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

Schippers, E.F.

CHAPTER 7

Cytokine response to endotoxin in individuals heterozygous for the Delta32 mutation of chemokine receptor CCR5

M. Heesen[1], E.F. Schippers[2], B. Bloemeke[3], D. Kunz[4] and J.T. van Dissel[2]

[1]Department of Anesthesia, Academic Medical Center, University of Amsterdam, Amsterdam, the Netherlands
[2]Department of Infectious Diseases, Leiden University Medical Center, Leiden, the Netherlands
[3]Department of Dermatology and [4]Clinical Chemistry and Pathobiochemistry, University Hospital of Aachen, Aachen, Germany

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Cytokine response to endotoxin in individuals heterozygous for the Δ32 mutation of chemokine receptor CCR5

Michael Heesen[a,*], Emile F. Schippers[b], Brunhilde Bloemeke[c], Dagmar Kunz[d], Jaap T. van Dissel[b]

[a]*Department of Anesthesia, Academic Medical Center, University of Amsterdam, Meibergdreef 9, 1100 DD Amsterdam, The Netherlands*
[b]*Department of Infectious Diseases, Leiden University Medical Center, Leiden, The Netherlands*
[c]*Department of Dermatology, University Hospital of Aachen, Pauwelsstrasse 30, 52057 Aachen, Germany*
[d]*Department of Clinical Chemistry and Pathobiochemistry, University Hospital of Aachen, Pauwelsstrasse 30, 52057 Aachen, Germany*

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Abstract

Studies of mice with a targeted disruption of the CCR5 gene suggest that the CC chemokine receptor 5 (CCR5) is a determinant of the cytokine response to endotoxin. In humans, a naturally occurring mutation of the CCR5 gene is a 32-basepair (bp) deletion which precludes the translation of the gene into a functional transmembrane protein. To evaluate the cytokine phenotype of heterozygosity for the 32 deletion, we studied the endotoxin-stimulated release of tumor necrosis factor-α, Interleukin (IL)-6, IL-8, IL-10, and IL-12 in whole blood ex-vivo of healthy volunteers and patients undergoing elective cardiac bypass surgery. This operation represents a major surgical trauma associated with ischemia-reperfusion-injury and triggers a profound inflammatory response. In these patients, cytokine plasma concentrations were measured during and after cardiac surgery. No difference was found between the frequencies of the observed and expected CCR5 genotypes in the groups of individuals studied. Furthermore, no significant difference in ex-vivo or peri- and postoperative cytokine plasma concentrations was detected between CCR5 wild-type homozygotes and individuals carrying one defective CCR5 allele. Our results indicate that heterozygosity for the 32 bp deletion of CCR5 is not associated with an altered cytokine response to endotoxin or to a major surgical trauma when compared with individuals homozygous for the wild-type allele.

Keywords: CC chemokine receptor 5; Cytokines; Endotoxin; Genetic polymorphism

1. Introduction

The seven-transmembrane spanning G protein-coupled receptor CCR5 binds the CC chemokines macrophage inflammatory protein-1α (MIP-1α), MIP-1β, and RANTES [1]. It is expressed on monocytes as well as CD45RO+ (memory) T cells [1,2]. Particular interest in this molecule comes from its role as an HIV co-receptor required for the infection of CD4 positive cells with macrophage-tropic HIV isolates [3]. A genetic variant of this molecule exists consisting of a 32-nucleotide deletion (Δ32) in the region encoding the second external loop [4]. This deletion causes a frame shift and the introduction of a premature stop codon. The protein encoded by this deleted allele is not translated [4]. Homozygosity for this defect was reported as a mechanism of resistance to HIV infection in multiply exposed individuals [4,5]. Heterozygosity for the Δ32 mutation is associated with a slower progression to AIDS and a longer disease-free survival compared with wild-type HIV infected individuals [5,6]. The frequency of the mutated allele is 10% in Caucasians [7]. Carriers of the Δ32 allele are healthy and, so far, under normal conditions no phenotype could be attributed to the heterozygous or homozygous genotype. This suggests that CCR5 function may be dispensable under normal conditions.

* Corresponding author. Tel.: +31-205-662-533; fax: +31-206-979-441.
E-mail address: m.heesen@amc.uva.nl (M. Heesen).

Studies in mice with a targeted disruption of this molecule presented evidence for a role of CCR5 in host defense against infection [8]. After intraperitoneal challenge with different concentrations of endotoxin CCR5 deficient mice had a higher survival rate than CCR5 wild types. Also, the LD_{50} value of endotoxin was significantly lower in wild-type compared with the knock-out mice (18.15 vs. 34.05 mg endotoxin/kg animal body weight). Moreover, peritoneal macrophages of CCR5 knock-out animals differed from their wild-type littermates with regard to the cytokine production. Macrophages of mice lacking CCR5 display reduced endotoxin-stimulated production of Interleukin (IL)-1β and IL-6. No difference in the release of tumor necrosis factor (TNF)-α was found between knock-out and wild-type mice [8]. Since these results of animal studies relate CCR5 expression to cytokine release, we hypothesized that heterozygosity for the Δ32 allele might be associated with differences in the cytokine response to endotoxin in humans as well. We studied healthy human volunteers and patients scheduled for elective cardiac surgery and evaluated the ex-vivo cytokine release after incubation of whole blood with endotoxin. In addition, peri- and postoperative cytokine plasma concentrations were determined in the patients undergoing cardiac surgery.

2. Results

Ninety blood donors and 122 cardiac surgery patients were genotyped for the CCR5 polymorphism. In both groups the frequencies of the wild-type (CCR5) allele and of the mutated Δ32 allele were found to be 93 and 7%, respectively. Thus, the genotype frequencies were 87% for CCR5/CCR5 and 13% for the heterozygous genotype (CCR5/Δ32). Statistical analysis did not reveal a difference between the frequencies of the observed genotypes and those of the expected genotypes, indicating that both study groups were in Hardy–Weinberg equilibrium.

The concentrations of the cytokines TNF-α, IL-1β, IL-6, and IL-8 obtained after stimulating whole blood of blood donors with 100 ng/ml endotoxin for 4 h are given in Table 1. Overall, no significant difference was found in the cytokine release of individuals homozygous for the CCR5 allele or CCR5/Δ32 heterozygotes.

Tables 2 and 3 summarize the data obtained from patients scheduled for elective cardiac surgery. In the whole blood samples taken before surgery and stimulated ex-vivo with various concentrations of endotoxin, a dose-dependent increase in TNF-α, IL-10, and IL-12 release was observed that did not differ between patients carrying either one of the CCR5 genotypes (Table 2). Furthermore, the concentrations of TNF-α, IL-10, and IL-6 in the blood of these patients during and after cardiac surgery (Table 3) did not reveal a significant difference between the CCR5/CCR5 and CCR5/Δ32 genotype, neither on release of the aortic cross-clamp nor on arrival at the intensive care unit (ICU) 2 h after completion of surgery.

Table 1
Whole blood cytokine response of healthy volunteers to endotoxin according to CCR5 genotypes

	CCR5/CCR5 ($n = 78$)	CCR5/Δ32 ($n = 12$)	p Values
TNF-α	2198 (1684–3709)	3278 (2237–3872)	0.345
IL-1β	381 (247–737)	280 (175–725)	0.544
IL-6	2876 (2329–4634)	3586 (2944–4368)	0.717
IL-8	1452 (833–2186)	1025 (582–2670)	0.430

Cytokine concentrations are ng/ml. Values are given as median with interquartile ranges (25th–75th percentile) in parentheses. Whole blood was stimulated with 100 ng/ml endotoxin (*E. coli* O5 : B55) for 4 h at 37 °C. Cytokine plasma concentrations were determined by measuring immunoreactivity by means of chemiluminescence.

3. Discussion

The main finding of the present study is that in humans the endotoxin-stimulated cytokine release did not differ between individuals homozygous for the wild-type CCR5 allele and persons heterozygous for CCR5 Δ32. This conclusion is based on two observations. Firstly, using an established and validated whole blood system to assess the release of TNF-α, IL-1β, IL-6, IL-8, and IL-12 after 4 h incubation with endotoxin, and IL-10 after 24 h stimulation, the cytokine release was not affected by CCR5 genotype. This assay avoids confounding factors including adherence-induced expression of cytokines that can be associated with isolation of certain cell populations such as monocytes. In addition, whole blood probably represents the most physiological environment for cytokine-producing cells. In a second approach, the cytokine release during and after surgery was determined in patients undergoing cardiac surgery. Open heart surgery with cardiopulmonary bypass triggers the release of pro- and anti-inflammatory cytokines into the systemic circulation [9,10]. Also in the peri- and postoperative blood samples, the cytokine concentrations did not differ according to the CCR5 genotype of the patients.

As a response to cardiac surgery plasma concentrations of TNF-α and IL-6 increased whereas IL-10 levels decreased. This finding is in agreement with previous reports [11–13]. The sources of plasma TNF-α are monocytes/macrophages and the myocardium [14]. IL-6 is produced by a variety of cells including monocytes, fibroblasts, and hepatocytes [15]. Wan et al. [16] identified the liver as the primary source of IL-10 in the perioperative phase of cardiac surgery.

In previous approaches to relate a phenotype to the Δ32 mutation, the concentrations of the natural ligands

Table 2
Ex-vivo cytokine release after endotoxin-stimulation of whole blood of patients before elective cardiac bypass surgery

10 ng/ml endotoxin		100 ng/ml endotoxin		1000 ng/ml endotoxin	
CCR5/CCR5	CCR5/Δ32	CCR5/CCR5	CCR5/Δ32	CCR5/CCR5	CCR5/Δ32
TNF-α 9323 (6930–12277)	8380 (7031–15203)	12589 (9034–17009)	13559 (8617–22320)	16975 (13408–22127)	18097 (12848–29217)
IL-10 798 (517–1128)	699 (409–1198)	1492 (1006–1962)	1397 (816–1985)	3475 (2543–4510)	3269 (2041–5070)
IL-12 1.0 (0.0–5.0)	3.5 (1.5–8.8)	2.0 (0.0–7.8)	5.5 (1.3–11.3)	7.0 (2.0–18.0)	12.0 (5.0–28.0)

Cytokine concentrations are in ng/ml. Values represent median with interquartile range (25th–75th percentile) in parentheses.
All *p*-values for differences between homozygous and heterozygous genotype > 0.40 (TNF-α $n = 106$ and 16; IL-10 $n = 101$ and 16; IL-12 $n = 102$ and 16).
Whole blood was incubated with endotoxin (*E. coli* 0111:B4) for 4 h (TNF-α, IL-12) or 24 h (IL-10) at 37 °C. Cytokine plasma concentrations were determined by ELISA.

of this receptor, the chemokines MIP-1α, MIP-1β, and RANTES were studied. Higher concentrations of these chemokines were reported in two individuals homozygous for the Δ32 mutation compared with wild-type homozygotes [17]. Yang et al. [18] studied chemokine concentrations in individuals homozygous for the wild-type and in Δ32 heterozygotes. This group was unable to find a difference in the chemokine synthesis after endotoxin stimulation.

In contrast, animal studies related the lack of the CCR5 molecule to the cytokine production. Mice with a targeted disruption of CCR5 showed a 50% reduction in the levels of IL-1β and IL-6 after endotoxin stimulation of peritoneal macrophages whereas no difference was observed for TNF-α [8]. We therefore hypothesized that the cytokine response may characterize the CCR5/Δ32 genotype. The Δ32 allele is a mutation introducing a premature stop codon and the deleted allele is not translated [4]. Carriers of the genotype CCR5/Δ32 thus lack one functional allele. One would expect that individuals carrying one Δ32 allele which does not code for an intact protein have about half the number of CCR5 molecules on the cell surface. However, the amount of CCR5 surface expression showed a 20-fold variation among individuals with two intact CCR5 alleles, using an MIP-1β binding assay or anti-CCR5 monoclonal antibodies [19,20]. The reasons for this high interindividual variability in wild-type homozygotes remain unclear. It cannot be excluded that genetic factors other than the Δ32 mutation determine the cellular density of CCR5. This high variance may by far outnumber the role of the defective CCR5 allele.

Among the reasons for the lack of association between cytokine release and CCR5 genotype, genetic polymorphisms of the cytokine genes have to be mentioned. Such genetic variations have been identified for all cytokines determined in our study [21–24]. These polymorphisms were shown to influence the synthesis of the respective cytokine. For instance, the TNF-α gene has a polymorphism at position −308 of the promoter region which regulates gene transcription [21]. The promoter region of the IL-1β gene bears two polymorphic sites, −31 and −511 [22]. The −31 polymorphism affects DNA binding and is in a linkage disequilibrium with the −511 site. Similarly, the promoter of the IL-6 gene was also found to carry a polymorphism consisting of a G/C substitution at position −174 [23]. In a reporter gene assay, the −174C oligonucleotide had a lower expression than the −174G construct suggesting a higher IL-6 synthesis by the G allele. Polymorphisms were also found in the IL-10 gene and associations with gene transcription were reported [24]. The study by Zhou et al. [8] suggested that in mice the concentrations of IL-1β and IL-6 are under the influence of the CCR5 polymorphism. It is not known

Table 3
Perioperative and postoperative plasma concentrations of various cytokines in patients undergoing elective cardiac bypass surgery

	Upon release of aorta cross-clamp		Upon arrival at ICU	
	CCR5/CCR5	CCR5/Δ32	CCR5/CCR5	CCR5/Δ32
TNF-α	2.4 (0.6–4.3)	2.7 (0.2–5.9)	4.3 (2.3–7.5)	5.1 (3.1–7.5)
IL-10	32 (11–81)	39 (6–207)	9 (4–26)	5 (0–17)
IL-6	39 (20–64)	35 (13–67)	300 (103–750)	146 (103–198)

Cytokine concentrations are in ng/ml. Values represent median with interquartile range (25th–75th percentile) in parentheses.
All *p*-values for differences between homozygous and heterozygous genotype > 0.40, except for IL-6 values upon arrival at ICU, $p = 0.068$.
Data on homozygous and heterozygous genotype available for TNF-α $n = 86$ and 16; IL-10 $n = 110$ and 21; IL-6 $n = 57$ and 9. Plasma cytokine levels were determined by ELISA.

whether the above-mentioned IL-1β and IL-6 polymorphisms which were described in humans also exist in mice. In humans, these cytokine genetic variations might outweigh the effect of a defective CCR5 molecule.

In conclusion, our study suggests that heterozygosity for the Δ32 mutation of CCR5 is not associated with a significant phenotype affecting the cytokine response to an infectious or inflammatory stimulus when compared with that in individuals homozygous for the wild-type allele.

4. Materials and methods

4.1. Subjects and cytokine measurements

Written informed consent from each individual and approval of the local medical ethics committee were obtained. Two groups of subjects were studied. The first group consisted of Caucasian healthy blood donors (aged 18–65 years). Twenty milliliters of heparin-anticoagulated blood was drawn and mixed 1 : 1 with RPMI 1640 (Gibco BRL, Karlsruhe, Germany) and stimulated with 100 ng/ml endotoxin (*Escherichia coli* O5 : B55, Sigma, St. Louis, MO) for 4 h at 37 °C. The plasma concentrations of TNF-α, IL-1β, IL-6, and IL-8 were determined by measuring immunoreactivity by means of chemiluminescence (Immulite, DPC Biermann, Bad Nauheim, Germany).

The second group were Caucasian patients undergoing elective cardiac surgery with cardiopulmonary bypass. On the morning just before surgery blood samples for the determination of ex vivo cytokine production were collected using heparin-anticoagulated tubes. Cytokine production was determined in whole-blood samples ex vivo as previously described [25]. 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 4 or 24 h at 37 °C. The operative procedures including premedication and anesthesia are described elsewhere [9]. During operation, blood samples were taken immediately upon release of the aorta clamp and upon arrival at the ICU 2 h after completion of surgery. Blood samples were collected in pyrogen-free ethylenediaminetetraacetic acid (EDTA) tubes and immersed in ice. Plasma was prepared by centrifugation at $3000 \times g$ for 5–10 min at 4 °C and stored at −70 °C. TNF-α, IL-10, IL-12, and IL-6 concentrations were determined with an ELISA technique (Central Laboratories for Bloodtransfusion, Amsterdam, The Netherlands and Medgenix diagnostics, Floury, Belgium) [26].

4.2. Genotyping for the CCR5 Δ32 polymorphism

Genomic DNA was isolated from peripheral blood cells using the QIAmp Blood kit (QIAgen, Hilden, Germany). Twenty nanograms of DNA was used in a 50 μl polymerase chain reaction (PCR) containing 1.5 mM $MgCl_2$ and 20 pmol of the forward primer 5′-ATCTCAAAAAGAAGGTCTTCATTAC-3′ and the reverse primer 5′-GAGTAGCAGATGACCATGACAA-3′, as described previously [5]. PCR conditions were as follows: 94 °C for 5 min followed by 35 cycles with 94 °C for 20 s, 55 °C for 30 s, 72 °C for 20 s, and a final elongation step (72 °C for 5 min). The PCR product was visualized on a 2% agarose gel. Based on the band size an allele was classified as wild-type (187 bp) or Δ32 (155 bp).

4.3. Statistical analysis

Data analysis was performed with the SPSS for Windows Release 10.0.7 program. The 2 × 2 table chi-square test was used to test for the Hardy–Weinberg equilibrium by comparing the expected with the observed genotype frequencies. Cytokine concentrations are given as median and interquartile range (25th–75th percentile). The Wilcox Mann–Whitney-test served for the comparison of cytokine data between the two CCR5 genotypes. Statistical significance was set at *p*-levels <0.05.

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References

[1] Raport CJ, Gosling J, Schweickart VL, Gray PW, Charo IF. Molecular cloning and functional characterization of a novel human CC chemokine receptor (CCR5) for RANTES, MIP-1β, and MIP-1α. J Biol Chem 1996;271:17161–6.

[2] Qin S, Rottman JB, Myers P, Kassam N, Weinblatt M, Loetscher M, et al. The chemokine receptors CXCR3 and CCR5 mark subsets of T cells associated with certain inflammatory reactions. J Clin Invest 1998;101:746–54.

[3] Dragic T, Litwin V, Allaway GP, Martin SR, Huang Y, Nagashima KA, et al. HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5. Nature 1996;381:667–73.

[4] Liu R, Paxton WA, Choe S, Ceradini D, Martin SR, Horuk R, et al. Homozygous defect in HIV-1 coreceptor accounts for resistance to some multiply-exposed individuals to HIV-1 infection. Cell 1996;86:367–77.

[5] Huang Y, Paxton WA, Wolinsky SM, Neumann AU, Zhang L, He T, et al. The role of a mutant CCR5 allele in HIV-1 transmission and disease progression. Nat Med 1996;2:1240–3.

[6] Rowe PM. CKR-5 deletion heterozygotes progress slower to AIDS. Lancet 1996;348:947.

[7] Martinson JJ, Chapman NH, Rees DC, Liu YT, Clegg JB. Global distribution of the CCR5 gene 32-basepair deletion. Nat Genet 1997;16:100–3.

[8] Zhou Y, Kurihara T, Ryseck R-P, Yang Y, Ryan C, Loy J, et al. Impaired macrophage function and enhanced T cell-dependent immune response in mice lacking CCR5, the mouse homologue of the major HIV-1 coreceptor. J Immunol 1998;160:4018–25.

[9] Wan S, LeClerc JL, Vincent JL. Inflammatory response to cardiopulmonary bypass: mechanisms involved and possible therapeutic strategies. Chest 1997;112:676–92.

[10] Wan S, LeClerc JL, Vincent JL. Cytokine responses to cardiopulmonary bypass: lessons learned from cardiac transplantation. Ann Thorac Surg 1997;63:269–76.

[11] Bouter H, Schippers EF, Luelmo SA, Versteegh MI, Ros P, Guiot HF, et al. No effect of preoperative selective gut decontamination on endotoxemia and cytokine activation during cardiopulmonary bypass: a randomized, placebo-controlled study. Crit Care Med 2002;30:38–43.

[12] Sablotzki A, Welters I, Lehmann N, Menges T, Gorlach G, Dehne M, et al. Plasma levels of immunoinhibitory cytokines interleukin-10 and transforming growth factor-beta in patients undergoing coronary artery bypass grafting. Eur J Cardiothorac Surg 1997;11:763–8.

[13] Marti F, Munoz J, Peiro M, Bertran E, Ferran C, Octavio C, et al. Higher cytotoxic activity and increased levels of IL-1 beta, IL-6, and TNF-alpha in patients undergoing cardiopulmonary bypass. Am J Hematol 1995;49:237–9.

[14] Meldrum DR. Tumor necrosis factor in the heart. Am J Physiol 1998;274:R577–R595.

[15] Heinrich PC, Castell JV, Andus T. Interleukin-6 and the acute phase response. Biochem J 1990;265:621–36.

[16] Wan S, LeClerc J-L, Vincent J-L. Cytokine response to cardiopulmonary bypass: lessons learned from cardiac transplantation. Ann Thorac Surg 1997;63:269–76.

[17] Paxton WA, Martin SR, Tse D, O'Brien TR, Skurnick J, VanDevanter NL, et al. Relative resistance to HIV-1 infection of CD4 lymphocytes from persons who remain uninfected despite multiple high-risk sexual exposure. Nat Med 1996;2:412–7.

[18] Yang JY, Togni M, Widmer U. Heterozygous defect in HIV-1 coreceptor CCR5 and chemokine production. Cytokine 1999;11: 1–7.

[19] Trkola A, Dragic T, Arthos J, Binley JM, Olson WC, Allaway GP, et al. CD4-dependent, antibody-sensitive interactions between HIV-1 and its co-receptor CCR-5. Nature 1996;384:184–7.

[20] Wu L, Paxton WA, Kassam N, Ruffing N, Rottman JB, Sullivan N, et al. CCR5 levels and expression pattern correlate with infectability by macrophage-tropic HIV-1, in vitro. J Exp Med 1997;185:1681–91.

[21] Wilson AG, Symons JA, McDowell TL, McDevitt HO, Duff GW. Effects of a polymorphism in the human tumor necrosis factor alpha promoter on transcriptional activation. Proc Natl Acad Sci USA 1997;94:3195–9.

[22] El-Omar EM, Carrington M, Chow W-H, McColl KE, Bream JH, Young HA, et al. Interleukin-1 polymorphisms associated with increased risk of gastric cancer. Nature 2000;404: 398–402.

[23] Fishman D, Faulds G, Jeffery R, Mohamed-Ali V, Yudkin JS, Humphries S, et al. The effect of novel polymorphisms in the interleukin-6 (IL-6) gene on IL-6 transcription and plasma IL-6 levels, and an association with systemic-onset juvenile chronic arthritis. J Clin Invest 1997;102:1369–76.

[24] Eskdale J, Gallagher G, Verweij CL, Keijsers V, Westendorp RG, Huizinga TW. Interleukin 10 secretion in relation to human IL-10 locus haplotypes. Proc Natl Acad Sci USA 1998;95:9465–70.

[25] De Groote D, Zangerle PF, Gevaert Y, Fassotte MF, Beguin Y, Noizat-Pirenne F, et al. Direct stimulation of cytokines (IL-1 beta, TNF-alpha, IL-6, IL-2, IFN-gamma and GM-CSF) in whole blood. I. Comparison with isolated PBMC stimulation. Cytokine 1992;4:239–48.

[26] van Dissel JT, van Langevelde P, Westendorp RG, Kwappenberg K, Frolich M. Anti-inflammatory cytokine profile and mortality in febrile patients. Lancet 1998;351:950–3.