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Original Article

New possible biomarkers for diagnosis of infections and diagnostic distinction between bacterial and viral infections in children

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ABSTRACT

Detailed information about patients with infections is required to ensure appropriate choice of treatment. Although white blood cell (WBC) counts, and C-reactive protein (CRP) levels are useful diagnostic indicators of infections, more rapid and easily assayed indicator(s) could improve diagnosis. Moreover, it is of pivotal importance to distinguish bacteria or viruses as causative pathogens. Overall, TLR2 and TLR4 expression levels in neutrophils derived from individuals (n = 118) with bacterial (n = 37) and viral (n = 34) infections were higher than those in control samples (n = 47). Significant higher levels of TNF-α in patients with both types of the infection were observed, and those of IL-4, IL-8, IL-10, and IL-12 also were observed in the present study. Levels of IL-2, IL-8, and IL-10 on day 1 post-viral infection were significantly higher than those on day 1 post-bacterial infection. Therefore, there is a possibility that IL-4, IL-8, IL-10, IL-12 and TNF-α might be biomarkers for infections, in addition to WBC counts and CRP levels, and that IL-2, IL-8 or IL-10 are potentially able to distinguish between bacterial and viral infections.

1. Introduction

In infections, the host innate immune response is characterized by the initial recognition of invading microbes by host “sentinel” cells via Toll-like receptors (TLRs) or other pattern recognition molecules [1]. The unlimited ability of the innate immune system to recognize a wide range of pathogens is controlled by limited numbers of microbial determinants expressed as pathogen-associated molecular patterns (PAMPs) on infective organisms [2,3]. The innate immune system uses germline-encoded receptors, pattern-recognition receptors (PRRs), which are capable of recognizing PAMPs. TLRs are a major family of PRRs involved in innate immune responses to infectious agents [4,5], and are expressed in various cell types, including circulating immune cells [5]. Among TLRs, TLR2 and TLR4 are expressed on immune cells, including peripheral neutrophils [6]; they are known as bacterial sensors, but are also reported to be involved in the detection of viral infections [7,8]. In addition, the interleukins, together with TNF-α and other chemokines, help to regulate inflammation and the intensity of the immune response, and play a key role in activating the adaptive immune response [9].

Neutrophils are very effective initial phagocytes, whose main function on activation is thought to be the clearance of infecting bacteria. To achieve this, these cells are equipped with a myriad of antimicrobial molecules, grouped into oxidative and non-oxidative systems. The complement system and neutrophil granulocytes are also important for eliminating bacterial or fungal infections [10,11].

Cytokines are produced by the immune system in response to invading pathogens [12]. A network of cytokine signals is essential in modulation of the inflammatory response, clearance of pathogens, and subsequent repair of infected tissues. Cytokines can be classified into two broad groups, based on their predominant functions; IL-1β, IL-1α, IL-6, IL-8, IL-17, MIP-1, and TNF-α are pro-inflammatory cytokines, whereas IL-4, IL-10, and TGF-β are anti-inflammatory cytokines [13–15]. However, this classification is not absolute, as many cytokines are capable of exerting both pro- and anti-inflammatory effects, depending on a variety of factors, such as immunological and clinical contexts [16].

Infections are characterized by signs and symptoms overlapping with other acute critical conditions, such as organ-specific infection syndromes. Laboratory parameters, such as white blood cell (WBC)

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counts, and C-reactive protein (CRP), provide additional diagnostic information. The distinction between bacterial and viral infections is clinically important, but often very difficult. This difficulty often leads to unnecessary treatment with antibiotics, which is unfortunate in the light of the growing problems with antibiotic resistance [17,18]. Currently used methods that may aid in the distinction between bacterial and viral infections are primarily WBC counts and CRP levels [19,20], which have typical clinical performances of 70%–80% sensitivity and specificity, resulting in a high rate of misdiagnosis [21]. Recently, the new bacterial infection markers, presepsin, procalcitonin, CD64 and proADM, have been described [22–25]. Presepsin, procalcitonin, and CD64 are used for the diagnosis of severe sepsis and septic shock, and proADM is used for prediction of the prognosis of bacterial infections; hence, these indicators are not suitable for the diagnosis of mild bacterial and viral infections in outpatients who do not require admission. For those patients, the development of more accurate laboratory methods is warranted.

Although there is accumulating evidence concerning the relationships among PAMPs, TLRs and levels of various cytokines in infections, differences in the roles of TLRs and levels of cytokines depending on whether an infection is bacterial or viral are not clear. The aim of this study was to analyze these in patients. Our studies revealed, for the first time, differences in TLR2 and TLR4 expression levels in peripheral neutrophils and variation in the pattern of plasma cytokine production between bacterial and viral infections.

2. Patients and methods

2.1. Study population

This study was reviewed and approved by the Toho University Ethics Committee. (permissions No. 19032 and No. 24003). Patients (n = 118) were enrolled at first visit to the pediatric outpatient department at Toho University Omori Medical Center and received medical treatment without being admitted to a hospital. Written informed consent was obtained from their parents. Patients were classified into three groups; 37 with bacterial infections, 34 with viral infections, and 47 without infections as controls, after diagnosis by a pediatrician. Patients in control group were clinically diagnosed with non-infectious and non-inflammatory diseases (e.g., umbilicus and inguinal hernia). No patients in any group developed immunological disorder. Patients in the bacterial and viral infection groups were further classified into those with samples taken on day 1 (range, ≥day 1 − <day 2), day 3 (≥day 3 − <day 4), or day 5 (≥day 5 − <day 6) after initial fever symptoms. These patients were not continuously examined and were all different individuals (i.e., more than one sample was not taken from any patient). All patients in both infection groups were judged as having “mild infection”, because they had recovered at the time of subsequent visits. Patient characteristics and clinical laboratory data are presented in Table 1 and causative microorganisms data are presented in Table 2.

2.2. Isolation of neutrophils

Whole blood samples (2 mL) were obtained from all patients before medical treatment. Each sample was centrifuged for separation of cells and plasma, and the plasma was stored at −80 °C until analysis of cytokines. Red blood cells in the cellular fraction were lysed with VersaLyse reagent (Beckman Coulter, Inc., CA, USA) for 10 min. The remaining cells were washed with PBS and neutrophils isolated using a Human CD66abce MicroBead kit (Miltenyi Biotec Inc., CA, USA). The isolation procedure was carried out following a published method [26] and isolated neutrophils were used immediately for quantitative analysis of TLR2 and TLR4 expression. The purity of the neutrophils was >99%.

2.3. Quantitative analysis of TLR2 and TLR4 expression

Neutrophils were stained with monoclonal antibodies CD282/TLR2-PE or CD284/TLR4-PE for 30 min at 4 °C.

The fluorescence staining of neutrophils was measured by FACSCalibur flow cytometry (Becton Dickinson, NJ, USA). Data were analyzed using FCS express 4 (De Novo Software, CA, USA).

2.4. Cytokine assays

Plasma IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-17, and TNF-α levels were measured using a Q-Plex Cytokine assay kit (Quansys Biosciences, NJ, USA), following the manufacturer's instructions. When cytokine levels were lower than the detectable limit, values were recorded as half of the Lower Limit of Detection.

2.5. Statistical analyses

Direct comparisons between any two groups were performed using the Mann–Whitney *U* test. P values < 0.05 were considered statistically significant. All statistical analyses were performed using GraphPad Prism version 6.0 (GraphPad Software, CA, USA).

3. Results

3.1. Clinical laboratory data in patients with bacterial or viral infection

In patients with bacterial infections, total white blood cell and neutrophil counts, and CRP levels were significantly higher than those in controls (Table 1). For patients with viral infections, white blood cell and neutrophil counts were significantly higher than those of controls in the early period of infection (day 1 post-infection); however, after day 3 post-infection counts decreased and were not significantly different to those of controls. CRP values in patients with viral infections were significantly higher than those of controls at all time points.

3.2. TLR2 and TLR4 expression levels in neutrophils from infected patients

TLR2 and TLR4 expression levels were significantly higher in neutrophils from infected patients compared to those in neutrophils of controls until day 5 post-infected outpatients with bacterial infections (Fig. 1). In patients with viral infections, expression levels of both TLR2 and TLR4 gradually increased over the course of the infections.

There was a significant difference in the level of TLR2 between bacterial and viral infection groups on day 3 post-infection (Fig. 1).

3.3. Patterns of cytokine production in infected patients

The level of IL-1α was significantly higher in samples from patients with bacterial infections than in those from controls until day 5 post-infection (Fig. 2). IL-1α, IL-1β and IL-2 cytokine levels were significantly elevated compared to those of controls on day 1 post-viral infection and a significantly higher level of IL-1β was also observed on day 3 post-viral infection. IL-4, IL-8, IL-10, IL-12, IL-17, and TNF-α levels were significantly higher in samples from patients with both bacterial and viral infections than in those from controls until day 5 post-infection, except for that of IL-17 on day 5 post-viral infection. IL-6 levels were significantly higher than those of

Table 1
Patient characteristics and laboratory data.

DPI	Male/female	Age (months) (range)	WBC ($\times 10^3/\mu L$)	NEUT ($\times 10^3/\mu L$)	CRP (mg/dL) (range)
Bacterial infections					
Day 1	8/2	65.6 (2–179)	12.9 ± 6.9**	8.4 ± 5.0**	2.1** (0.2–8.8)
Day 3	4/10	58.7 (4–152)	9.6 ± 3.9	5.2 ± 3.0*	3.4** (0.1–5.8)
Day 5	6/7	54.2 (1–120)	11.7 ± 4.6**	6.6 ± 2.8**	3.5** (0.2–15.5)
Viral infections					
Day 1	6/6	60.8 (3–181)	9.7 ± 3.1**	6.8 ± 3.3**	0.6** (0–1.8)
Day 3	8/4	47.8 (1–145)	7.0 ± 3.1	3.2 ± 2.7	0.8** (0–3.1)
Day 5	7/3	52.9 (7–155)	7.8 ± 4.5	2.8 ± 2.2	0.9** (0.1–4.2)
Control (without infections)					
	29/18	46.9 (0–193)	7.4 ± 2.6	2.7 ± 1.1	0.03 (0–0.2)

Age and CRP data are expressed as means and ranges. WBC and NEUT data are expressed as means ± SD. *p < 0.05, vs control. **p < 0.01, vs control. DPI, WBC, NEUT, and CRP indicate days post infection, white blood cells, neutrophils, and C-reactive protein, respectively.

Table 2
Causative microorganism data.

Bacterial infections (n = 37)		Viral infections (n = 34)	
Day 1	**n**	**Day 1**	**n**
Haemophilus influenzae	3	*Enterovirus*	6
Campylobacter jejuni	3	*Respiratory syncytial virus*	5
Mycoplasma pneumoniae	2	*Influenza virus*	1
Escherichia coli	1	**Day 3**	
Streptococcus pyogenes	1	*Respiratory syncytial virus*	7
Day 3		*Enterovirus*	2
Haemophilus influenza	4	*Influenza virus*	1
Campylobacter jejuni	3	*Cytomegalovirus*	1
Streptococcus pneumoniae	3	*Adenovirus*	1
Mycoplasma pneumoniae	2	**Day 5**	
Escherichia coli	1	*Respiratory syncytial virus*	4
Staphylococcus aureus	1	*Epstein-Barr virus*	3
Day 5		*Influenza virus*	2
Haemophilus influenza	3	*Cytomegalovirus*	1
Streptococcus pneumoniae	3		
Mycoplasma pneumoniae	3		
Branhamella catarrhalis	2		
Escherichia coli	1		
Campylobacter jejuni	1		

controls until day 5 post-bacterial infection and that was significantly higher than those of controls only on day 1 post-viral infection. Levels of IL-5 were significantly higher only on day 1 post-viral infection, compared with those of controls.

There were significant differences in levels of IL-2, IL-8, and IL-10 on day 1 post-infection between bacterial and viral infected groups (Fig. 2).

The concentration of IL-8 on day 1 post-viral infection was significantly higher than those on days 3 and 5. The concentration of IL-17 on day 5 post-viral infection was significantly lower than those on days 1 and 3 (Fig. 2).

4. Discussion

In the present study, to clarify whether or not the period after initial fever symptoms (infections) influences the expression levels of TLRs and cytokines in plasma samples, we grouped patients roughly into days 1, 3, and 5 post-infection.

We analyzed the expression levels of TLR2 and TLR4 in neutrophils from children and levels of cytokines in plasma samples from outpatients. An interesting report concerning the innate immune response to infection and the role of cytokines in relation to disease severity indicated that sera levels of cytokines were considerably higher in severe than in mild infections or healthy controls [9]. In general, severe infections develop from mild infections (which comprise the majority of outpatient cases). However, there are few reports analyzing data from outpatients to investigate the expression patterns of TLRs and cytokines relative to time post-infection. In this study, for the first time, we analyzed these factors in outpatients with mild infections.

In patients with viral infections, plasma levels of the cytokines analyzed in this study, other than TNF-α, were considerably higher in the early stage of infection. Cytokine concentrations were generally highest on admission and those of IL-1, IL-6, IL-8, and IL-10, which act as acute-phase proteins, decreased rapidly after admission, in contrast with the more gradual kinetics of CRP [27–29]. These data suggest that cytokines, other than TNF-α and CRP, are unlikely to be useful biomarkers for viral infections. As TNF-α increases vascular permeability and killing of intracellular pathogens [30], up-regulation of TNF-α in plasma may be expected to persist during infections. In patients with bacterial infection, concentrations of IL-8, IL-17, and TNF-α were higher than those of controls until day 5 post-infection. One possible reason for these observations may be the role of these cytokines in increasing the migration and function of neutrophils. In addition, IL-6, which influences levels of CRP, and IL-12, which regulates production of INF-γ, also remained high until day 5

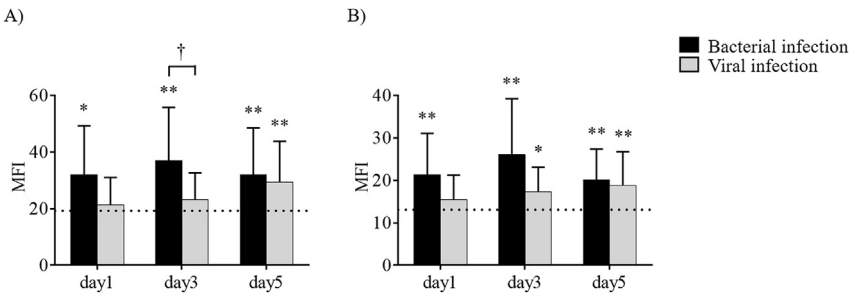

Fig. 1. Expression levels of TLR2 and TLR4 in neutrophils derived from patients with bacterial and viral infections. A) Expression levels of TLR2. B) Expression levels of TLR4. Dotted line indicates the mean values of control samples from patients without infections. Data are expressed as means ± SD. * and ** indicate $p < 0.05$ and $p < 0.01$ versus control, respectively. † indicates $p < 0.05$ versus different type infection.

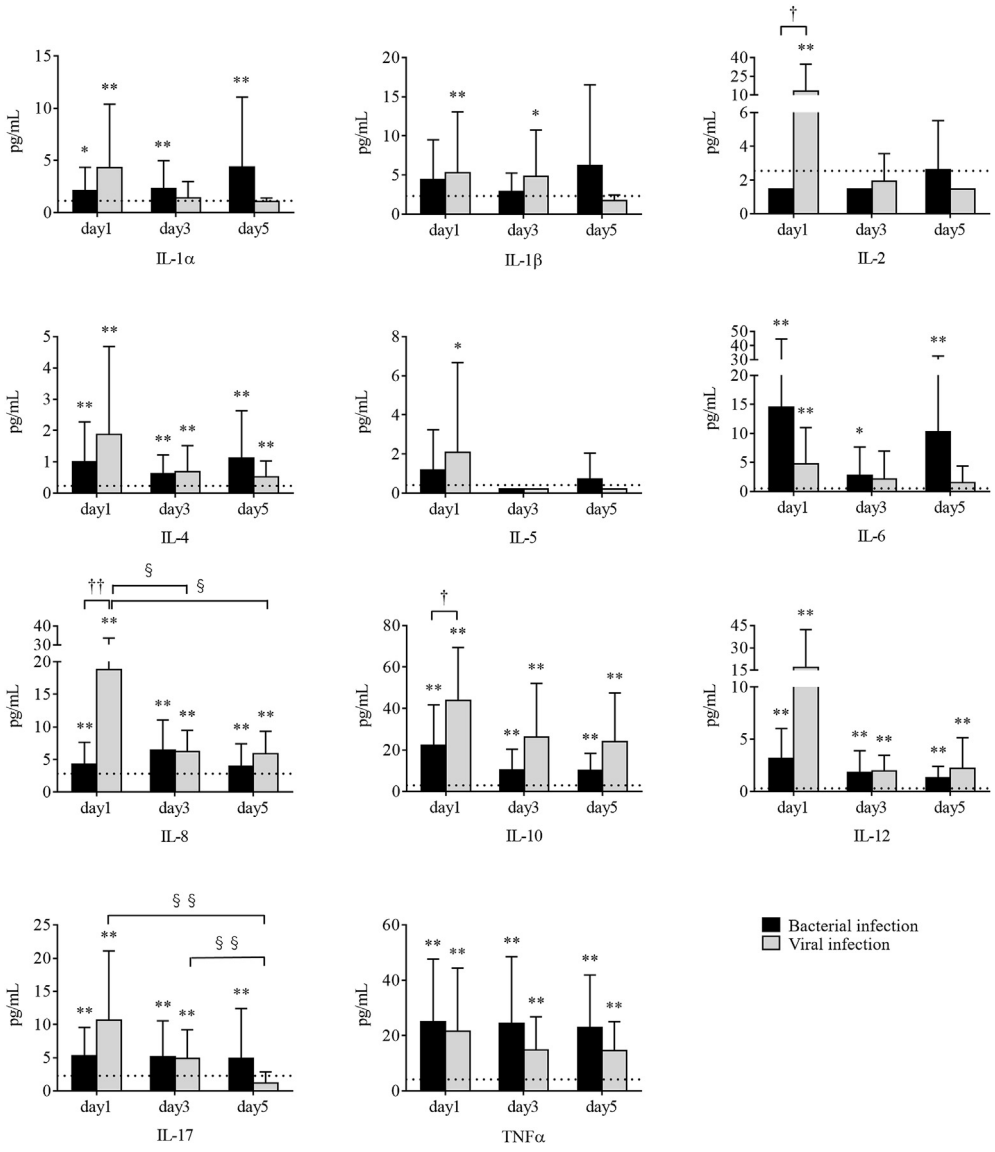

Fig. 2. Cytokine levels in patients with bacterial and viral infections. Plasma IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-17, and TNF-α levels were measured using a commercial ELISA kit. The dotted line indicates the mean values from control patients without infections. Data are expressed as means ± SD. * and ** indicate $p < 0.05$ and $p < 0.01$ versus control, respectively. † and †† indicate $p < 0.05$ and $p < 0.01$ versus different type infection, respectively. § and §§ indicate $p < 0.05$ and $p < 0.01$ versus different day, respectively.

post-infection. Yu X. et al. [9] reported that IL-6 is an important pro-inflammatory cytokine, elevated in the sera of patients with inflammatory diseases; therefore, although our samples were from a different source (plasma, rather than sera), our results appear to be consistent with their findings.

The results demonstrate that IL-4, IL-8, IL-10, IL-12, and TNF-α levels elevated in the plasma of patients with viral infections, and that IL-17 levels elevated only for the early stage of viral infections. In addition, on the basis of the present data, assays of plasma levels of IL-2, IL-8 or IL-10 may be able to distinguish between bacterial and viral infections, which may assist in ensuring appropriate choice of treatment.

Neutrophils are markedly increased in the circulation and tissues during bacterial or fungal infections [31,32]. In contrast, although they are the first and predominant immune cell population recruited to an affected site after viral infection, their contribution to antiviral defense is much less appreciated [33]. The beneficial role of neutrophils has been confirmed in viral infection models [34,35]. The present study confirmed that neutrophil counts in samples from outpatients with bacterial infections were higher than those in controls. Furthermore, in viral infections neutrophil counts were also higher than those of controls in the early stage of infection. These results indicate that neutrophils may play a pivotal role in, not only bacterial, but also viral infections, at the early stage of infection.

Among the various PRRs, TLR2 and TLR4 have attracted the most attention, because they mediate recognition of bacterial components and drive the antibacterial functions of neutrophils [36]. In addition, influenza A virus has been shown to upregulate TLR2 expression in neutrophils, thus increasing the production of H_2O_2 in response to TLR2 ligands such as peptidoglycans [37]. The present study showed that the expression level of TLR2 in patient samples might increase in the presence of sufficient numbers of pathogens. These data suggest that TLR2 expression may be a marker for bacterial or viral infections. Since the expression patterns of TLR4 in patients were similar to those of TLR2, TLR4 may also be a biomarker for infections.

The polarization and plasticity of innate immune cell populations has been reported [38]. By comparing various aspects of the innate immune response, it appears that increased neutrophil polarization towards a pro-inflammatory phenotype (increased IL-12 and decreased IL-10) is responsible for improved clearance of pathogens in the periphery [39], indicating that neutrophil polarization influences bacterial clearance after infections. The present study showed that IL-10 levels remained relatively elevated until day 5 after both bacterial and viral infections in patients. In contrast, levels of IL-12 were high in patients with early stage viral infections and less so in patients with bacterial infections. Taken together, these data show that differences in the degree of infection and the timing of the assay may influence cytokine levels.

Finally, our observations provide a possibility that, not only TLR2 and TLR4 expressions on neutrophils, but also TNF-α, may be biomarkers for infections, in addition to the known indicators, WBC counts and CRP, and furthermore IL-2, IL-8, or IL-10 on the early stage may be a biomarker for differentiation between bacterial and viral infections. However, since the results have not been verified using analysis methods such as the area under the receiver operating characteristic (ROC) curve, present study was only proposed the possibility of new indicators. On the other hand, no additional blood samples are required to assay TLRs and cytokines in the plasma of patients, since the drawing of whole blood is routine in patients with suspected infections. In addition, levels can be measured using a commercial kit, which provides results simply and rapidly. In near future, larger scale study might be required to confirm present results because the number of samples was small in the present study.

Conflicts of interest statement

All authors: No potential conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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