IL-10 gene down-expression in circulating mononuclear cells during 36h infusion of drotrecogin-α activated: a pilot study

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ABSTRACT

Introduction: To investigate the gene expression of interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α) and interleukin-10 (IL-10) in circulating mononuclear cells harvested from septic shock patients on drotrecogin-α activated (DAA) in order to determine whether this treatment has any effect on the inflammation phase.

Methods: We have conducted a prospective cohort study in two intensive care departments. Blood samples were collected at inclusion (T1) and 36h later (T2) to measure plasma cytokines and the changes in intracellular TNF-α, IL-10 and IFN-γ mRNA expressions using RT-qPCR. Thirty two septic shock patients were included: 16 with DAA at 24 µg/kg/h/96h (DAA+) and 16 control (DAA−) eligible but contraindicated for DAA because of low platelet count.

Results: The basal characteristics were similar in both groups: mortality (50%), plasma cytokine concentrations, and baseline IFN-γ, TNF-α and IL-10 mRNA expressions (DAA+ vs. DAA−). At T2, there was a significant IFN-γ gene down-regulation in DAA+ but not in DAA− patients (-0.34 [-0.62;+1.54] vs. +1.41 [+0.35;+5.87], p=0.008). In survivors, DAA administration was associated with a down-expression of both IFN-γ (-0.65 [-0.93; -0.48] vs. +0.7 [-0.04;+1.26], p=0.01) and IL-10 (-0.78 [-0.92; -0.6] vs. -0.18 [-0.68;+0.46], p=0.038). In the non-survivors, DAA infusion was associated with IL-10 over-expression when compared with survivors (+0.54 [-0.35;+11.52] vs. -0.78 [-0.92; -0.6], p<0.001).

Conclusion: In this study, lack of IL-10 gene down-expression despite a 36-h infusion of DAA is an ominous sign in septic shock patients suggesting that DAA is not able to reverse outcome. Our results suggest how that DAA can significantly decrease the expression of anti-inflammatory cytokines expression in septic shock patients, and IL-10 or IFN-γ gene down-expression could represent be markers of DAA response.
Introduction

To improve outcome, continuous infusion of drotrecogin-α activated (DAA) is recommended over 96h at a rate of 24µg/kg/h in septic shock patients as early as possible [1]. This dosage has raised cost / effectiveness concerns. Yet, since DAA has been made commercially available, in vitro studies have highlighted many more properties for this molecule and it can no longer be ignored [2]. A convenient biomarker of its “best use” would be welcome to select patients who could truly benefit from this treatment. This test has never been done for several reasons: (1) single and serial measurements of plasma concentrations of inflammation biomarkers are inconsistent and do not reliably predict outcome in septic patients [3], and (2) since the effect of infused DAA probably changes according to the endogenous concentration of activated protein C at any given moment, it is currently difficult to know if patients are responding to this drug and whether DAA dosage should be adapted when treatment is started without monitoring its plasma concentration over 96h—a complicated and expensive procedure.

In addition, there are methodological pitfalls in septic shock trials relating to the diversity of sources of infection and the exact time it started. Also, the inflammatory response triggered by pathogen-associated molecular patterns through Toll-like receptors (TLR) activates a fast response by the nuclear factor kB (NF-kB) pathway. Because the cellular signaling of TLRs can be modified by polymorphism [4] and the subunits of NF-kB expression may be affected by DAA [5], participants in DAA clinical trials should have TLRs and NF-kB expressions as similar as possible so that groups can be appropriately compared. Only then will genes targeted by the NF-kB complex be expressed on a sound basis of
comparison. Although, conclusions would be more reliable, large clinical trials taking all these parameters into account would be much too expensive.

This study was conducted in real-life conditions of septic shock management to address the following question: does DAA have any tangible effect on the early pro-inflammatory response to septic shock, measured as a change in TNF-alpha (TNF-α), interferon-gamma (INF-γ), and interleukin-10 (IL-10) mRNA expressions in circulating mononuclear cells (CMNCs) harvested from patients and true controls? If so, this would provide an early indication of improvement, and perhaps a DAA efficiency biomarker.

**Patients, Materials and Methods**

This study was approved by our institutional review board for human research and informed written consent was obtained from each participant. Patients with inherited or acquired immunodeficiency were excluded.

Over 1 year, we included 16 consecutive patients following the criteria of septic shock according to the definition of the American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference [6] treated with DAA (DAA+ group) at the standard dosage (24µg/kg/h for 96h), resulting in a mean plasma DAA concentration of 45 ng/mL of DAA [2]), and 16 patients as a control group fulfilling septic shock criteria and eligible for DAA but contraindicated (platelet count <30,000/mm³ and/or uncontrolled bleeding).

We defined T1 as the moment within 12h after fulfilling septic shock criteria and before giving DAA; T2 was set 36h later. The Simplified Acute Physiology Score II [7], the Logistic Organ Dysfunction score [8] and 28-day mortality were recorded.
Plasma concentrations of IL1-beta, IL-2, IL-4, IL-5, IL-6, IL-8, TNF-α, IL-10, IFN-γ and IL-12 were measured (CBA Human inflammation kit, BD Biosciences, San José, CA, USA) and lympho-monocytes subpopulations determined by flow cytometry (Becton Dickinson, Rungis, France).

Aliquots of 20 ml of whole blood were drawn in EDTA and, CMNC were isolated by Ficoll gradient centrifugation (Eurobio, Les Ulis, France) to obtain aliquots of 10^6 cells. RNA extraction and reverse transcription and quantitative real-time PCR were performed with a gene reporter (HMBS) as reported [9]. The primers for NF-kB sub-units (p50, p65 and IκBa) were purchased from Genome Express, Montreuil, France. For cytokines transcriptional expression (TNF-α, IL-10 and IFN-γ) QuantiTect Primer Assays primers (Qiagen, Courtaboeuf, France) were used. Gene expression was assessed using the 2-DDCT method as reported [10].

The TLR-2 (G2408A) and TLR-4 (A12874G and C13174T) polymorphisms were detected using a hybridization probe assay according to reported method [4].

**Statistical analysis**

T1 and T2 gene expressions are expressed according to previous studies [6]. Values are expressed as means ± SD or as median and interquartile range [25-75]. For cytokines gene expression, results are expressed as fold change calculated as the ratio (T2-T1)/T1. Kinetic data were studied with analysis of variance for repeated measurements (Friedman’s two-way analysis). Comparisons of the mean were made by non-parametric tests (Mann-Whitney U-test or Kruskal-Wallis). Association with the genotype was sought with logistic regression. p < 0.05 was considered as significant.
Results

Clinical and basic biological characteristics (Table 1)
Clinical characteristics of the groups are shown in Table 1. No difference between DAA+ group and control group was found concerning SAPS II (respectively 62.7 ± 2.8 vs 61.5 ± 3.7), LOD score (respectively 9.3 ± 0.8 vs 8.4 ± 0.8) and ICU stay (respectively 32.9 ± 8.8 vs 54.2 ± 19.3 days). Source of infection, types of germ and number of septicaemia were statistically non significant between the two groups (Table 1). Mortality rate was 50% in both groups.

TLRs polymorphisms
To assess whether TLRs polymorphism had a role in DAA response, we searched for TLR-2 and TLR-4 polymorphisms in the DAA+ and DAA− groups. Results are indicated in Table 1. No significant difference of distribution was found between DAA group and control group regarding TLR-2 or TLR-4 polymorphisms. Concerning TLR-4 we observed a similar prevalence of the double mutation Asp299Gly Thr399Ile in both DAA and control groups (Table 1) as previously described [11].

Lymphocyte subsets
No statistical change was found between the DAA+ or DAA− groups regarding CD4+ or CD8+ T cells subsets. No change of T cell CD4/CD8 ratio was found over time or between groups (data not shown).

Pro and anti-inflammatory cytokine profiles
To establish Th1/Th2 profiles, several cytokines were measured in serum at T1 and T2: IL-1beta, IL-2, IL-4, IL-5, IL-6, IL-8, TNF-α, IL-10, IFN-γ and IL-12. Only IL-1beta, IL-6, IL-8, TNF-α, IL-10, IFN-γ showed values above detection threshold. No significant change of cytokine profile was found over time between both studied groups and regarding TLR-4 or TLR-2 polymorphisms (Table 2). Concerning TNF-α, IL-10, IFN-γ, we did not find any correlation with their intracellular mRNA expression and their serum levels of TNF-α, IL-10 and IFN-γ throughout the study.

**Evolution of intracellular mRNA expressions** (Figures 1 and 2)

From T1 to T2, the fold changes of mRNA expressions of p50, p65, and IkBa NF-kB subunits were not significantly different between groups or between survivors and non survivors (data not shown).

At T1, IFN-γ, TNF-α and IL-10 mRNA expressions were similar in both groups (DAA+ vs. DAA−). At T2, there was a significant IFN-γ gene down-regulation in DAA+ but not in DAA− patients: -0.34 [-0.62;+1.54] vs. +1.41 [+0.35;+5.87] respectively, p=0.008 (Figure 1).

When we analysed the survival/non-survival subgroups, we found that in the survivors, DAA administration was associated with a down-expression of both IFN-γ (-0.65 [-0.93;-0.48] vs. + 0.7 [-0.04;+1.26], p=0.01) and IL-10 (-0.78 [-0.92;-0.6] vs. -0.18 [-0.68;+0.46], p=0.038) (Figure 2A). In non survivors, there was no significant difference of expression for the three cytokines expression with or without DAA (Figure 2B). Nevertheless, in the non–survivors, DAA infusion was associated with IL-10 overexpression (+0.54 [-0.35;+11.52] vs. -0.78 [-0.92;-0.6], p=0.038) and IFN-γ over-expression (+1.07 [+0.22;+02.79] vs. −0.65[-0.14;-0.78], p<0.001) when compared with the survivors (Figure 2C).
Finally, there were no differences between groups for TNF-α gene expression regardless of the outcome.

Discussion

In our septic shock patients with clinical and biological characteristics as similar as possible to real-life conditions, a continuous infusion of DAA resulted in changes worthy of note in the IFN-γ gene expression, suggesting that the drug had an early anti-inflammatory effect on the IFN-γ gene expression, yet IL-10 was not significantly reduced. Moreover, when survival was also considered, IL-10 dampened the pro-inflammatory reaction of early septic shock in the survivors, whereas it failed to do so in the non-survivors at the same point of treatment. The lack of IL-10 gene expression decrease by CMNCs is associated with an ominous outcome even if full supportive treatment is provided. These data suggest that: (1) DAA infusion interferes in vivo with IFN-γ and IL-10 gene expressions through NF-kB-independent modulation, (2) continued IL-10 and IFN-γ gene expression despite a 36-h infusion of DAA may indicate that a standard dosage of DAA fails to affect outcome.

Increased plasma concentrations of cytokines such as IFN-γ, TNF-α or IL-10 is a hallmark of septic shock and has negative consequences for recovery, although no clinically relevant thresholds have been proposed [3,12]. However, data on plasma cytokine concentrations do not seem to determine outcome because the exact onset of sepsis-driven cytokines release is usually not clearly definable in septic patients [13]. Therefore, in this study we checked cytokine gene expression by CMNCs, and confirm that changes to them occur without consequences on plasma levels in accordance with previous studies [14]. The most significant drawbacks of this approach are the routine availability of the RT-qPCR technique in a hospital laboratory and the time required to obtain the lab test result, which
nevertheless takes a few hours (<6h) with commercially available techniques of molecular biology.

Our data are in partial accordance with in vitro studies that claim that DAA up-regulates IL-10 in LPS-stimulated human monocytes [15]. Yet in vitro the DAA concentration required is significantly higher (120ng/mL) than that achieved in vivo with standard infusion (45 to 52ng/mL) [2,16]. In our setting, IL-10 gene expression was dramatically increased in the non-surviving patients given DAA suggesting either a greater IL-10 gene up regulation by DAA in patients with poor prognosis, or simply that DAA has no effect on high IL-10 up regulation by sepsis itself. Since the baseline gene expression levels were not significantly different between groups whatever the outcome and treatment, we suggest that DAA has an influence that is not necessarily powerful.

One of the limitations of our study is that it was underpowered but our hypothesis can not be tested in a random fashion in a large population of patients since DAA administration availability can not be declined refused in the absence of contraindication for ethical reasons.

Furthermore, in septic shock DAA may actually require a higher infusion rate than the standard one to reach cellular efficiency (i.e., dampening IL-10 gene expression). Conversely, DAA may have no effect on patients with continuous IL-10 expression despite a 36h infusion. Hence, maintaining DAA for 96h as recommended would be questionable. In both situations, the level of IL-10 up regulation in CMNCs after 36h DAA infusion may become a biomarker of efficiency. The time required to obtain the IL-10 gene expression ratio is technically feasible during the working hours of the laboratory: consequently, in a patient infused 36h with a T2 test indicating a lack of IL-10 and IFN-γ gene down-regulation, the standard DAA dosage could be reduced after approximately the half scheduled dosage has been infused (which would result in a 50% saving of the drug). This warrants confirmation by a prospective randomized placebo study including only patients with IL-10 over expression.
Conclusion

If IL-10 and/or IFN-γ gene down expression fail to occur despite 36h of DAA infusion in septic shock patients, maintaining treatment for 96h as recommended becomes questionable. IL-10 and IFN-γ gene expressions by CMNCs could become a biomarker of DAA efficiency in this setting.

Key messages

• drotrecogin-α (activated) has early anti-inflammatory effects at the transcriptional level on circulating mononuclear cells

• Checking the transcriptome of cytokines/chemokines in immune cells could be a new approach for monitoring the effect of drotrecogin-α (activated) in sepsis

Author’s contributions

TL and PB contributed to conception, analysis and interpretation of data. AL contributed to acquisition of data. PB and FS wrote the manuscript. MPG revisited it for biological contents. FS and PO have given final approval.

Competing interests

The authors declare that they have no competing interests.

Acknowledgement

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References


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<th>DAA – (n=16)</th>
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<tr>
<td>Age (yr)</td>
<td>67.4 ± 4.0</td>
<td>67.9 ± 2.7</td>
<td>NS</td>
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DAA + refers to the group receiving drotrecogin alpha (activated), and DAA – refers to the group not receiving it.
### Table 1: Patient Characteristics

Data are means +/- SD. The respective p values result from the non-parametric Kruskal-Wallis test, while associations with the genotype were searched with logistic regression. SAPS II: Simplified Acute Physiology Score II on admission; LOD score: Logistic Organ Dysfunction score; TLRs: Toll like receptors; NS: non significant.

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<th>DAA+  n=16</th>
<th>DAA− n=16</th>
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<tr>
<td></td>
<td>T1 (pmol/mL)</td>
<td>T2 (pmol/mL)</td>
<td>T1 (pmol/mL)</td>
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<tr>
<td><strong>IL-1</strong></td>
<td>32.1[17.1-54.1]</td>
<td>30.1[16-31]</td>
<td>39.6[20.8-58.3]</td>
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<tr>
<td></td>
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<td>DAA−</td>
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<tr>
<td>IL-6(pmol/mL)</td>
<td>824.5[161.5-507.5]</td>
<td>140.7[38.8-265]</td>
<td>1885[1102.1-2500]</td>
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<tr>
<td>IL-8(pmol/mL)</td>
<td>623[102.5-2348.3]</td>
<td>110[31.6-159.8]</td>
<td>402.3[289.1-754.1]</td>
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<tr>
<td>IL-10(pmol/mL)</td>
<td>47[26.2-54]</td>
<td>12.4[8.6-23.3]</td>
<td>90[44.8-1048.8]</td>
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**Table 2** Serum cytokines level of DAA+ and control groups. Data are expressed as median [IQR 25-75]. DAA+: septic shock patients treated with DAA. DAA−: septic shock patients contraindicated to rhAPC treatment. T1: time-point before DAA infusion; T2: time-point 36h after infusion of DAA. The p value: analysis of variance for repeated data (T1 and T2). NS: non significant.

**Fig. 1** Evolution of IFN-γ, TNF-α and IL-10 mRNA expressions in CMNCs from DAA + (n=16) and DAA− patients (n=16) between inclusion (T1) and after a 36h DAA infusion (T2). Box and whiskers plots show median and interquartile range [25-75]. Fold change is calculated as the ratio (T2-T1)/T1. DAA: drotrecogin alpha activated. IFN-γ expression is significantly reduced in the DAA+ group (*: p=0.008, Mann-Whitney test) than in the control group.

**Fig. 2** Evolution of IFN-γ, TNF-α and IL-10 mRNA expressions in CMNCs from DAA + (n=16) and DAA− patients (n=16) between inclusion (T1) and after a 36 h DAA infusion (T2). Box and whiskers plots show median and inter quartile range [25-75]. Fold change is calculated as the ratio (T2-T1)/T1. DAA: drotrecogin-α activated.

A. In survivors (n=8) there is a significant lower expression of IFN-γ in the DAA+ group (*: p=0.01, Mann-Whitney test) and also a significant decrease of IL-10 expression in survivors (DAA+ group) than in survivors of controls DAA− (**: p=0.038, Mann-Whitney test). B. In
non-survivor groups (n=8) there is no significant difference of cytokines expression between DAA+ and control groups. C. When we compare survivors versus non survivors, there is a significant over-expression for both IFN-γ and IL-10 in DAA+ non survivors compared to DAA+ survivors (§: p = 0.038, #: p < 0.001 respectively, Mann-Whitney test).