively correlated ($r = 0.203$; $p = 0.023$ and $r = 0.287$; $p = 0.001$, respectively), indicating that these parameters describing LPS sensitivity and maximal production capacity, respectively, can be estimated by measuring either TNF-α or IL-10. Interleukin-10 concentrations in patients experiencing endotoxemia in vivo negatively correlated with the IL-10 levels produced upon stimulation with 1000 ng/mL LPS as well as the estimated IL-10_{max} ex vivo. In vivo, a positive correlation between the TNF-α concentration at time-point 2 and the IL-10 concentration at time-point 3 was found, consistent with an important contribution of the magnitude of TNF-α release upon the subsequent IL-10 production. Carriers of the IL-10 promoter −1330G, −1082A, −819T, −592A (GATA) haplotype had lower IL-10 production ex vivo upon stimulation with 10 and 100 ng/mL LPS and higher EC_{50} values (the estimated LPS concentration at which 50% of the maximal IL-10 response is reached) as compared to carriers of the other haplotypes combined, indicating decreased LPS sensitivity ex vivo. These individuals did not differ from the others in interleukin-10 production capacity upon stimulation with a high LPS concentration (i.e., 1000 ng/mL) and the estimated IL-10_{max} values, were similar, indicating unimpaired maximal IL-10 production capacity ex vivo. Carriers of the IL-10 promoter AGCC haplotype had lower EC_{50} values as compared to carriers of the other haplotypes combined, indicating increased LPS sensitivity ex vivo. In accordance with this finding, carriers of the AGCC haplotype had higher circulating IL-10 levels in vivo. The common TLR4 polymorphisms (Asp299Gly and Thr399Ile) were associated with slightly higher IL-10 production capacity ex vivo and in vivo, however, this was not statistically significant. Our results indicate that polymorphisms in the proximal IL-10 promoter region are associated with in

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* Corresponding author. Tel.: +31 71 526 2613; fax: +31 71 526 6758.
E-mail address: e.f.schippers@lumc.nl (E.F. Schippers).

vivo and ex vivo LPS sensitivity. The contribution to the inter-individual variation, however, is limited since the variation between individuals in LPS sensitivity and IL-10 production capacity can only partly be attributed to these IL-10 promoter polymorphisms.

Keywords: Cardiopulmonary bypass; Endotoxemia; Lipopolysaccharides; Toll-like receptor-4; IL-10 promoter

1. Introduction

Interleukin-10 (IL-10) is an important immunoregulatory cytokine. It is involved in the regulation of inflammatory responses through direct influence over tumor necrosis factor (TNF)-α production. Also, IL-10 plays an important role in the course of infectious diseases, for instance, the severity to which meningococcal meningitis progresses is associated with serum IL-10 concentrations, such that a high serum IL-10 level was observed in patients with a poor or fatal outcome, whereas patients with mild disease and a good prognosis had lower serum IL-10 levels [1−3]. In Gram-negative infection, the presence of lipopolysaccharide (LPS) in the circulation plays a pivotal role in the release of IL-10. Human monocytes, the main producers of IL-10, exhibit substantial inter-individual differences in IL-10 production upon LPS stimulation ex vivo [4]. A study investigating the inter-individual variation of IL-10 production following LPS stimulation of whole blood cultures ex vivo suggested that the differences in IL-10 production capacity can to a large extent be explained by differences in genetic background [5]. The inter-individual differences in produced IL-10 could not simply be explained by corresponding differences in TNF-α production, implicating that the differences in the individual's ability to produce IL-10 are not simply reflecting differences in the individual's ability to produce TNF-α [5]. It has been suggested that differential transcription is the principle mechanism of the inter-individual differences in IL-10 production [6]. Indeed, the IL-10 production is proportionally related to mRNA production and not to the half-life of IL-10 [7]. Given the fact that the differences in IL-10 production are most likely transcriptionally regulated, the IL-10 promoter region has been an important target for investigation [6]. In the IL-10 promoter, 11 single nucleotide polymorphisms (SNPs) have been described. Studies of SNPs in the proximal 1.1 kb have yielded the existence of a relative small number of haplotypes [8−10]. The dimorphic polymorphisms (G or A at position −1330, G or A at position −1082, C or T at position −819, C or A at position −592) are in preferential allelic association, namely AGCC, GACC and GATA haplotypes. It has been shown that the differences in the production capacity of IL-10 ex vivo are associated with IL-10 haplotypes [6]. Furthermore, the SNP in the IL-10 promoter at position −2849 was associated with differences in IL-10 production ex vivo upon stimulation with 1000 ng/mL LPS and transcriptional activity [11−13]. So far, other known SNPs in the IL-10 promoter have not been evaluated in this way.

Toll-like receptor-4 (TLR4) is part of a large family of transmembrane proteins and is believed to be crucial in mediating LPS effects. TLR4 is expressed on monocytes and macrophages, and to a lesser extent on lymphocytes and other cell types [14,15]. The recently described association between the Asp299Gly polymorphism in TLR4 and Gram-negative septic shock suggests a functional defect in TLR4 leading to increased susceptibility to Gram-negative bacteremia [16].

Cardiopulmonary bypass surgery leads to perioperative endotoxemia in most patients, and this procedure may serve as a model to study the association of genetic polymorphism and endotoxin mediated IL-10 production in vivo. The most immunoreactive reactive component of endotoxin is lipid A, being a structural element of LPS.

The aims of the current study were to assess the inter-individual variation in IL-10 production upon whole blood stimulation with LPS, to determine the correlation between production rates and various SNPs in the IL-10 promoter region as well as the TLR4 coding region, and to determine how much of the inter-individual variation could be attributed to these genetic factors. We also studied the inter-individual variation in the in vivo IL-10 production following cardiopulmonary bypass surgery in the same way.

2. Results

2.1. Patients

We studied 159 consecutive patients undergoing elective cardio-thoracic surgery with cardiopulmonary bypass. The patient characteristics have been described previously [17]. Briefly, there was a predominance of male patients (66%), the median age for males and females was 65 and 67, respectively ($p = 0.091$). Active smoking occurred in 35 patients (22%) whereas 20 patients (12%) had diabetes mellitus. Surgical procedures were extensive; 20 patients (12%) underwent coronary artery bypass surgery (CABG) combined with valve replacement, 49 patients (30%) underwent valve replacement only, whereas 89 patients (54%) underwent CABG only. Eight patients underwent other surgical procedures, mainly aortic surgery.

2.2. IL-10 promoter genotyping

Typing for the IL-10 single nucleotide polymorphisms was successful in most patients; in a minority of the samples no definite typing could be established (Table 1). As previously described the 4 defined proximal dimorphic polymorphisms (A or G at position −1330, G or A at position −1082, C or T at position −819, C or A at position −592) resulted in three allelic associations, namely AGCC, GACC, and GATA haplotypes, resulting in six subsequent biallelic haplotypes (Table 1). The haplotype frequencies were similar to the frequencies described previously [9].

2.3. TLR4 genotyping

The TLR4 Asp299Gly and Thr399Ile substitutions were successfully determined in all the patients. The Asp299Gly substitution was found in 17 patients (10.7%), 16 patients were heterozygous and 1 patient was homozygous. Except for 1 patient the TLR4 Asp299Gly substitution was accompanied by the TLR4 Thr399Ile substitution. Two patients were identified with an isolated TLR4 Thr399Ile substitution (Table 1). The patient homozygous for the Asp299Gly substitution was also homozygous for the Thr399Ile substitution.

2.4. Ex vivo IL-10 measurements

Blood samples for ex vivo LPS stimulation were available from 125 patients. In general, we found a dose dependent IL-10 production upon stimulation of whole blood with increasing concentrations of LPS. Median IL-10 levels upon stimulation with 0 ng/mL LPS were below or at the lower level of detection in the majority of patients [median 3.0 pg/mL (IQR: 3.0−3.0)], and no differences were found between the various groups (data not shown). Median IL-10 levels upon stimulation with 10, 100 and 1000 ng/mL LPS were 801 (IQR: 506−1221), 1482 (IQR: 996−1974) and 3493 (IQR: 2534−4673) pg/mL, respectively (Table 2). For each patient we estimated the dose−response characteristics EC_{50} and E_{max} (i.e., EC_{50} is the estimated LPS concentration at which 50% of the maximal IL-10 response is reached, and E_{max} is the estimated maximal IL-10 production), as described in the methods.

We first investigated whether any association was present between the IL-10 promoter polymorphisms and the levels of IL-10 production upon stimulation with the different LPS concentrations, and the estimated dose−response characteristics (i.e. EC_{50} and E_{max}) ex vivo. As shown in Table 2, the level of IL-10 produced following ex vivo LPS stimulation was not statistically different in the six biallelic genotypes of the proximal IL-10 promoter and the distal SNPs. However, carriers of the GATA haplotype (homozygous and heterozygous carriers combined), as compared to carriers of the other haplotypes combined, had lower IL-10 levels after stimulation with 10 and 100 ng/mL LPS [619 (IQR: 431−1011) versus 826 (IQR: 575−1142), $p = 0.041$ and 1253 pg/mL (IQR: 808−1707) versus 1651 (IQR: 1194−2042), $p = 0.006$, respectively, Fig. 1). Furthermore, these patients had significantly higher EC_{50} values [159 (IQR: 97−293) versus 122 ng/mL (IQR: 67−190), $p = 0.027$, Fig. 2], indicating decreased LPS sensitivity. More interestingly, the IL-10 concentration after stimulation with the highest LPS concentration (i.e. 1000 ng/mL), and the estimated E_{max} were not different in the two groups [3357 (IQR: 2111−4517) versus 3529 (IQR: 2634−4738), $p = 0.377$ and 3966 pg/mL (IQR: 2174−5345) versus 3737 (IQR: 2844−5585), $p = 0.767$, respectively]. However, homozygous carriers of the GATA haplotype, as compared to all other patients combined, had higher EC_{50} and E_{max} values [230 (IQR: 141−502) versus 134 ng/mL (IQR: 78−196), $p = 0.013$ and 5122 pg/mL (IQR: 3758−7555) versus 3605 (IQR: 2533−5242), $p = 0.035$, respectively]. On the other hand, patients carrying the AGCC haplotype (homozygous and heterozygous carriers combined), as compared to carriers of the other haplotypes combined, had slightly lower EC_{50} values [125 (IQR: 66−204) versus 159 ng/mL (IQR: 106−230), $p = 0.041$], indicating higher LPS sensitivity (Fig. 2).

Patients carrying the Asp299Gly polymorphism as compared to carriers of wild-type alleles, had somewhat higher IL-10 concentrations upon stimulation with 100 and 1000 ng/mL LPS [1799 (IQR: 1237−2058) versus

Table 1
IL-10 promoter haplotype/genotype in 159 patients undergoing elective cardiac surgery with cardiopulmonary bypass

IL-10 promoter genotype, *n* (%)		
−1330, −1082, −819, −592/−1330, −1082, −819, −592		
AGCC/AGCC		31 (20)
AGCC/GACC		42 (27)
AGCC/GATA		30 (20)
GACC/GACC		16 (10)
GATA/GATA		15 (10)
GACC/GATA		20 (13)
IL-10 promoter genotype, *n* (%)		
−3575	AT	70 (51)
	TT	50 (37)
	AA	16 (12)
−2849	GG	72 (55)
	AG	56 (42)
	AA	4 (3)
−2763	CC	61 (46)
	AC	61 (46)
	AA	11 (8)
TLR4 haplotype, *n* (%)		
299+/399+		140 (88)
299−/399+[a]		1 (1)
299−/399−		16 (10)
299+/399−		2 (1)

[a] One out of 16 patients homozygous, all others heterozygous.

Table 2
IL-10 production ex vivo on whole blood stimulation with LPS according to IL-10 and TLR4 genotype/haplotype[a]

			10 ng/mL	100 ng/mL	1000 ng/mL	IL-10_{max}	$EC_{50[LPS]}$
All patients ($n = 125$)			801 (506–1121)	1482 (996–1974)	3493 (2534–4673)	3817 (2712–5445)	139 (82.6–226)
IL-10 promoter genotype (n)							
−1330/−1082/−819/−592							
AGCC/AGCC		(28)	819 (518–1195)	1476 (921–2042)	3341 (2411–4259)	3526 (2454–4823)	145 (60.4–195)
AGCC/GACC		(30)	922 (630–1129)	1666 (1309–2161)	3722 (2856–5353)	3985 (3100–6056)	105 (75.8–161)
AGCC/GATA		(24)	646 (409–1081)	1129 (787–1734)	2765 (1898–4216)	3352 (1986–4694)	134 (67.0–268)
GACC/GACC		(12)	800 (698–1001)	1799 (1189–2025)	3680 (2826–4540)	3959 (3124–5225)	144 (71.8–192)
GATA/GATA		(12)	741 (478–963)	1446 (1043–1520)	4088 (3185–5906)	5122 (3758–7555)	230 (141–502)
GACC/GATA		(17)	508 (393–982)	1108 (691–1869)	3493 (1694–4985)	3965 (2064–6092)	145 (109–254)
IL-10 promoter genotype (n)							
−3575	AT	(55)	826 (516–1188)	1492 (968–2036)	3386 (2531–4840)	3542 (2348–5793)	117 (75.2–223)
	TT	(40)	757 (490–1009)	1378 (989–1878)	4075 (2819–5012)	4792 (3176–6403)	167 (132–300)
	AA	(13)	813 (482–1179)	1680 (760–2089)	3228 (1951–3852)	3470 (2406–4431)	67 (23.9–145)
−2849	GG	(60)	665 (489–1027)	1482 (979–1992)	3868 (2704–5498)	4627 (3135–6685)	165 (103–262)
	AG	(42)	832 (445–1221)	1360 (760–1866)	3044 (1812–3826)	3330 (1960–4425)	113 (42.1–235)
	AA	(3)	869 (813–1135)	1814 (1783–1887)	4130 (3528–4259)	4693 (3587–4849)	144 (59.8–147)
−2763	CC	(48)	678 (478–1009)	1412 (973–1992)	3798 (2363–5025)	4213 (2700–6403)	150 (95.9–224)
	AC	(49)	855 (510–1164)	1430 (1005–1834)	3444 (2553–4243)	3966 (2798–4727)	139 (79.8–267)
	AA	(9)	813 (370–1269)	1680 (664–1950)	3341 (1562–4735)	3470 (1767–5393)	144 (89.3–221)
TLR4 haplotype (n)							
299+/399+		(109)	801 (490–1148)	1450 (930–1940)	3395 (2411–4673)	3587 (2373–5345)	133 (76.8–226)
299−/399+		(1)	1080 (−)	2251 (−)	7381 (−)	9582 (−)	301 (−)
299−/399−[b]		(13)	716 (526–950)	1783 (1228–1979)	4130 (3345–4511)	4693 (3627–5813)	144 (109–241)
299+/399−		(2)	852 (664–1040)	1887 (1633–2140)	4476 (3722–5229)	5130 (4230–6031)	152 (143–160)

[a] IL-10 concentrations are in pg/mL. Values are expressed as median values and interquartile range between parentheses. Data on 0 ng/mL LPS not shown, the majority of IL-10 levels were below or at lower detection limit, and now differences were found between the various groups.
[b] One out of 13 patients homozygous, all others heterozygous.

1458 pg/mL (IQR: 957–1956), $p = 0.174$ and 4171 (IQR: 3351–4856) versus 3457 pg/mL (IQR: 2425–4685), $p = 0.083$, respectively]. The same trend was observed when comparing carriers of the Thr399Ile polymorphism with carriers of wild-type alleles [1783 (IQR: 1245–2006) versus 1451 pg/mL (IQR: 944–1969), $p = 0.177$ and 4130 (IQR: 3357–4567) versus 3426 pg/mL (IQR: 2418–4690), $p = 0.111$, respectively]. In

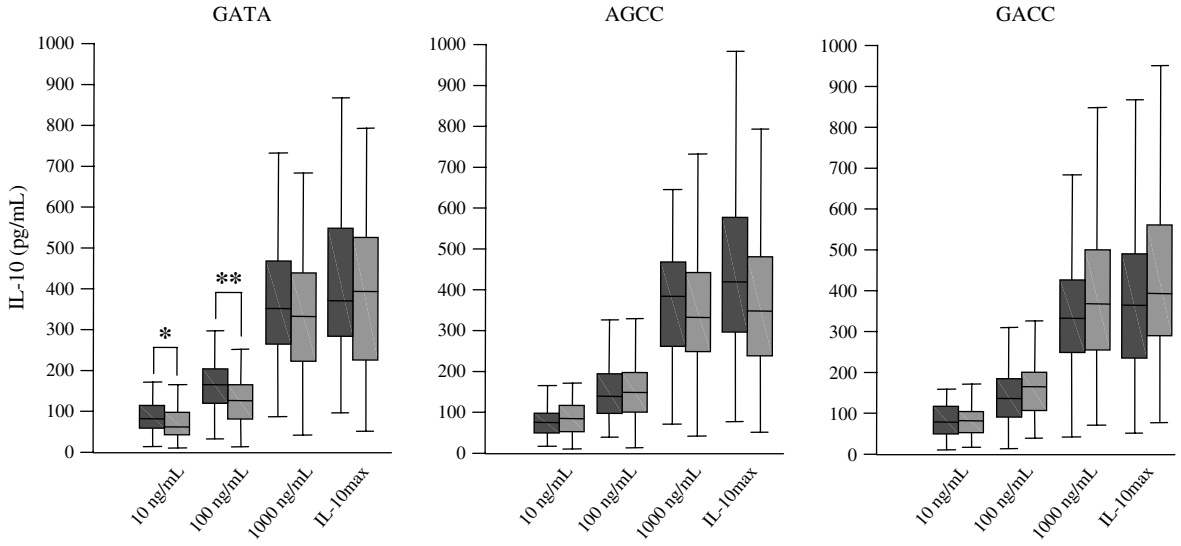

Fig. 1. IL-10 production upon whole blood stimulation with 10, 100 and 1000 ng/mL LPS respectively, and the estimated IL-10_{max}. Left panel represents carriers of the GATA haplotype (lighter box plots) as compared with all others. Middle panel represents carriers of the AGCC haplotype (lighter box plots) as compared with all others. Right panel represents carriers of the GACC haplotype (lighter box plots) as compared with all others. The box plots display the median, the 25th and 75th percentiles, and the smallest and largest IL-10 concentrations that are not outliers. IL-10 was measured by enzyme-linked immunosorbent assay; the detection limit was 4 pg/mL. $*p = 0.041$, $**p = 0.006$.

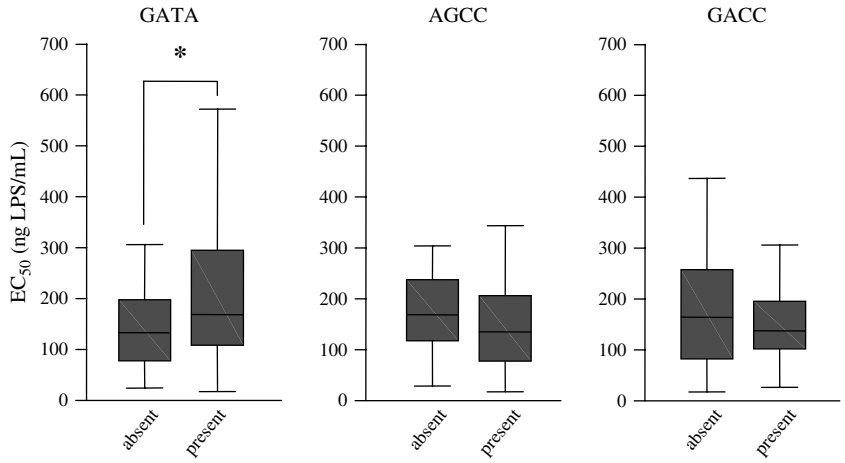

Fig. 2. Estimated EC_{50} values, calculated as described in the methods. Left panel represents carriers of the GATA haplotype (right box plot) as compared with all others. Middle panel represents carriers of the AGCC haplotype (right box plot) as compared with all others. Right panel represents carriers of the GACC haplotype (right box plot) as compared with all others. The box plots display the median, the 25th and 75th percentiles, and the smallest and largest EC_{50} values that are not outliers. $*p = 0.027$.

accordance with these findings, the estimated IL-10 E_{max} was higher in the carriers of either the Asp299Gly or the Thr399Ile polymorphism as compared to wild-type allele carriers [4771 (IQR: 3682–6506) versus 3604 pg/mL (IQR: 2399–5404), $p = 0.064$ and 4693 (IQR: 3738–6031) versus 3596 pg/mL (IQR: 2386–5425), $p = 0.085$, respectively]. Although none of the described differences reached the level of statistical significance, the consistency in the observations suggest an increased in vitro IL-10 production capacity at high concentrations of LPS in patients carrying either the Asp229Gly or the Thr399Ile polymorphism. No differences in EC_{50} value were found between the various groups (all $p > 0.38$).

2.5. In vivo endotoxin measurements

Perioperative blood samples for endotoxin measurements were available from most patients and have been described in detail previously [17]. Briefly, endotoxin levels were ≥ 5 pg/mL directly before anesthetic induction in 4 out of 152 patients (2.6%). On aorta declamping (time-point 2), 30 min into reperfusion (time-point 3) and on arrival at the ICU (approximately 2 h after surgery, time-point 4) 74 out of 142 patients (52.1%), 71 out of 143 patients (49.7%) and 54 out of 151 patients (35.8%) had endotoxin levels ≥ 5 pg/mL, respectively. In 85 out of 143 patients (59.4%) the endotoxin level at either time-point 2 and/or 3 was ≥ 5 pg/mL. Median endotoxin levels increased from below the level of detection to 5.0 and 4.9 pg/mL at aorta declamping and 30 min into reperfusion, respectively, and decreased afterwards to 4.2 pg/mL on arrival at the ICU.

2.6. In vivo IL-10 measurements

Plasma IL-10 concentrations before anesthetic induction were below or at the lower level of detection in the majority of patients [median 2.0 pg/mL (IQR: 2.0–4.0)]. On aorta declamping, 30 min into reperfusion and on arrival at the ICU were 32 (IQR: 10–88), 37 (IQR: 11–87) and 8.0 (IQR: 4.0–26) pg/mL, respectively (Table 3). No differences in IL-10 levels at any of the time-points were found between the six biallelic genotypes of the proximal IL-10 promoter and the distal SNPs, or the TLR4 haplotypes and genotypes (all $p > 0.17$). However, carriers of the AGCC haplotype (homozygous and heterozygous carriers combined), as compared to carriers of the other haplotypes combined, had slightly higher IL-10 levels at time-points 2 and 3 [35.0 (IQR: 11.0–88.5) versus 19.5 pg/mL (IQR: 6.5–49.0), $p = 0.053$ and 43.0 (IQR: 13.0–112) versus 24.0 pg/mL (IQR: 10.0–62.3), $p = 0.038$, respectively]. Furthermore, when combining measurements from consecutive time-points (i.e. time-point 2 and 3 or 2, 3 and 4 combined, respectively) the differences were more clear [43.0 (IQR: 12.0–106) versus 23.5 pg/mL (IQR: 10.0–55.3), $p = 0.005$ and 24.0 (IQR: 7.0–72.0) versus 16.0 pg/mL (IQR: 5.0–39.3), $p = 0.016$, respectively]. Also, homozygous carriers of the GATA haplotype, as compared to all other patients combined, had lower IL-10 at time-points 2 and 3 [17.0 (IQR: 4.3–28.3) versus 35.0 pg/mL (IQR: 11.0–89.0), $p = 0.026$ and 20.0 (IQR: 10.0–33.0) versus 39.0 pg/mL (IQR: 11.0–105), $p = 0.065$, respectively]. Furthermore, when combining measurements from consecutive time-points (i.e. time-point 2 and 3 or 2, 3 and 4 combined, respectively) the differences were more clear [18.0 (IQR: 5.5–32.0) versus 37.5 pg/mL (IQR: 11.0–106), $p = 0.04$ and 13.5 (IQR: 4.3–26.8) versus 23.0 pg/mL (IQR: 7.0–67.5),

Table 3
Perioperative IL-10 production according to IL-10 and TLR4 genotype/haplotype[a]

		Anesthetic induction	Aorta declamping	30 min into reperfusion	On arrival at the ICU
All patients		2.0 (2.0–4.0)	32 (10–88)	37 (11–87)	8.0 (4.0–26)
		$n = 150$	$n = 137$	$n = 138$	$n = 148$
Endotoxemia[b]					
Negative		2.0 (2.0–4.0)	19 (8.0–68)	33 (11–92)	5.5 (4.0–25)
		$n = 56$	$n = 55$	$n = 54$	$n = 56$
Positive		2.0 (2.0–4.0)	34 (11–103)	41 (12–89)	7.0 (2.0–24)
		$n = 82$	$n = 80$	$n = 82$	$n = 81$
IL-10 promoter genotype					
−1330, −1082, −819, −592/−1330, −1082, −819, −592					
AGCC/AGCC		2.0 (2.0–4.0)	48 (14–207)	44 (16–203)	9.0 (2.0–30)
		$n = 31$	$n = 29$	$n = 28$	$n = 31$
AGCC/GACC		2.0 (2.0–4.0)	32 (8.3–80)	37 (11–88)	6.0 (2.0–29)
		$n = 40$	$n = 36$	$n = 37$	$n = 39$
AGCC/GATA		2.0 (2.0–4.0)	53 (13–87)	45 (16–83)	9.0 (4.0–22)
		$n = 29$	$n = 24$	$n = 25$	$n = 28$
GACC/GACC		2.0 (2.0–2.5)	14 (6.0–46)	11 (6.0–63)	5.0 (2.0–17)
		$n = 14$	$n = 15$	$n = 15$	$n = 15$
GATA/GATA		2.0 (2.0–4.0)	17 (4.3–28)	20 (10–33)	5.0 (4.0–22)
		$n = 15$	$n = 14$	$n = 15$	$n = 15$
GACC/GATA		2.0 (2.0–3.0)	35 (17–168)	36 (20–177)	6.5 (2.5–25)
		$n = 17$	$n = 15$	$n = 14$	$n = 16$
IL-10 promoter genotype					
−3575	TA	2.0 (2.0–4.0)	43 (10–102)	50 (11–159)	7.0 (2.8–30)
		$n = 67$	$n = 60$	$n = 59$	$n = 66$
	TT	2.0 (2.0–4.0)	25 (8.0–50)	24 (10–60)	9.0 (4.0–25)
		$n = 46$	$n = 43$	$n = 43$	$n = 45$
	AA	2.0 (2.0–3.5)	23 (9.8–209)	28 (11–190)	6.0 (2.0–26)
		$n = 16$	$n = 14$	$n = 15$	$n = 16$
−2849	GG	2.0 (2.0–4.0)	32 (8.0–76)	34 (11–81)	9.0 (4.0–26)
		$n = 68$	$n = 62$	$n = 61$	$n = 67$
	AG	2.0 (2.0–4.0)	49 (11–128)	41 (11–140)	7.0 (2.0–31)
		$n = 54$	$n = 49$	$n = 50$	$n = 53$
	AA	3.0 (2.0–5.5)	11 (4.0–73)	12 (8.0–55)	7.0 (2.8–20)
		$n = 4$	$n = 3$	$n = 3$	$n = 4$
−2763	CC	2.0 (2.0–4.0)	28 (8.0–57)	33 (11–63)	9.0 (4.0–26)
		$n = 57$	$n = 51$	$n = 51$	$n = 56$
	AC	2.0 (2.0–4.0)	49 (11–135)	42 (11–194)	8.0 (2.0–28)
		$n = 59$	$n = 54$	$n = 54$	$n = 58$
	AA	2.0 (2.0–4.0)	39 (9.0–212)	36 (11–249)	5.0 (4.0–30)
		$n = 11$	$n = 10$	$n = 10$	$n = 11$
TLR4 haplotype					
299+/399+		2.0 (2.0–4.0)	32 (10–90)	33 (11–109)	8.0 (2.0–27)
		$n = 132$	$n = 122$	$n = 122$	$n = 130$
299−/399+		2.0 (−)	80 (−)	42 (−)	8.0 (−)
		$n = 1$	$n = 1$	$n = 1$	$n = 1$
299−/399−[c]		4.0 (2.0–4.0)	43 (10–50)	43 (21–67)	9.0 (5.0–17)
		$n = 15$	$n = 12$	$n = 13$	$n = 15$
299+/399−		2.0 (2.0–2.0)	9.0 (2.0–16)	21 (5.0–37)	7.5 (6.0–9.0)
		$n = 2$	$n = 2$	$n = 2$	$n = 2$

[a] IL-10 concentrations are in pg/mL. Median values and interquartile range between parentheses. Lower detection limit for IL-10 and endotoxin were 4.0 and 3.0 pg/mL, respectively.
[b] Positive defined as having an endotoxin level of ≥ 5 pg/mL at aorta declamping and/or 30 min into reperfusion, negative defined as an endotoxin level of <5 pg/mL at both the time-points.
[c] One patient out of 15, 12, 13 and 15 homozygous, respectively, all others heterozygous.

$p = 0.010$, respectively]. To determine whether the patterns of IL-10 production over time were different between carriers the proximal IL-10 promoter haplotypes or their six subsequent biallelic genotypes, we used a repeated measurements model as described in the methods. For this analysis log-transformed IL-10 levels were used as dependent variable. Since the time-point proved to be the most significant variable in this model

we used this variable as a fixed-factor, leaving baseline measurements (time-point 1) out of the analysis. To investigate the influence of other relevant variables (i.e. TNF-α and endotoxin concentrations) they were introduced in the model. We did not find statistically significant differences in the pattern of IL-10 production over time between any of the proximal IL-10 promoter haplotypes and the distal SNPs.

The median IL-10 levels were lower at all post-operative time-points in carriers of the 299 and 399 wild-types alleles as compared to carriers of the Asp299Gly or the Thr399Ile polymorphisms respectively, however, this was not statistically significant (all $p > 0.37$). The patterns of IL-10 production over time were not different between carriers of the substitutions as compared to the wild-types.

2.7. Correlations between endotoxin and IL-10 levels

A positive correlation between endotoxin and IL-10 levels was observed when analysing all paired samples ($n = 572$, $r = 0.433$; $p = <0.001$). After excluding either all paired samples at time-point 1 or all paired samples with endotoxin and/or IL-10 level below the level of detection (i.e., endotoxin and IL-10 level below 3.0 and 4.0 pg/mL, respectively) or both, the correlation decreased but remained statistically significant ($n = 424$, $r = 0.115$; $p = 0.018$, $n = 395$, $r = 0.333$; $p = <0.001$ and $n = 350$, $r = 0.132$; $p = 0.013$, respectively). Furthermore, median IL-10 levels, at all post-operative time-points were higher in patients having endotoxemia (defined as having an endotoxin level of >5 pg/mL at aorta declamping and/or 30 min into reperfusion) as compared to patients without endotoxemia, however, this difference did not reach the level of statistical significance (all $p > 0.14$, Table 3).

2.8. Correlations between ex vivo IL-10 production and in vivo IL-10 concentrations

Since in vivo IL-10 concentrations were found to correlate with the level of endotoxemia after aorta declamping, suggesting a causative role for endotoxin in the IL-10 production, it seems plausible to investigate whether a correlation could be found between the in vitro IL-10 production upon LPS stimulation and the in vivo IL-10 concentrations during endotoxemia. A significant negative correlation between IL-10 concentration at aorta declamping and 30 min into reperfusion with ex vivo IL-10 production using 1000 ng/mL LPS was found ($r = -0.303$; $p = 0.001$ and $r = -0.210$; $p = 0.030$, respectively). Also, a significant negative correlation was found between IL-10 concentration at aorta declamping with ex vivo IL-10 production using 100 ng/mL LPS ($r = -0.216$; $p = 0.024$).

Furthermore, we found a negative correlation between IL-10 concentration at aorta declamping and 30 min into reperfusion with the estimated IL-10_{max} ($r = -0.305$; $p = 0.001$ and $r = -0.225$; $p = 0.018$, respectively). After dividing the patients into two groups according to the presence or the absence of endotoxemia (endotoxemia positive patients, defined as patients having an endotoxin level $>$ 5 pg/mL at aorta declamping and/or 30 min into reperfusion or endotoxemia negative patients defined as patients having an endotoxin level $<$ 5 pg/mL at both the time-points) the correlations were only significant in the endotoxemia positive patients. In the patients having endotoxemia, a negative correlation between IL-10 concentration at aorta declamping and 30 min into reperfusion with ex vivo IL-10 production upon stimulation with 1000 ng/mL LPS was found ($r = -0.389$; $p = 0.001$ and $r = -0.282$; $p = 0.023$, respectively). In accordance with this finding the same negative correlation was observed between IL-10 concentration at aorta declamping with ex vivo IL-10 production upon stimulation with 100 ng/mL LPS ($r = -0.280$; $p = 0.023$). Moreover, a significant negative correlation between IL-10 concentration at aorta declamping and 30 min into reperfusion with the estimated IL-10_{max} was found ($r = -0.377$; $p = 0.002$ and $r = -0.279$; $p = 0.022$, respectively).

2.9. Correlations between IL-10 and TNF-α production ex vivo and in vivo

Data on ex vivo TNF-α production and in vivo TNF-α concentrations have been published previously [17]. Herein, we investigated whether any correlations existed between the ex vivo IL-10 and TNF-α production and in vivo concentration were present (Table 4).

We observed positive correlations between TNF-α and IL-10 production ex vivo upon stimulation with all the LPS concentrations tested (Table 4, r ranging from 0.305 to 0.563, all $p < 0.007$). Furthermore, the estimated TNF-α EC_{50} and IL-10 EC_{50}, as well as the TNF-α_{max} and IL-10_{max} were positively correlated ($r = 0.203$; $p = 0.023$ and $r = 0.287$; $p = 0.001$, respectively). The observed positive correlation between TNF-α and IL-10 EC_{50} values, being markers of LPS determined by ex vivo TNF-α and IL-10 production, respectively, indicates that LPS sensitivity influences ex vivo TNF-α and IL-10 production in the same direction.

In vivo no significant correlations were observed between TNF-α and IL-10 concentrations at any of the time-points (Table 4). We find this remarkable since one might expect a positive correlation, especially at the time-points at which endotoxemia occurs most significantly. We hypothesized that the correlation might be absent due to the fact that TNF-α is released early upon endotoxemia, whereas IL-10 is released later and peaks when TNF-α levels already decrease. This hypothesis

Table 4
Correlations between in vivo and in vitro IL-10 and TNF-α production

			IL-10								
			In vivo				In vitro				
			Anesthetic induction	Aorta declamping	30 min into reperfusion	On arrival at the ICU	10 ng/mL	100 ng/mL	1000 ng/mL	IL-10_{max}	$EC_{50[LPS]}$
TNF-α	In vivo	Anesthetic induction	$r = 0.358$ $p = <0.001$	$r = 0.144$ $p = 0.139$	$r = 0.172$ $p = 0.077$	$r = 0.208$ $p = 0.029$	$r = 0.163$ $p = 0.127$	$r = 0.084$ $p = 0.436$	$r = 0.127$ $p = 0.240$	$r = 0.117$ $p = 0.274$	$r = -0.018$ $p = 0.866$
		Aorta declamping	$r = 0.166$ $p = 0.091$	$r = 0.018$ $p = 0.854$	$r = 0.073$ $p = 0.463$	$r = 0.166$ $p = 0.091$	$r = 0.135$ $p = 0.220$	$r = 0.069$ $p = 0.534$	$r = 0.186$ $p = 0.093$	$r = 0.166$ $p = 0.128$	$r = -0.006$ $p = 0.955$
		30 min into reperfusion	$r = 0.222$ $p = 0.021$	$r = -0.069$ $p = 0.479$	$r = -0.072$ $p = 0.457$	$r = 0.258$ $p = 0.007$	$r = 0.215$ $p = 0.049$	$r = 0.129$ $p = 0.243$	$r = 0.168$ $p = 0.128$	$r = 0.131$ $p = 0.230$	$r = -0.042$ $p = 0.703$
		On arrival at the ICU	$r = 0.189$ $p = 0.049$	$r = -0.087$ $p = 0.375$	$r = -0.109$ $p = 0.265$	$r = 0.078$ $p = 0.416$	$r = 0.257$ $p = 0.016$	$r = 0.128$ $p = 0.236$	$r = 0.115$ $p = 0.293$	$r = 0.093$ $p = 0.389$	$r = -0.105$ $p = 0.332$
	In vitro	10 ng/mL	$r = -0.413$ $p = <0.001$	$r = -0.237$ $p = 0.012$	$r = -0.220$ $p = 0.021$	$r = 0.046$ $p = 0.613$	$r = 0.563$ $p = <0.001$	$r = 0.480$ $p = <0.001$	$r = 0.305$ $p = 0.001$	$r = 0.213$ $p = 0.017$	$r = -0.269$ $p = 0.002$
		100 ng/mL	$r = -0.400$ $p = <0.001$	$r = -0.211$ $p = 0.026$	$r = -0.186$ $p = 0.051$	$r = 0.077$ $p = 0.399$	$r = 0.491$ $p = <0.001$	$r = 0.468$ $p = <0.001$	$r = 0.242$ $p = 0.007$	$r = 0.153$ $p = 0.089$	$r = -0.342$ $p = <0.001$
		1000 ng/mL	$r = -0.381$ $p = <0.001$	$r = -0.288$ $p = 0.002$	$r = -0.250$ $p = 0.008$	$r = 0.068$ $p = 0.458$	$r = 0.498$ $p = <0.001$	$r = 0.493$ $p = <0.001$	$r = 0.390$ $p = <0.001$	$r = 0.318$ $p = <0.001$	$r = -0.174$ $p = 0.052$
		TNF-α_{max}	$r = -0.363$ $p = <0.001$	$r = -0.271$ $p = 0.004$	$r = -0.225$ $p = 0.018$	$r = 0.066$ $p = 0.471$	$r = 0.478$ $p = <0.001$	$r = 0.482$ $p = <0.001$	$r = 0.360$ $p = <0.001$	$r = 0.287$ $p = 0.001$	$r = -0.208$ $p = 0.020$
		$EC_{50[LPS]}$	$r = -0.153$ $p = 0.094$	$r = 0.057$ $p = 0.551$	$r = 0.078$ $p = 0.414$	$r = 0.014$ $p = 0.875$	$r = -0.387$ $p = <0.001$	$r = -0.239$ $p = 0.007$	$r = -0.122$ $p = 0.123$	$r = -0.055$ $p = 0.540$	$r = 0.203$ $p = 0.023$

was substantiated by the observation of a positive correlation between TNF-α concentration at time-point 3 and IL-10 production at time-point 4 (i.e. on average 2 h later, $r = 0.258$; $p = 0.007$), indicating some contribution of the TNF-α release magnitude on the subsequent IL-10 production.

3. Discussion

The main findings of the present study are that the circulating IL-10 concentrations following cardiopulmonary bypass in patients undergoing cardio-thoracic surgery correlate positively with the intensity of endotoxemia during the reperfusion phase upon aortic declamping. Patients carrying the AGCC allele had slightly higher post-operative IL-10 levels as compared to carriers of all other haplotypes combined. Homozygous carriers of the GATA allele had lower post-operative IL-10 levels as compared to all other patients. Furthermore, AGCC allele carriers had lower IL-10 EC_{50} values (i.e. higher LPS sensitivity ex vivo) as compared to patients carrying the other alleles, whereas carriers of the GATA allele showed the opposite (i.e. higher EC_{50} values; lower LPS sensitivity). For both the AGCC and the GATA allele carriers, the maximal induced IL-10 production upon ex vivo LPS stimulation was similar as compared to the other groups. The in vivo IL-10 release during reperfusion (i.e. at aorta declamping and 30 min into reperfusion) in patients experiencing endotoxemia was significantly correlated with the IL-10 production ex vivo upon stimulation with 1000 ng/mL and the estimated maximal IL-10 production capacity (the $IL\text{-}10_{max}$), no correlation was found between the IL-10 EC_{50} ex vivo with in vivo IL-10 levels.

The model we choose, i.e. measuring naturally occurring endotoxemia and subsequent cytokine release in vivo in patients undergoing cardio-thoracic surgery, enables us to study the inter-individual differences in these responses and relate these to genetic factors. In a previous publication we described the TNF-α response in the same way. We found significant endotoxemia in over half of the patients undergoing cardiac surgery with cardiopulmonary bypass which is in accordance with previous studies [18,19]. At the peak of endotoxemia, upon aortic declamping and 30 min into reperfusion, we observed a significant positive correlation between circulating endotoxin levels and IL-10 production, indicating a role of endotoxin in the perioperative release of IL-10. However, although we found a significant correlation, it's magnitude is limited, suggesting that many other factors apart from endotoxin play a role in the variation of IL-10 production in these patients.

Since we used 3 different concentrations of LPS in our ex vivo whole blood assay we were able to calculate descriptive parameters of the dose–response relation for each patient. Also, to estimate spread relative to the mean, the coefficients of variation (standard deviation [SD] divided by the mean) were calculated for all LPS concentrations. The values were 50.4%, 46.4% and 94.8% for 10, 100 and 1000 ng/mL LPS, respectively. This variation is similar to the variation found in other populations and exceeds the variation that can be explained by the laboratory variation only, which is estimated at 9.9–12.3% [4]. This indicates that the variations we found between subjects are to be explained by other factors than laboratory variation only. Since we find it feasible to assume that the LPS concentration needed for the release of 50% of the maximally evoked IL-10, the EC_{50}, is a marker of LPS sensitivity we evaluated to what extent this parameter could presage the in vivo response to endotoxin. Theoretically, important inter-individual differences in the dose–response characteristics of IL-10 production upon LPS stimulation can be overlooked by measuring IL-10 production upon stimulation with only one single (supra-physiological) LPS concentration.

We found that the level of ex vivo IL-10 production upon stimulation with 1000 ng LPS/mL was significantly correlated with the in vivo production upon significant endotoxemia. Thus, the ex vivo LPS stimulation assay is a predictor of the in vivo release of IL-10 during endotoxemia after cardiac surgery. Remarkably, however, the correlation is inverse; high ex vivo IL-10 production capacity is associated with low in vivo circulating IL-10 levels. The explanation of this finding is puzzling. To our knowledge no other study investigated the correlation between endotoxin mediated IL-10 production ex vivo and in vivo. With respect to the validity of the comparison of the ex vivo and in vivo IL-10 production, and the conclusions drawn, several factors should be taken into account. In our analysis we correlated IL-10 concentrations in vivo with in vitro production capacity. In these analyses the time factor is rather artificially ignored to a certain extent. In vivo the time between exposure to the stimulus (endotoxin) and measurement of the cytokine was several hours at the maximum. In vitro, however, we measured the IL-10 concentration at 24 h after adding LPS. In a previous study it was demonstrated that after 18 h of ex vivo stimulation the IL-10 mRNA levels reached the highest level making measurement of IL-10 after 24 h as indicator of maximal IL-10 production capacity logical [7]. Day-to-day intra-individual variation of IL-10 concentrations after 24 h of stimulation was low (about 10%). Giving the wide range of IL-10 production levels between individuals this measurement seems a good basis to demonstrate genetically defined variation [6]. We measured the ex vivo IL-10 production in a blood sample drawn several hours before the in vivo production capacity was measured, probably making the variation

due to day-to-day variation even smaller. Although in all patients a standardized anesthesia protocol was followed, differences in the use of certain medical devices and medications might have influenced our results. Also, not all patients experienced the same amount of endotoxin stimulus in vivo. Although most patients had significant endotoxemia at the later time-points, some did not. To be able to study the relationship between endotoxin and IL-10 release we specifically studied their relation in patients with significant endotoxemia. We found even stronger correlations in the patients experiencing endotoxemia, indicating the relevance of endotoxin as stimulus in the in vivo IL-10 production. Furthermore, for the in vitro stimulation assay we used crude *Escherichia coli* LPS rather than purified lipid A, the ligand for TLR4. Recent studies indicate that these preparations can be contaminated with relevant traces of other bacterial products, i.e. lipoproteins, rather than lipid A, that signal through other receptors deriving from the TLR family, especially TLR2 [20,21]. This might have complicated our conclusions on the role of the TLR4 genotype. However, our in vivo model reflects a physiological exposure to naturally occurring endotoxemia. Also, in Gram-negative bacterial infection it is likely that the innate immune system is confronted with a mixture of endotoxins. One study reported post-operative down-regulation of TLR2 and TLR4 receptors on PBMC's after abdominal surgery [22]. No data exist on TLR receptor down-regulation in cardio-thoracic surgery with extracorporal bypass.

Since several studies indicated a role of genetics in cytokine release we investigated the influence of the various known polymorphism in the IL-10 promoter. The frequencies of the various genotypes were similar to those found in other populations (Table 1) [9,10,13,23–28]. We found that the carriers of the GATA haplotype had lower LPS sensitivity and IL-10 production ex vivo, furthermore, homozygous GATA carriers also had lower IL-10 production in vivo. On the other hand, carriers of the AGCC haplotype had higher sensitivity to LPS. In a study of twins by Reuss et al. the AGCC haplotype was associated with higher (around 20%) transcriptional activity in a reporter gene assay using luciferase as reporter [23]. The study emphasizes the importance of the SNP at position −1082 in the transcriptional activity of the proximal promoter. It is suggested that an A at position −1082 confers optimal binding affinity for the transcriptional factor PU.1 that inhibits gene expression, resulting in an increased transcriptional activity when a G is present at position −1082. However, in the study by Reuss et al. levels of produced IL-10 upon whole blood stimulation using 100 ng/mL LPS were not different between the various haplotypes [23]. An explanation for this finding could be that the LPS concentration used in their assay was too high to pick up the differences we found at the lower LPS concentrations. As mentioned before, we found no differences in IL-10 levels using the highest LPS concentration (1000 ng/mL). The phenotype of increased LPS sensitivity we found ex vivo in AGCC haplotype carriers, is further supported by the significantly higher circulating IL-10 levels following cardiopulmonary bypass in the AGCC haplotype carriers as compared to carriers of the other haplotypes combined. At this time-point the endotoxin levels peaked in most patients. In a study conducted by Suárez et al. constitutive IL-10 levels were measured in peripheral blood of healthy volunteers and were correlated to SNPs in the proximal promoter region. In that study, the GCC genotype was found significantly more often in the group with high IL-10 levels ($\geq$ 2 pg/mL), again indicating the importance of a G at position −1082 [24]. Constitutive IL-10 gene expression (measured by IL-10 mRNA detection from whole blood samples) was higher in subjects carrying the GCC/GCC genotype as compared to the non-GCC carrying genotypes [24]. In a study performed by Fijen et al. no differences were found in IL-10 production between the −1082G/A genotypes in an experimental human endotoxemia model in healthy subjects [29]. However, looking more closely to their findings in a rather small group of healthy subjects, a trend is visible towards higher IL-10 levels in carriers of a G at position −1082. No other study investigated the role of IL-10 polymorphisms in endotoxemia mediated IL-10 production capacity in vivo.

The common TLR4 polymorphisms (Asp299Gly and Thr399Ile) are associated with slightly higher IL-10 capacity ex vivo, however, this did not reach the level of statistical significance. The frequencies of the various genotypes were similar to those found in other populations (Table 1) [30–34]. To our knowledge no other study investigated the correlation between TLR4 polymorphisms and the ex vivo or the in vivo endotoxin mediated IL-10 production. The common TLR4 polymorphisms (Asp299Gly and Thr399Ile) are not associated with differences in IL-10 production capacity ex vivo and in vivo.

Although a type II error could be responsible for overlooking differences in IL-10 production capacity between the other IL-10 promoter and the TLR4 polymorphisms studied, our data suggest that their role, if present, is limited, and cannot explain the large inter-individual differences found in IL-10 capacity, which was recently estimated at around 50% [23]. The demographic characteristics of the cardiac surgery patients were similar to other groups of patients described in the literature [35,36], making direct extrapolation of our findings possible.

In summary, based on our results and the findings in the literature, we propose that it is likely that the IL-10

promoter polymorphism at position −1082 base pairs relative to the transcriptional start site plays a functional role in LPS mediated IL-10 production capacity in vitro and in vivo. The common TLR4 polymorphisms do not interact with factors determining IL-10 production capacity in vivo and ex vivo. Although part of the large inter-individual variation found in the in vivo and ex vivo responses to LPS can be explained by known polymorphisms in the IL-10 promoter region, overall their effects are limited and can only explain about 5−10% of the variation. Further studies are needed to determine to what extent the remaining inter-individual variation found in the IL-10 response to LPS is determined by genetic or random, environmental factors.

4. Materials and methods

4.1. Patients

The study was performed at the Leiden University Medical Center, an 800-bed secondary and tertiary referral hospital. To be eligible for enrolment, the patients had to be aged 18 years or older and being scheduled for elective cardiac surgery with cardiopulmonary bypass between July 1, 1998, and December 30, 1999. We obtained institutional approval from the local medical ethics committee (protocol #P168/96). Each patient gave a written, informed consent. The patients studied were 159 consecutive patients undergoing elective cardiac surgery with cardiopulmonary bypass. Of these, 122 had been included in a previously published study on the effect of selective gut decontamination (SGD) on endotoxemia and cytokine activation [37]. Of these 122 patients 24 received preoperative SGD consisting of polymyxin B and neomycin. In the previously mentioned study, perioperative endotoxemia and subsequent cytokine activation were found not to be influenced by the SGD regimen. Also, the results were not different when these patients were excluded from the analysis. Patients were followed-up throughout their stay in the hospital. Demographic characteristics were systematically collected for all the patients entering the study.

4.2. Study design

On the day of surgery blood samples for ex vivo whole blood LPS stimulation, using pyrogen-free tubes (Kabi-Vitrum, Amsterdam, The Netherlands), and DNA extraction were drawn before anesthesia. Blood samples for the determination of in vivo endotoxin and cytokine levels were collected from each patient before anesthetic induction, on aorta declamping, 30 min into body reperfusion (i.e. 30 min after stopping the extra corporal perfusion), and at the ICU admission (approximately 2 h after surgery). In a previous pilot study, we found that endotoxemia occurs most likely at these time-points [37].

4.3. Whole blood LPS stimulation

Cytokine production was determined in whole blood samples ex vivo as previously described [38]. Briefly, whole blood samples were mixed 1:1 with RPMI 1640 (Gibco, Germany) and lipopolysaccharide (*E. coli* 0111:B4, Difco, Detroit, MI) was added to final concentrations of 10, 100 and 1000 ng/mL and cells were stimulated for 24 h at 37 °C under 5% CO_2 atmosphere. Interleukin-10 concentrations were measured in the supernatant as described below.

4.4. Endotoxin measurements

Blood for endotoxin determination was collected in pyrogen-free tubes (Kabi-Vitrum, Amsterdam, The Netherlands) and the platelet rich plasma was prepared by centrifugation. Endotoxin was determined by a quantitative photometric assay with end-point measurement as described elsewhere [39]. The assay's lower detection limit for endotoxin was about 3.0 pg/mL [39].

4.5. IL-10 measurements

Blood for determination of IL-10 was collected in pyrogen-free ethylenediaminetetraacetic acid (EDTA) tubes (Chromogenics, Amsterdam, Netherlands) and immersed in ice. Plasma was prepared by centrifugation at 3000*g* for 5−10 min at 4 °C and stored at −70 °C. Interleukin-10 concentrations in the supernatants of the ex vivo whole blood assay and the samples from the in vivo IL-10 production were analysed at completion of the study. Interleukin-10 concentrations were determined with a standard ELISA technique (Central Laboratories for Bloodtransfusion, Amsterdam, The Netherlands; Medgenix diagnostics, Floury, Belgium); the lower detection limit for IL-10 was 4.0 pg/mL.

4.6. DNA isolation

DNA was isolated from citrate blood from each patient, red blood cells were lysed with three volumes of lysis buffer (1.55 M NH_4Cl, 0.1 M $KHCO_3$, 0.01 M EDTA, pH 7.4); the remaining cells were incubated for 17 h at 37 °C with SDS and proteinase K. The DNA was extracted using phenol and chloroform as described by Maniatis et al. [40].

Table 5
Sequence and specificity of biotin labeled sequence-specific oligonucleotides and critical washing temperature (CWT) in °C

Polymorphism	Primer sequence (5′ → 3′)	CWT	Specificity
−1330	ACCTAGGTCAATGTTCCTCC	52	G
	GGAGGAACACTGACCTAGGT	54	A
−1082	TTCTTTGGGAGGGGGAAG	51	G
	ACTTCCCCTTCCCAAAGAA	52	A
−819	CAGGTGATGTAACATCTCTGTGC	62	C
	GCACAGAGATATTACATCACCTGT	63	T
−592	CCGCCTGTCCTGTAGGAA	50	C
	TTCCTACAGTACAGGCGGG	52	A

4.7. Typing of the proximal IL-10 promoter polymorphism

The polymorphic regions at positions −1330, −1082, −819 and −592 were evaluated by PCR amplification using the following primers: 5′-CCCTGACTATA-GAGTGGCAG-3′ (forward) and 5′-GTGCTGAGC-TGTGCATGCCT-3′ (reverse). The single nucleotide polymorphisms were determined using Allele Specific Hybridization (ASH-blot) as previously described [10]. Briefly, the PCR products were dot blotted on the membranes (Hybond−N^+, Amersham, Buckinghamshire, UK) and hybridized overnight at 42 °C with the specific biotin labeled oligoprimer. After hybridization the blot was washed with ASH-buffer at the critical temperature. Sequences of the sequence-specific oligoprimers and their respective critical washing temperatures are given in Table 5.

4.8. Typing of distal IL-10 promoter polymorphisms

Typing for the IL-10 single nucleotide polymorphisms at positions −3575, −2849 and −2763 was performed by PCR amplification. Forward and reverse primers used are given in Table 6 [28]. For the positions −2849 and −2763 a second, nested-PCR was performed using P2BR as reverse primer (Table 6). Primers were purchased from Biosource International (Foster City, CA). PCR amplifications were performed using the Thermolyne Amplitron II Thermal Cycler (Barnstead/Thermolyne, Dubuque, IA). PCR-amplified products were electrophoresed on a 10% polyacrylamide gel to detect the size differences of the fragments between the polymorphisms, following digestion with a specific restriction enzyme (Table 6).

4.9. TLR4 mutation analysis

The presence of the TLR4 Asp299Gly and Thr399Ile mutations was determined as previously described [17].

4.10. Calculation of ex vivo LPS/IL-10 dose−response

For each patient two characteristics (i.e. EC_{50} and E_{max}) of the LPS-induced IL-10 release were calculated by non-linear regression with the dose−response model according to the Hill equation (Eq. (1)).

$$E_N = E_{N,max} \times C/(EC_{50} + C) \quad (1)$$

where E_N is the observed IL-10 production at a given LPS concentration C, EC_{50} is the estimated LPS concentration at which 50% of the maximal IL-10 response is reached, and $E_{N,max}$ is the estimated maximal IL-10 production. In a previous pilot study we found that measuring the IL-10 production at the LPS concentration we used (i.e. 10, 100 and 1000 ng/mL) was sufficient to calculate the dose−response characteristics EC_{50} and $E_{N,max}$ (E.S., unpublished observation, report in preparation).

4.11. Statistical analysis

IL-10 concentrations in vivo and ex vivo in different haplotype groups and genotypes were not distributed

Table 6
Primers sequences and restriction enzymes used for detection of the polymorphisms in the IL-10 gene promoter and size of the PCR products and restriction fragments

Polymorphism	Primer sequence (5′ → 3′)	PCR product (bps)	Enzyme	Size	Allele
−3575	GGTTTTCCTTCATTTGCAGC	231	*Bam*HI	121 + 110	TT
	ACACTGTGAGCTTCTTGAGG			231	AA
−2849	CTGTAATCTCAGCACTCTGG	193	*Alw*I	153 + 40	GG
	ACCAAGTCTGGCCCTTGGAA/GTTCAAGCCATTCTCCTGCC			193	AA
−2763	CTGTAATCTCAGCACTCTGG	193	*Dde*I	114 + 71 + 8	CC
	ACCAAGTCTGGCCCTTGGAA/GTTCAAGCCATTCTCCTGCC			185 + 8	AA

normally, and so non-parametric testing has been used throughout. IL-10 concentrations are quoted as the median with interquartile ranges between parentheses. For statistical analysis blood samples with IL-10 and endotoxin levels below the limit of detection were entered as half of the value of the lower detection limit (2.0 and 1.5 pg/mL, respectively). To determine whether polymorphisms in the IL-10 promoter were associated with levels of in vivo IL-10 production, repeated measurements models (SPPS 11.0, mixed) were used to determine whether the patterns of IL-10 production over time were different between polymorphisms. For this analysis log-transformed IL-10 and endotoxin levels were used. The ex vivo and in vivo IL-10 production characteristics between the various haplotypes and genotypes were compared using Kruskal–Wallis test, individual groups were compared by the Mann–Whitney *U* test. Correlations were assessed non-parametrically using Spearman correlation test. Statistical significance was tested two-tailed, with the α set to 0.05.

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