Journal of
Medical Microbiology & Diagnosis

Campbell and Mark, J Med Microb Diagn 2014, 3:4
DOI: 10.4172/2161-0703.1000164

Research Article Open Access

Differential Systemic MyD88-Dependent vs. MyD88-Independent Cytokine Ratio by Etiologic Agent in Adults with Severe Community-Acquired Pneumonia

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Abstract

Purpose: Classifying bacteria early, before cultures results are available, would improve the choice of initial antibiotics in patients with severe pneumonia. TLR2 and TLR4 transduce signal through the MyD88-dependent pathway to stimulate IL-8 and TNF-α production. TLR4 can also signal through a MyD88-independent pathway to stimulate RANTES and IFN-β production. As gram-negative bacteria primarily activate TLR4, while gram-positive bacteria also activate TLR2, differential cytokine expression would be expected depending on specific bacterial etiologies.

Methods: Admission serum samples from 53 patients admitted to Medical Intensive Care Unit at the University of Maryland Medical Center between January 2006 and September 2013 were assayed for IL-8, RANTES, TNF-α, and IFN-β levels using the University of Maryland cytokine core laboratory and commercial ELISA kits. Cytokine levels and the ratio of MyD88-independent to MyD88-dependent cytokines, ([IFN-β] X [RANTES])/([IL8] X [TNF-α]) were compared to the culture identified organisms.

Results: 14 gram-negative and 17 gram-positive pneumonias were identified. None of the individual cytokine demonstrated statistically significant differences between the gram-negative and gram-positive infections. The ratio of MyD88-independent/MyD88-dependent cytokines was 111.1 ± 34.9 in gram-negative infections, 29.9 ± 8.3 in gram-positive infections, with p=0.04.

Conclusions: Serum MyD88-independent to MyD88-independent cytokine ratios significantly discriminated gram-negative from gram-positive pneumonia. As technology improves our ability to generate panels of cytokines quickly from clinical specimens, a strategy of pooled cytokine ratios based on underlying pathophysiology, as was done in our study, could guide clinicians in critical early antibiotic choices.

Keywords: Pneumonia; Cytokines; Tumor necrosis factor-alpha; Interferon-beta; Interleukin-8; Regulated on activation, Normal T cell expressed and secreted; Gram- negative; Gram-positive; Innate immunity

Introduction

The lung is physiologically in contact with the outside world during each breath; humans inhale up to one million bacteria per day in some environments, providing the source for the majority of pulmonary infections [1]. The respiratory tract employs a variety of defense mechanisms to maintain sterility of the lung parenchyma. These include upper airway filtering of inspired air, coughing, mucous and mucociliary clearance, barrier protection by airway epithelial surfaces, adaptive immunity, and the innate immune system.

The innate immune system includes cell surface receptors that function as pattern recognition receptors (PRRs). PRRs recognize pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs) to initiate immune responses [2]. Examples of PRRs include NOD-like receptors (NLRs), RIG-I-like receptors (RLRs), and t [3]. The TLRs are a group of 10 type I transmembrane receptors, initially described in *Drosophila melanogaster*, that function as PRR's [4]. They are expressed in airway epithelial cells [5] and antigen-presenting cells [6]. Individual TLRs differ in their ligand specificity; TLR4 is known to bind gram-negative bacterial lipopolysaccharide (LPS), various heat shock proteins, Lipid A, paclitaxol, and the respiratory syncytial virus F protein [7]. TLR2 binds gram-positive bacterial molecules such as lipoteichoic acid (LTA), lipoproteins, mycobacteria cell-wall lipoarabinomannan [7], and the atypical LPS found in *Liptosipra* and *Porphyromonas* species [8].

TLR2 and TLR4 signaling have been investigated in a variety of models [9,10]. All TLRs except TLR3 can transduce a receptor-ligand binding event by activation of intracellular myeloid differentiation factor 88 (MyD88) [11]. Downstream of MyD88, IL-1R-associated kinase (IRAK) and TNF-receptor-associated factor 6 (TRAF6) [12] activate TAK1 and MAP kinase kinase 6 (MKK6), which in turn activate NF-κB, JNK, and p38 [13]. NF-κB is a critical regulator of IL-8 [14,15], and MyD88 knockout mice do not produce TNF-α, IL-1-6. MyD88 knockouts do not manifest endotoxic shock in response to LPS, indicating that this pathway is essential for the biological response to this signal [16].

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Received July 14, 2014; **Accepted** September 23, 2014; **Published** September 25, 2014

Citation: Campbell D, Mark JC (2014) Differential Systemic MyD88-Dependent vs. MyD88-Independent Cytokine Ratio by Etiologic Agent in Adults with Severe Community-Acquired Pneumonia. J Med Microb Diagn 3: 164. doi:10.4172/2161-0703.1000164

TLR4 can also transduce signal via a MyD88-independent pathway. Ligand receptor interaction results in association with TRIF and TRAM, leading to activation of interferon- regulatory factor 3 (IRF3) [17]. IRF3 induces IFN-β [3,18] and RANTES [19,20] production. These mediators are not produced upon stimulation of the MyD88-dependant pathway and are not blocked by the MyD88 pathway inhibitor TGF-α [21]. In murine *Pseudomonas aeurginosa* pneumonia, MyD88 knockout mice do not up-regulate TNF-α [22]. In contrast, MyD88 knockout mice retain the ability to produce RANTES with TLR stimulation [23], and MyD88 knockout bone marrow derived cells retain the ability to make INF-β [24].

TLR2 has been shown to play a role in gram-positive bacterial pneumonia defense. Knapp et al. investigated the role of TLR2 in murine pneumococcal pneumonia. TLR2 expression in murine lung tissue was found to increase following inoculation with *S.pneumoniae* in wild type mice. Alveolar macrophages from wild type mice inoculated with *S.pneumoniae* produced significantly higher amounts of TNF-α and KC than TLR2 knockout mice, indicating that TLR2 is important in the macrophage response to pneumococcus [25]. *S. pneumoniae* induced TLR2 mRNA expression, IL-8 expression, and NF-κB activity in HRK-293 cells [15].

TLR4 deficient mice have reduced survival and increased lung bacterial burden as compared to wild type mice in experimental *Klebisella pneumonia* [26]. Non-typeable *Haemophilus influenzae* is known to have both the TLR4 ligand LPS, as well as several lipoproteins which are TLR2 ligands. In mouse models of pneumonia with this pathogen TLR4 but not TLR2 was shown to be important for early cytokine and chemokine production, suggestingthat TLR4 pathways are important in the innate immune defense of this particular gram-negative pathogen [27]. In murine models of *Acinetobacter baumanni* pneumonia, TLR4 knockout mice were found to have higher bacterial loads, impaired cytokine and chemokine response, and delayed lung inflammation as compared to wild type controls [28]. These studies support a key role for TLR4 in the response to gram- negative pneumonia.

Given the differential roles of TLR2 and TLR4 in gram-negative vs. gram-positive infections, we reasoned that the differential downstream cytokine production from the MyD88-independent (INF-β and RANTES) and MyD88-dependent (IL-8 and TFN-α) could be used in a rapid assay to distinguish these pneumonias. Further, as there is typically wide variability in individual cytokine levels in patients, we hypothesized that the ratio ([INF-β] X [RANTES]/[IL-8] X [TNF-α]) might be a better indicator of pneumonia type than isolated cytokine levels. We sought to test this in patients with severe pneumonia at the University of Maryland.

Subjects, Materials and Methods

Study design and measurements

A portion of the patients enrolled in this study were enrolled under protocol H-23204, while the later patients (2010 and beyond) were enrolled under protocol HP-00043013, both approved by the University of Maryland IRB. Included were adults 18 years of age and older admitted to the University of Maryland Medical Intensive Care Unit (MICU) with an admission diagnosis of severe pneumonia (community or hospital acquired). Candidate patients were identified as soon as possible after admission by daily review of recent admissions with the physician in charge of the MICU and step down units. Candidate patients were enrolled after giving informed consent, most commonly by the patient's durable power-of-attorney. Exclusion criteria were: Chronic ventilation or presence of a tracheostomy, severe immunosuppression (AIDS, metastatic cancer, neutropenia), active tuberculosis, hematocrit less than 20%, prothrombin time greater than 3.0 INR, platelets <20 k/mm^3, unable to obtain informed consent, or denied permission by the patients clinical physician. Data collected on each enrolled patient were: Apache II score in the first 24 hours after presentation, age, sex, race, maximum temperature in first 24 hours of admission, and all culture results. A culture was considered positive if it was from a respiratory, pleural, or blood source, and was specifically treated with antibiotics by the clinical physician. Positive cultures were recorded as gram-negative or gram-positive on microbiologic characterization of the organism in the culture. A third group included patients where no organism was definitively identified, or other organisms were caustive (fungal, mycobacterium, viral).

Each patient had one serum sample taken as close to the time of admission as possible, typically within 12 hours and universally within 24 hours. Serum was frozen at -80°C until assayed in the University of Maryland Cytokine Core Facility for IL-8, TNF-α, and RANTES (www.cytokines.com). INF-β was measured using a commercially available (PBL Biomedical Laboratories, Piscatawy, NJ) sandwich ELISA kit using standard company protocol.

Data analysis

Baseline characteristics for enrolled patients were tabulated for all variables measured, and groups were compared using paired t-test assuming unequal variance for continuous variables. Pearson chi-square was used for categorical data. Individual cytokine levels were compared between groups. Additionally, a composite continuous

	N =	ALL Mean	STD	N =	Gram (-) Mean	SEM	N =	Gram (+) Mean	SEM	Unknown	P**=N=Mean SEM		
Age	53	58.8	18	14	56.9	5.4	17	56.1	4.4	22	62.0	3.5	0.91
Apache II	53	21.9	7.1	14	24.8	1.5	17	23.5	1.6	22	18.8	1.6	0.56
T_{max} within 24 hrs. of admission	53	100.7	1.9	14	101.1	0.6	17	101.1	0.4	22	100.1	0.4	0.97
		F	M		F	M		F	M		F	M	
Gender	53	25	28	14	5	9	17	6	11	22	14	8	0.99
		Black	White		Black	White		Black	White		Black	White	
Ethnicity*	50	30	20	11	7	4	17	8	9	22	15	7	0.86

Note: *There were 2 asians and 1 hispanic not included above.
**P values calculated by type t-test for all continuous variables.
For categorical variables, P values are by Pearson Chi- square. All p values compare gram-negative vs. gram-positive groups

Table 1: Clinical Characteristics of enrolled patients.

Subject	Pathogen	Source*	Gram type
1	Enterococcus sp.	Sputum	+
2	*S. pneumonia*	Blood	+
3	NONE	NONE	0
4	NONE	NONE	0
5	*P. aeruginosa*	Blood, sputum	-
6	NONE	NONE	0
7	MRSA	Sputum	+
8	MRSA	BAL	+
9	NONE	NONE	0
10	Streptococcus sp., Viridans	Pleural	+
11	*P. aeruginosa*	Sputum	-
12	NONE	NONE	0
13	*E. Coli*	Blood	-
14	*K. pneumonia, S. maltophilia*	BAL	-
15	Streptococcus sp., α-hemolytic	BAL	+
16	MSSA	BAL	+
17	NONE	NONE	0
18	NONE	NONE	0
19	*C. koseri*	BAL	-
20	*S. maltophila, A. baumannii*	Sputum	-
21	NONE	NONE	0
22	*Klebsiella pneumonia*	Catheter tip	-
23	NONE	NONE	0
24	NONE	NONE	0
25	*S. pneumonia*	Sputum	+
26	*L. pneumophila*	BAL (DFA)	-
27	NONE	NONE	0
28	*Candida albicans*	Blood	0
29	NONE	NONE	0
30	MSSA/sputum	Sputum	+
31	NONE	NONE	0
32	MSSA	Blood	+
33	Streptococcus sp., group G	Sputum	+
34	*P. aeruginosa*	Sputum	-
35	MRSA	Sputum	+
36	MTB	Sputum	0
37	NONE	NONE	0
38	NONE	NONE	0
39	MRSA	Blood	+
40	*P. mirabilis*	Blood	-
41	NONE	NONE	0
42	Streptococcus sp., group F	Sputum	+
43	VRE	VRE/blood	+
44	NONE	NONE	0
45	NONE	NONE	0
46	*K. pneumonia*	BAL	-
47	*P. aeruginosa*	Blood	-
48	NONE	NONE	0
49	*P. aeruginosa*	BAL	-
50	NONE	NONE	0
51	MRSA	Pleural	+
52	*E. Coli, P. mirabilis*	BAL	-
53	*S. pneumonia*	Sputum	+

*BAL=bronchoalveolar lavage culture
MRSA=Methicillin-resistant *S. Aureus*
MSSA=Methicillin-sensitive *S. Aureus*
VRE=Vancomycin-resistant Enterococcus sp.
DFA=fluorescent antibody test

Table 2: Etiologic organism, site of positive culture, and gram classification of each subject.

variable "ratio" was calculated for each subject, and was calculated as the product of INF-β and RANTES levels, divided by the product of IL-8 and TNF-α levels. Cytokine and ratio levels were compared between groups by t-test assuming unequal variance.

Results

Dataset

Two patients initially enrolled were dropped from the study. One patient was an intravenous drug user who did not have a central line-blood drawing was impossible. The second patient's sample was mislabeled in a bin with blood from other patients, and could not be unambiguously identified. Both of these patients were excluded from the analysis. Therefore, 53 patients were enrolled in and completed the study between January, 2006 and September, 2013. Demographics on each analyzed subject are shown in Table 1. There were 20 (38%) whites, 30 (57%) blacks and 3 (5%) other who averaged 58.8 ± 18 years of age. 28 (53%) of the subjects were male, and the overall group had an average Apache II score of 21.9 ± 7.1. Tmax averaged 100.7 ± 1.9. There were no significant differences between the gram-positive and the gram-negative groups in any of these data.

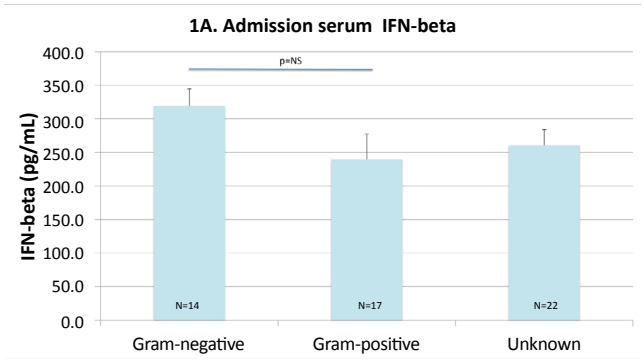
Table 2 shows culture results by organism, source, and gram stain type for each subject. There were 14 gram-negative, 17 gram-positive, and 22 gram-unknown/other infections.

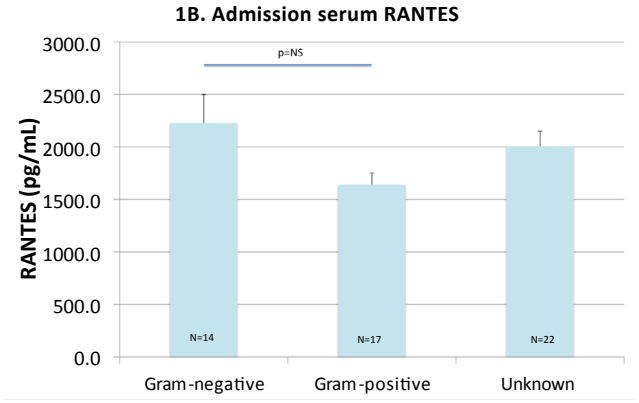
Cytokine levels for patient serum samples are tabulated in Table 3, and shown graphically in Figure 1. None of the cytokines measured demonstrated a statistically significant difference between the gram-negative and the gram-positive groups. However, generating a ratio of MyD88-independent cytokines to MyD88-dependent cytokines [INF-β] X [RANTES] / [IL-8] X [TNF-α] demonstrated a significant difference between the groups, 111.1 ± 34.9 for gram-negative vs. 29.9 ± 8.3 for gram-positive, with a p value of 0.04.

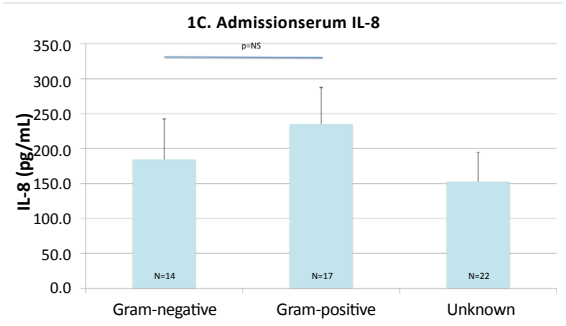
Discussion

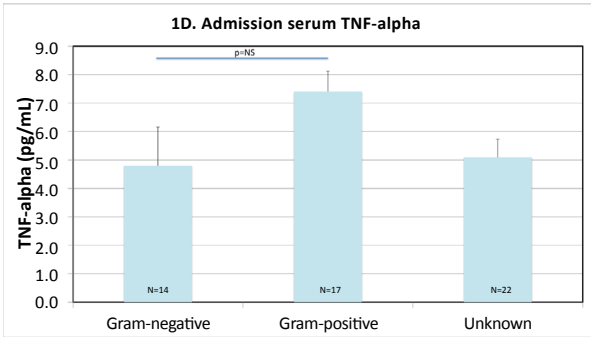
Few studies have been able to use cytokine levels as a clinically useful tool in infectious diseases. One study looking at IL-1β, IL-10, and IL-8 in 47 patients with pneumonia was able to discriminate *K. pneumoniae* infection based on cytokine levels [29]. However, IL-6, IL-8, and CRP levels were unable to distinguish varied pathogens in 87 cases of childhood pneumonia [30]. Variability in TLR activation and subsequent cytokine production with different infectious diseases has made the clinical use of cytokine measurement problematic. The mechanisms behind this variability are myriad and complex.

TLR2 has been shown to bind to multiple ligands, including the lipoteichoic acid (LTA) found in gram-positive bacteria, but also glycolipids, lipopeptides, and lipoproteins [31]. Though gram-negative bacteria are thought to primarily activate TLR4 with LPS, TLR2 receptors can be activated by other gram-negative bacterial products. Infection with *P. aeruginosa* can have paradoxical effects on IL-8 and RANTES levels. The organism is known to increase IL-8 levels in human airway epithelial cells by secreting small molecular weight factors [32]. However, RANTES is degraded by the metalloproteases, alkaline protease, and elastase secreted by *P. aeruginosa* infecting airway epithelial cells, whereas IL-8 is much more resistant to proteolysis [33]. In human neutrophils, TLR2 and TLR4 mediated production of IL-8 was shown to be initially induced directly by LTA but with larger scale production coming from a second phase, mediated by endogenous TNF-α [34]. The majority of LTA-induced neutrophil activation was independent of the TLR2 and TLR4- mediated pathways, but relied on









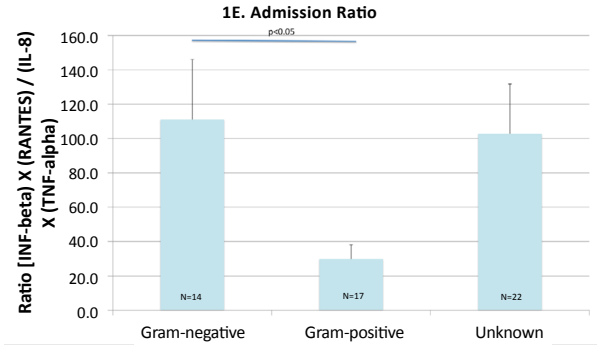


Figure 1(A-E): Bar graph of admission INF-β ,RANTES, IL-8, and TNF-α levels, all in pg/mL, and the ratio of ([INF-β] X [RANTES]/[IL-8] X [TNF-α]), no units, grouped by etiology of the pneumonia. Error bars are S.E.M.

	Gram-unknown mean	Gram-unknown SEM	Gram-negative mean	Gram-negative SEM	Gram-positive mean	Gram-positive SEM	**P-value
IFN-β	260.4	23.5	319.3	25.3	239.5	37.8	0.09
RANTES	2007.3	144.2	2229.5	268.6	1641.0	110.9	0.06
IL-8	153.1	41.7	184.3	58.2	235.0	52.5	0.52
TNF-α	5.1	0.6	4.8	1.4	7.4	0.7	0.11
Ratio* **	102.8	29	111.1	34.9	29.9	8.3	0.04

*All values are pg/mL of serum (except Ratio values, which is unitless).
**P values calculated by type 3 t-test for all continuous variables.
***Calculated as [IFN-β] X [RANTES] / [IL-8] X [TNF-α].

Table 3: Summary of mean admission levels* of MyD88-dependent (IL-8, TNF-β) and MyD88-independent (RANTES, IFN-α cytokines and the ratio. These data are shown graphically in Figure 1.

CD14. Levels of IL-8 were able to be attenuated by IL-10 in the second phase, likely due to its regulatory effect on TNF-α. These data suggest that cytokine levels in various infections change markedly over the course of infection, and have multiple mechanisms responsible for their production.

In vivo data from real human infections is even more variable. A recent study measuring TLR2 and TLR4 expression in elderly patients with high mortality infections found that expression of both TLRs were decreased in patients who died compared to survivors [35]. TLR2 and TLR4 expressions were positively correlated with IL-1, IL-6, and TNF-α levels, but had no correlation with CRP, APACHE II score or CURB-65 score.

Furthermore, a prospective study of 1,895 patients of hospitalized with CAP and sepsis published in 2013 showed that among 423 small molecules detected in serum in the emergency department, the levels of 70 of those differed significantly between survivors and nonsurvivors, and two major molecules stimulated increased TNF-αand IL-1β. Despite these results, a metabolite-based prognostic model to predict 90 day mortality performed only modestly (AUC=0.67, 95% CI 0.48-0.81) [36].

Given such variation in individual cytokine levels in infectious diseases, we demonstrated that a composite index of TLR2 to TLR4 related cytokines functioned much better to discriminate the clinically important question of whether the causative organism is gram-positive or gram-negative. As technology for measurement of cytokine levels improves, the use of fast, point-of-care analysis of cytokine ratios could significantly aid in the management of these difficult and sick patients.

Limitations

A significant number of patients enrolled in this study did not have a positive culture result, and their exclusion from the analysis of gram-negative vs. gram-positive cytokines may have skewed our results. However, in the majority of common clinical infectious diseases, no etiologic agent is identified by culture, and nothing can be done to account for any bias that may be introduced by this clinical limitation.

This study was performed as a proof-of-principle investigation. An increased number of cytokine and other inflammatory mediators, with a broad statistical approach to combining the measurements could result in even better performance, and may even allow determination of specific infectious species. Multiplexed analysis of clinical specimens could significantly increase the utility of this strategy.

Conclusion

The statistically higher ratio of MyD88-independent to MyD88-dependent cytokine levels in patients with culture proven gram-negative vs. gram-positive infection supports our original hypothesis. A ratio of cytokines, designed utilizing known pathophysiologic pathways, was a much more robust discriminator of infection type than individual cytokine levels taken individually, and might have important clinical implications in the care of patients with severe pneumonia.

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