Extended report:

**Aberrant TNF secretion by whole blood in healthy subjects with a history of reactive arthritis**

Time course in adherent and non-adherent cultures

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Abstract

Objectives Pathogenesis of reactive arthritis (ReA) apparently involves aberrations in innate immune functions such as monocyte tumour necrosis factor (TNF) generation. Our aim was to investigate TNF production of healthy subjects with previous yersinia-triggered ReA.

Methods Study comprised HLA-B27-positive subjects with previous ReA (B27+ReA+), and B27+ReA- and B27-ReA- subjects (n=15 each). Whole blood TNF production was induced by LPS, which binds to CD14/TLR4 on monocyte surface, or by a combination of phorbol 12-myristate 13-acetate (PMA) and Ca$^{2+}$ ionophore A23187, which activates monocytes independently of cell-surface receptors. To further evaluate the possible role of adhesion-mediated signaling on TNF production, blood samples were incubated in adherent or non-adherent conditions. TNF levels in culture supernatants were measured using an automated immunoassay analyser. The CD14(-159)C/T genotype was determined by a cycle minisequencing method.

Results B27+ReA+ supernatants showed higher TNF levels than did B27+ReA- supernatants in PMA/A23187 wells in 2-hour (P=0.004) and 4-hour (P=0.001) cultures. Rapid initial TNF release took place in adherent but not in non-adherent conditions. This adhesion-associated difference was higher in B27+ReA+ than in B27+ReA- or B27-ReA- group in response to PMA/A23187 (P-values<0.001), and higher in B27+ReA+ than in B27-ReA- group in response to LPS (P=0.021). CD14(-159)T was associated with elevated LPS-induced TNF secretion allele dose-dependently (P=0.030).

Conclusions Subjects recovered from yersinia arthritis show enhanced TNF production, which may be regulated at the level of monocyte adhesion.

Keywords

ADHESION; INNATE IMMUNITY; REACTIVE ARTHRITIS; TNF
Introduction

Reactive arthritis (ReA) is a sterile joint inflammation, complicating an extra-articular infection, most commonly gram-negative Chlamydia urethritis, or Yersinia, Salmonella, Campylobacter or Shigella enteritis, especially among HLA-B27 positive individuals [1]. The pathogenesis of ReA is unclear but may involve aberration(s) in adaptive immune response, for instance, presentation of arthritogenic self-peptides to CD8+ T cells by HLA-B27, or formation of HLA-B27 dimers recognized by CD4+ T cells [2]. However, evidence has accumulated to show that also innate immune reactions, which represent early stages of the host defense against the arthritogenic microbes [3], may play a role in the development of ReA. Innate immune system receptors, like CD14/Toll-like receptor -complexes on monocytes and macrophages, recognize lipopolysaccharides (LPS) and other pathogen-associated molecular patterns. Binding of LPS activates intracellular signaling, which leads to activation of transcription factors and generation of immunoregulatory cytokines, such as tumour necrosis factor (TNF). Low TNF secretion by blood mononuclears may be related to chronic ReA [4], and, in general, may interfere with eradication of bacterial infection at its early stage [5-7].

Several factors may affect monocyte TNF production. Firstly, circulating monocytes are heterogeneous in terms of function and maturity [8]. In healthy subjects, most monocytes are strongly positive for CD14 and negative for the Fcγ-III receptor CD16. However, in patients with generalized inflammation, up to 50% of monocytes express weakly CD14 and co-express CD16. These CD14+CD16+ monocytes exhibit enhanced TNF production capacity [9] and are major TNF producers in the blood [10]. Secondly, signaling via CD11b/CD18 complex, a β2-integrin, which mediates monocyte adhesion to endothelium and non-endothelial surfaces [11; 12], may modify TNF production and monocyte survival [13; 14]. Finally, the CD14(-159)C→T polymorphism has been reported to increase monocyte CD14 expression [15; 16].

Because leukocyte migration from the circulation into the joint requires leukocyte adherence to vascular endothelium and extracellular matrix proteins, we reasoned that leukocyte activation might be regulated at the level of CD11b/CD18 complex signaling. Furthermore, in patients with systemic inflammation, phagocytes are activated in the blood but do not necessarily adhere to the endothelium [17]. To mimic these adherent and non-adherent in vivo conditions we used concurrently adherent cell culture wells and non-adherent tubes, respectively. Using this setting we studied, firstly, whether the induction of TNF release is abnormal in healthy subjects with a history of Yersinia-triggered ReA, and, secondly, the effect of cell adherence on TNF production. Monocytes in whole blood samples were stimulated by a combination of Ca2+ ionophore A23187 and phorbol 12-myristate 13-acetate (PMA), cell-surface receptor-independent activators of the protein kinase C (PKC) pathway, or by LPS.

Materials and methods

Subjects
Forty-five native Finnish volunteers were recruited. Fifteen HLA-B27 positive subjects had had *Yersinia*-triggered reactive arthritis with complete recovery (B27+ReA+ group, Table 1). They had participated in clinical studies of acute ReA [18] and disease outcome [19]. *Yersinia* antibodies were examined after recovery during follow-up mean 8.5 years after the acute disease, and in all patients they had decreased to normal levels. Fifteen of the healthy reference subjects (10 women, 5 men, median age 47 years, range 32-64) were HLA-B27 positive (B27+ReA- group), and 15 (10 women, 5 men, median age 53, range 43-58) HLA-B27 negative (B27-ReA- group). Individuals with anti-inflammatory medication or signs of infection at the time of donation were excluded from the study. Lack of subclinical systemic inflammation was verified using a high sensitivity CRP assay: median serum CRP level (range) was 0.75 µg/mL (0.30-5.80) for B27+ReA+, 0.80 µg/mL (0.20-8.40) for B27+ReA-, and 0.50 µg/mL (0.20-1.80) for B27-ReA- group. The subjects gave their informed consent.

Table 1 Subjects with previous *Yersinia*-triggered reactive arthritis.
Characteristics at the time of acute disease and follow-up visit.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of women/men</td>
<td>10/5</td>
</tr>
<tr>
<td>Age at study time, years, median (range)</td>
<td>57 (39-71)</td>
</tr>
<tr>
<td>Acute arthritis</td>
<td></td>
</tr>
<tr>
<td>Age at onset, years, median (range)</td>
<td>31 (15-45)</td>
</tr>
<tr>
<td>Number of patients positive for the HLA-B27 antigen</td>
<td>15</td>
</tr>
<tr>
<td>Number of inflamed joints, median (range)</td>
<td>6 (2-15)</td>
</tr>
<tr>
<td>Number of patients with extra-articular symptoms</td>
<td>11</td>
</tr>
<tr>
<td>Urethritis</td>
<td>9</td>
</tr>
<tr>
<td>Iritis</td>
<td>4</td>
</tr>
<tr>
<td>Conjunctivitis</td>
<td>1</td>
</tr>
<tr>
<td>Erythema nodosum</td>
<td>1</td>
</tr>
<tr>
<td>Duration, months, median (range)</td>
<td>3 (1-8)</td>
</tr>
<tr>
<td>White blood cell count <em>10^9</em>/L, median (range)</td>
<td>10.8 (5.6-15.2)</td>
</tr>
<tr>
<td>Erythrocyte sedimentation rate, mm/h, median (range)</td>
<td>91 (16-140)</td>
</tr>
<tr>
<td>Lowest Hb, g/L, median (range)</td>
<td>116 (99-139)</td>
</tr>
<tr>
<td>Women</td>
<td>118 (103-140)</td>
</tr>
<tr>
<td>Reciprocal agglutination titre for <em>Yersinia enterocolitica</em> O:3 or O:9, median (range)</td>
<td>2560 (640-20480)</td>
</tr>
<tr>
<td>Follow-up visit</td>
<td></td>
</tr>
<tr>
<td>Period since diagnosis of acute arthritis, years, median (range)</td>
<td>18 (15-25)</td>
</tr>
<tr>
<td>Number of fully recovered patients</td>
<td>15</td>
</tr>
</tbody>
</table>

1 Normal range 4-10
2 Normal range 1-10
$^3$ Normal range 125-160 in women, 135-180 in men

$^4$ Normal <160
Reagents

Pyrogen-free heparin (Lövens, Ballerup, Denmark); Dulbecco’s phosphate buffered saline (PBS) and RPMI 1640 medium (both from Life Technologies Ltd., Paisley, UK); *Escherichia coli* O111:B4 lipopolysaccharide (LPS); phorbol 12-myristate 13-acetate (PMA) and Ca\(^{2+}\) ionophore A23187 (all from Sigma, St. Louis, MO, USA); TNF Sample Diluent (Diagnostic Products, Los Angeles, CA, USA); sterile AB serum (Finnish Red Cross, Helsinki, Finland); fluoresceine isothiocyanate (FITC) conjugate of mouse anti-CD11b mAb (IgG1, clone BEAR 1), phycoerythrin-CY5 (PC5) conjugate of anti-CD16 mAb (IgG1, clone 3G8) and PC5 conjugate of irrelevant mouse mAb IgG1 (clone 679.1Mc7) (all from Immunotech, Marseille, France); R-phycoerythrin (RPE) conjugate of anti-CD14 mAb (IgG2a, clone TÜK4) and RPE conjugate of irrelevant mAb (IgG2a, clone DAK-G05) (both from DAKO A/S, Glostrup, Denmark); FACS lysing solution and QuantiBRITE PE standards (Becton Dickinson, San Jose, CA, USA); dNTPs (Pharmacia, Uppsala, Sweden), AmpliTaq Gold DNA polymerase and PCR buffer (Perkin Elmer, Boston, MA, USA); shrimp alkaline phosphatase and exonuclease I (USB, Cleveland, OH, USA), DyNAzyme DNA polymerase II and DyNAzyme buffer (Finnzymes, Espoo, Finland), \(^{3}H\)-labeled dCTP (specific activity 53 Ci/mmol) and dTTP (96 Ci/mmol) (Amersham Life Science, Little Chalfont, Buckinghamshire, UK); and primers (Sigma-Genosys, Pampisford, Cambridgeshire, UK).

Blood samples

Three blood samples from each subject were obtained by phlebotomy. The first was collected into a Falcon polypropylene tube (Becton Dickinson, Lincoln Park, NJ, USA) containing heparin 10 IU/mL blood, and placed on an ice-water bath. Twelve 100-µL aliquots were used immediately for the whole blood culture assay, and two 25-µL aliquots for flow cytometry. The second sample was collected into an EDTA(K3) tube (Vacutainer No. 367657, Becton Dickinson), aliquoted into Cryo tubes (Greiner GmbH, Solingen, Germany), and stored at -70°C for nucleic acid analysis. The third was collected into a EDTA(K3) tube for differential cell count.

Methods

Experimental design

In preliminary experiments, TNF dose responses to LPS and combinations of PMA and A23187 were tested on whole blood samples diluted 1:10 in RPMI 1640. LPS at 1 µg/mL, and PMA plus A23187 both at 5 µM were found as convenient and comparable stimuli and chosen for the actual experiments.

Preliminary studies revealed that TNF levels reached the plateau by 4 hours of incubation. This agrees with previous studies indicating that in whole blood assay, TNF mRNA is induced in response to LPS in 1 to 2 hours [20] and the initial TNF levels in the culture supernatants peak at 2 to 6 hours [21-24]. Later waves of cytokine production may occur, dependent on the cytokines released within the first 1 to 4 hours of incubation [25]. We concluded to have two time points, at two and four hours, to observe the time course of LPS- or PMA/A23187-dependent TNF production.
LPS was from Escherichia coli, and not from Yersinia enterocolitica, to exclude the possibility that the patients have low levels of yersinia-specific antibodies, which can respond with the polysaccharide moiety of Yersinia-LPS [26]. The proinflammatory properties of the lipid A moiety of LPS from Yersinia enterocolitica or Escherichia coli appear comparable [27].

**Whole blood culture assay**

LPS (400 µg/mL PBS), PMA and Ca²⁺ ionophore A23187 (both at 5 mM in 99.5-% ethanol) stock solutions were stored at -20°C. The 100-µL aliquots of heparinized blood were added into polypropylene tubes (No. 352063, Becton Dickinson) or cell culture wells (No. 3515, Corning Inc., Corning, NY, USA) pre-supplemented with: 1) 800 µL of RPMI 1640 and 100 µL of LPS (finally at 1 µg/mL), or 2) 900 µL of RPMI and 1 µL of PMA and 1 µL of A23187 (both finally at 5 µM), or 3) 900 µL of RPMI 1640 alone. Two identical sets of cultures, one for 2-hour and the other for 4-hour incubation, were made in both test tubes and cell culture wells, and incubated at +37°C in 5% CO₂. The culture supernatants were snap-frozen and stored at -70°C. TNF levels were determined within two months. The cell pellet was suspended in 50 µL of ice-cold AB serum and used for flow cytometry.

**Cell-surface staining of leukocytes**

The aliquots of heparinized blood and cell pellet in AB serum were stained with pretitrated amounts of FITC-anti-CD11b, RPE-anti-CD14, and PC5-anti-CD16, as described previously [28]. Flow cytometric data acquisition was performed within 4 hours.

**Flow cytometry**

A FACSort flow cytometer (Becton Dickinson) and CellQuest software were used. QuantiBRITE PE bead standards (Becton Dickinson) were run weekly. The data were acquired and analysed using QuantiCalc software (Verity Software House, Topsham, ME, USA) as described previously [28]. According to the manufacturer, the ratio of fluorochrome to protein is 1. Consequently, the number of PE molecules bound is equal to anti-CD14 antibody binding capacity (ABC) of the cell.

**DNA isolation and CD14 genotyping**

The whole blood samples were thawed and DNA was isolated using QIAamp DNA Blood Midi Kit (QIAGEN, Hilden, Germany).

The CD14(-159)C/T polymorphism was genotyped using a cycle minisequencing technique as described previously [29]. Primers flanking the CD14-159 polymorphism were 5’-CCTGGAAATATTGCAATGAAGGATG-3’ and 5’-CCAGGAGACACAGAACCCTAGATGC-3’.

The PCR products were diluted 1:2 in DyNAzyme buffer and transferred onto new 96-well PCR plates (Advanced Biotechnologies) containing reagents for the cycle minisequencing reaction. The primer construct was 5’-Biotin-GCAGAATCCTTCCCTGTTACGG-3’, and the nucleotides were ³H-labeled dCTP and dTTP.

After cycling, the contents of the wells were transferred into plate wells with covalently attached streptavidin (SA cov. Scintiplate 1450-511, Wallac, Turku, Finland). After binding, CPMs of the bound primers were calculated using a beta counter (Microbeta 1450, Wallac, Turku, Finland). The ratio of the two signals was used for genotyping.

**Determination of TNF**
The culture supernatants were thawed and diluted 1:5 in TNF Sample Diluent. TNF levels were measured using Immulite, a chemiluminescent immunoassay system (Diagnostic Products). The detection limit was 4 pg/mL. The intra-assay variation was 9% and the interassay variation 12%. The results were corrected for dilution and background. To evaluate the time course of TNF production, the kinetic ratio = TNF level at 4 hours / TNF level at 2 hours was calculated.

**Differential cell count**

Cell counts were collected by an automatic analyser.

**Data analysis**

To characterize the trends of the whole blood assay, the data obtained with B27-ReA-group were analysed. B27+ReA- group was excluded because of the possibility of clinically silent sacroiliitis related to B27 positivity [30]. Two dependent samples were compared by Wilcoxon signed ranks test. Secondly, differences between the three subject groups were evaluated using Kruskal-Wallis test. Post hoc analyses were performed by Mann-Whitney U test. Effect of *CD14*(-159)T allele dose on TNF production was tested using Jonckheere-Terpstra test for linear trend, and T allele prevalence in the groups by Fisher’s exact test. The analyses were done using SPSS Version 10.07 (SPSS, Chicago, IL, USA). A P-value less than 0.05 was considered significant.

**Results**

**TNF production of 15 healthy HLA-B27 negative subjects**

**LPS**

Results depicted in Fig. 1A and Table 2 show that in cell culture wells supporting cell adhesion, LPS-induced TNF levels at 4 hours did not differ from respective values at 2 hours, resulting in median kinetic ratio of 1.0. In test tubes (Fig. 1B, Table 2), which do not support cell adhesion, TNF production was slower, the TNF levels being significantly higher at 4 hours than at 2 hours (P=0.001), and the median kinetic ratio 4.5.

In comparison of cell culture wells and test tubes, the former showed higher TNF levels at 2 hours (P=0.005) indicating again that adhesion enhanced early stage TNF release, whereas 4-hour levels were comparable indicating that adhesion did not affect maximal LPS-receptor-independent TNF production.

**Combination of PMA and Ca^{2+} ionophore A23187**

In cell cultures stimulated with PMA and A23187 (Fig. 1C and 1D, Table 2), both in adherent and non-adherent conditions, TNF levels at 4 hours were higher than at 2 hours resulting in median kinetic ratio of 1.7.

In comparison of cell culture wells and test tubes, the former had higher TNF levels at 2 hours (P=0.015) indicating again that adhesion enhanced early stage TNF release, whereas 4-hour levels were comparable indicating that adhesion did not affect maximal LPS-receptor-independent TNF production.
**TNF production of HLA-B27 positive subjects with previous yersinia arthritis**

*Time course of TNF production*

Median monocyte count of the blood samples was $0.4 \times 10^9/L$ in each subject group. In adherent cell cultures treated with PMA and A23187 (Table 2, Fig. 2), B27+ReA+ group showed higher TNF levels than did B27+ReA- group at both 2 hours ($P=0.004$) and 4 hours ($P=0.001$).

In non-adherent cultures containing PMA and A23187, median TNF levels did not differ between the groups, but the kinetic ratio was higher in B27+ReA+ group than in B27+ReA- group ($P=0.002$) or B27-ReA- group ($P=0.008$) (Table 2, Fig. 2).
Table 2: TNF levels in whole blood culture supernatants. Comparison of HLA-B27 positive subjects with previous reactive arthritis (B27+ReA+, n=15), and HLA-B27 positive (B27+ReA-, n=15) and negative (B27-ReA-, n=15) reference subjects.

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>TNF level, pg/mL, median (range)</th>
<th>Kruskal-Wallis test</th>
<th>Kinetic ratio, median (range)</th>
<th>Kruskal-Wallis test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B27+ReA+</td>
<td>B27+ReA-</td>
<td>B27-ReA-</td>
<td></td>
</tr>
<tr>
<td>Adherent culture wells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LPS 2</td>
<td>426.5 (165.5-822.0)</td>
<td>257.0 (126.0-685.0)</td>
<td>297.0 (138.5-1440.0)</td>
<td>P=0.145</td>
</tr>
<tr>
<td>2 hours</td>
<td>334.0 (164.0-1185.0)</td>
<td>335.5 (151.0-880.0)</td>
<td>278.5 (175.0-1050.5)</td>
<td>P=0.395</td>
</tr>
<tr>
<td>4 hours</td>
<td>1122.5 (644.5-3535.0)</td>
<td>660.0 (328.0-2290.0)</td>
<td>745.0 (366.5-1810.0)</td>
<td>P=0.017</td>
</tr>
<tr>
<td>2 hours</td>
<td>1780.0 (1070.0-20430.0)</td>
<td>1115.0 (729.5-1889.0)</td>
<td>1210.0 (664.0-3125.0)</td>
<td>P=0.005</td>
</tr>
<tr>
<td>PMA 3 and A23187 4</td>
<td>196.0 (69.0-540.0)</td>
<td>183.5 (59.0-490.0)</td>
<td>170.0 (73.0-895.0)</td>
<td>P=0.749</td>
</tr>
<tr>
<td>2 hours</td>
<td>1134.5 (575.0-2503.5)</td>
<td>855.0 (457.5-2039.5)</td>
<td>765.0 (91.0-3510.0)</td>
<td>P=0.316</td>
</tr>
<tr>
<td>4 hours</td>
<td>480.0 (24.0-1365.0)</td>
<td>500.0 (100.5-2235.0)</td>
<td>615.0 (222.0-1150.0)</td>
<td>P=0.491</td>
</tr>
<tr>
<td>Non-adherent test tubes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LPS 2</td>
<td>1.0 (0.4-1.9)</td>
<td>1.0 (0.6-2.5)</td>
<td>1.0 (0.6-1.5)</td>
<td>P=0.912</td>
</tr>
<tr>
<td>2 hours</td>
<td>1.7 (1.0-5.8)</td>
<td>1.7 (0.8-2.7)</td>
<td>1.7 (1.4-2.1)</td>
<td>P=0.796</td>
</tr>
<tr>
<td>4 hours</td>
<td>5.3 (2.9-14.4)</td>
<td>4.5 (3.1-12.9)</td>
<td>4.5 (0.5-9.7)</td>
<td>P=0.567</td>
</tr>
<tr>
<td>PMA 3 and A23187 4</td>
<td>3.0 (0.9-46.4)</td>
<td>1.5 (0.2-3.0)</td>
<td>1.7 (0-10.7)</td>
<td>P=0.003</td>
</tr>
</tbody>
</table>

Post hoc (Mann-Whitney U test):

- Bacterial lipopolysaccharide 1 µg/mL: P=0.004; B27+ReA+ > B27+ReA-
- Phorbol 12-myristate 13-acetate 5 µM: P=0.001; B27+ReA+ > B27+ReA-
- Ca²⁺ ionophore A23187 5 µM: P=0.002; B27+ReA+ > B27+ReA-
- P=0.008; B27+ReA > B27-ReA-
**Effect of adhesion on TNF production**

To explore whether the effect of adhesion on TNF release differed between B27+ReA+ and reference groups, the difference between TNF levels in cell culture well and respective test tube was calculated. Median difference between the groups was significant in 2-hour PMA/A23187 cultures (P<0.001, Kruskal-Wallis test), and *post hoc* analysis revealed that the difference was significantly higher in B27+ReA+ group than in B27+ReA- group or B27-ReA- group (P-values<0.001, Mann-Whitney U test).

In LPS-stimulated conditions, the respective median difference in TNF levels after 2 hours differed between the groups (P=0.048). In *post hoc* analysis these differences were higher in B27+ReA+ group than in B27-ReA- group (P=0.021).

**TNF production in relation to CD14(-159) genotype**

TNF levels calculated per 10^5 CD14-positive monocytes in whole blood culture increased in a T allele dose-dependent manner in response to LPS in test tubes at 4 hours (Fig. 3); median (range) was 2317.0 pg (1500.0-3271.0) in CC, 2702.0 pg (1538.0-3734.0) in CT, and 3114.5 pg (1866.0-4107.0) in TT group (P=0.030, Jonckheere-Terpstra test).

TNF levels and kinetic ratios obtained with PMA plus A23187 were not associated with $CD14(-159)C/T$.

T allele prevalence was 36.7 % in B27+ReA+, 40.0 % in B27+ReA- and 56.7 % in B27-ReA- group, and did not differ significantly between the groups.

**Monocyte CD14 expression**

There were no significant differences in median CD14 fluorescence intensity of monocytes in whole blood samples between B27+ReA+ (median ABC 14434, range 7629-17342), B27+ReA- (14295, 10194-19286) and B27-ReA- (13232, 6926-20046) groups. Median proportion of CD14+CD16+ monocytes in whole blood was comparable in B27+ReA+ (4.2 %, 1.0-13.4), B27+ReA- (4.3 %, 0.6-6.8) and B27-ReA- (2.5 %, 0.5-8.8) groups.

**Discussion**

In adherent whole blood cultures, samples of HLA-B27 positive healthy subjects with previous *Yersinia* arthritis released more TNF in response to direct activation of the PKC pathway, stimulated by the combination of PMA and Ca^{2+} ionophore A23187, than did samples of HLA-B27 positive reference subjects. In agreement with a previous study [31], the $CD14(-159)T$ allele increased LPS-induced TNF production in an allele dose-dependent manner. However, the frequency of $CD14(-159)T$ and the proportion of CD14+CD16+ monocytes in whole blood samples were comparable in the subject groups. Whereas these results do not explain the difference in TNF levels in PMA/A23187 wells between arthritis and reference subjects, adhesion may provide an explanation. When adherent and non-adherent PMA/A32187 culture conditions were compared, the former increased 2-hour TNF
production more in arthritis group than in the reference groups. Additionally, kinetic ratio (TNF level at 4 hours / TNF level at 2 hours) of non-adherent PMA/A23187 cultures was higher in arthritis group in comparison with the reference groups. These results raise a question if abnormal signaling upon adhesion plays a role in the enhanced TNF induction in arthritis group. Of note, signaling via CD11b/CD18, the major molecule mediating monocyte adhesion to cell culture well surfaces [11; 12], enhances TNF production [13] and prolongs monocyte survival [14]. Thus, some aberration(s) in CD11b/CD18 signaling may explain the increased TNF production.

Transferring the HLA-B27 gene to epithelial cells [32] or monocytic cells [33] apparently increases cytokine generation in response to bacteria or bacterial LPS, suggesting a direct role of HLA-B27. In the present study, the enhanced TNF production capacity, revealed by PMA/A23187 stimulation as described above, was associated with presence of previous arthritis, not with HLA-B27. However, in LPS-stimulated cultures, adherent conditions increased TNF levels more in HLA-B27 positive arthritis group in comparison with HLA-B27 negative but not with HLA-B27 positive reference group. This suggests that HLA-B27 positivity and enhanced LPS receptor-mediated response may be independent but additive factors operating in the pathogenesis of reactive arthritis. It is of interest that low TNF secretion by mononuclears in response to phytohemagglutinin may correlate with chronicity of ReA [4]. So it is possible that vigorous TNF response of adhered phagocytes provokes clinical joint inflammation, but, on the other hand, helps to overcome the disease more quickly.

A novel feature of our assay was that TNF production was measured concurrently in adherent and non-adherent cultures. After LPS-induction, TNF production differed dramatically between these conditions. TNF levels remained stable in adherent cell culture wells after 2 hours indicating only a rapid initial burst of TNF. In non-adherent conditions, TNF levels were lower than in wells at 2 hours but release continued thereafter. These findings suggest that adhesion-mediated signals for TNF production are stimulatory at the early stage but not at the advanced stage of the response, or, in general, that the course of the response is more controlled in adhesive conditions. The more restrained TNF production of adhered phagocytes may be aimed at protecting endothelial cells from undue exposure to TNF in vivo. An intriguing possibility is that circulating monocytes of patients with systemic inflammation lack adhesion-mediated inhibitory signals, and, thus, generate inappropriately high amounts of pro-inflammatory cytokines, which activate vascular endothelium. This would promote leukocyte adhesion and inflammation-mediated end organ injury [34].

These preliminary results indicate that HLA-B27 positive healthy subjects with previous Yersinia arthritis show enhanced TNF production, which may be regulated at the level of monocyte adhesion. Whether this innate immune aberration derives from abnormal CD11b/CD18 signaling warrants further studies.

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Competing interest statement

The authors declare they have no competing interests.

Ethics approval

Study protocol was approved by the ethics committee of internal disorders.

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References


**Figure legends**

**Figure 1**

TNF in whole blood culture supernatants of HLA-B27 negative subjects without reactive arthritis (n=15). TNF levels after incubation in bacterial lipopolysaccharide 1 µg/mL A, in cell culture wells, B, in test tubes; TNF levels after incubation in the combination of phorbol 12-myristate 13-acetate 5 µM and Ca²⁺ ionophore A23187 5 µM C, in cell culture wells, D, in test tubes.

**Figure 2**

Scattergrams of TNF levels in whole blood culture supernatants. TNF levels after incubation in the combination of PMA and A23187 A, in cell culture wells for 2 hours, B, in cell culture wells for 4 hours, C, in test tubes expressed as kinetic ratios. Horizontal lines indicate the medians. Note the logarithmic scale in A and B, and the cutting and scaling of the vertical axis in C. For explanation of the subject groups and details of the assay conditions, see Table 2.

**Figure 3**

TNF production / 100 000 monocytes in whole blood in relation to CD14(-159) genotype. Whole blood samples were incubated in bacterial lipopolysaccharide 1 µg/mL in test tubes for 4 hours.
P = 0.004; B27+ReA+ > B27+ReA-
P = 0.001; B27+ReA+ > B27+ReA-
P = 0.002; B27+ReA+ > B27+ReA-
P = 0.008; B27+ReA+ > B27-ReA-
CD14(-159)

T NF, pg/100000 monocytes

- CC (n=24)
- CT (n=8)
- TT (n=13)

P=0.030; TT > CT > CC

TNF, pg/100000 monocytes

CD14(-159)

CC (n=24)
CT (n=8)
TT (n=13)
Aberrant TNF secretion by whole blood in healthy subjects with a history of reactive arthritis. Time course in adherent and non-adherent cultures
Krista Anttonen, Arto Orpana, Marjatta Leirisalo-Repo and Heikki Repo

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