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Profile of Tumor Necrosis Factor Alpha Levels in Childhood Malignancy with Febrile Neutropenia

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ABSTRACT

Infection is a significant cause of morbidity and mortality in childhood malignancy with Febrile Neutropenia (FN). Tumor Necrosis Factor-Alpha (TNF-α) is involved in host defense against bacterial invasion. However, changes in TNF-α levels with the possibility of bacterial infection confirmed by blood culture are still unclear. The study aimed to evaluate TNF-α levels in childhood malignancy with FN who had blood cultures with a control group. Observational cross-sectional analysis during January-October 2020 at Dr. Soetomo General Academic Hospital, Surabaya. Childhood malignancy with FN episodes as the case group and nonfebrile neutropenia as the control. TNF-α levels examination used plasma with the Enzyme-Linked Immunosorbent Assay (ELISA) sandwich method. Blood culture results were obtained from the patient's medical record. The differences in TNF-α levels in the case groups and control were analyzed by the T-square test for two independent samples or Mann-Whitney U according to the data distribution. There were 18 cases group with 30 FN episodes and 15 controls. There were 8(26.66%) positive and 22(73.33%) negative blood cultures from 30 FN episodes. The mean TNF-α levels in the positive blood culture cases group and control: 14.72±5.77 and 9.78±2.74 pg/mL, and the median (min-max) negative blood cultures: 12.19 (7.01-25.70) pg/mL. There was no significant difference in TNF-α levels in the positive and negative blood culture cases group (p=0.527), but there was a significant difference in the control (p=0.049 and p=0.027). Therefore, TNF-α levels cannot be used as a marker of bacterial infection in the case groups.

Keywords: TNF-α, positive blood cultures, negative blood cultures

INTRODUCTION

Infection is one of the leading causes of morbidity and mortality in childhood malignancy with Febrile Neutropenia (FN). Neutropenia is known to be the leading risk factor for bacterial infection.[1] Proinflammatory cytokine as Tumor Necrosis Factor-Alpha (TNF-α) secreted by various cells also compromises the host's defense mechanism and releases an inflammatory response towards bacterial infection.[1,2] The increase of cytokines in the circulation supports the presumption that there is an infection and has the potential to be an early screening marker for infection in child patients with malignancy and FN, but changes in TNF-α levels with the possibility of bacterial infection are confirmed by blood culture are still unclear.[1,2] Several studies reported various results of TNF-α levels in the same condition. Xia *et al*., Surbatovic *et al*., and Araujo *et al*. reported an increase in TNF-α in those conditions.[3,4] Siegmund *et al*. reported the reverse results, TNF-α showed significantly lower concentration in patients with FN.[5] Blood cultures as the standard diagnosis of bacterial infection is known to have a low diagnostic value, depending on whether the patient has received prophylaxis antibiotics, the volume and time of blood collection and the number of bottles collected.[6]

American Society of Clinical Oncology dan Infectious Disease Society of America (ASCO/IDSA) and the European Society for Medical Oncology (ESMO) define FN as having a temperature ≥ 38.3°C in one oral measurement or a temperature of ≥ 38.0°C for more than one hour with an Absolute Neutrophil Count (ANC) < 500 cell/μL or a decrease of ANC from 500 cell/μL in the following 24-48 hours.[7,8] The episode of FN is defined as the point where the FN criteria are fulfilled and ends when the requirements are no longer fulfilled.[9] A febrile neutropenia can cause a delay and dose reduction of chemotherapy, disturbing the success of therapy; lengthening hospitalization; increasing care cost, morbidity, and mortality.[10]

The relationship between circulating TNF-α levels and the possibility of bacterial infection confirmed by culture examinations in childhood malignancy

patients with FN is still unknown and controversial, with limited research, especially in Dr. Soetomo General Academic Hospital, Surabaya.

This study's purpose is to evaluate the TNF-α concentrations in childhood malignancy patients with FN that have blood culture results and in the control group.

METHODS

This cross-sectional observational analytic study was conducted during January-October 2020 in the Inpatient Pediatric Ward and Clinical Pathology Installation UNAIR Medical Faculty/Dr. Soetomo General Academic Hospital, Surabaya. The target population was all childhood malignancy patients. This research's case group was the population with an FN episode and fulfilled the inclusion and exclusion criteria. The study's specimen was peripheral blood of the control group in an EDTA tube for TNF-α. Blood culture results were obtained from the patient's medical record as the routine workup for FN in children with malignancy. Child patients without fever (nonfebrile) during the same care period fulfilled the inclusion criteria for the control group, underwent TNF-α levels examination, and were the control group without blood cultures. Inclusion criteria for patients with malignancy and FN were as follows: age between 1-16 years old, are willing to be included in the study by signing an informed consent or were represented by their parent/guardian, and had blood culture results. The exclusion criteria were the onset of fever was > 12 hours.

Blood samples in the EDTA tube were centrifuged at 1000x g for 15 minutes to get 100 µL of plasma. Plasma was collected and stored at -80 C for less than three months for TNF-α examination collectively following the recommendation of the instrument. TNF-α was calculated using the sandwich Enzyme-linked immunosorbent Assay (ELISA) method from the Elabscience® reagent kit.[11] Quality control of TNF-α was conducted using an ELISA reader, and control was done before samples were analyzed. Quality control was also conducted every time the instrument was calibrated. Results were stated well if they were in the control range.[11]

TNF-α levels in the positive and negative blood cultures; and control group were analyzed using the T-square test for two independent samples if the data were normally distributed or Mann-Whitney U for data that were not normally distributed. The normality test was conducted using the Shapiro-Wilk test. The same procedure was done for research characteristics, age, ANC, and temperature.

This research has been approved by the Health Research Ethics Committee of Dr. Soetomo General Academic Hospital, Surabaya, with the ethical approval number 1931/KEPK/IV/2020.

RESULTS AND DISCUSSIONS

The case group comprises 18 kids, eight boys, and ten girls, with various malignancy diagnoses. Several of them had more than one episode of FN. As many as 30 episodes of FN in the case group were used as samples and fulfilled the inclusion criteria. Fifteen people were appointed as the control group, consisting of 9 males and 6 females with various malignancy diagnoses, malignancy in the case group was dominated by Acute Lymphoblastic Leukemia (ALL) as much as 13 (72.22%), the rest were Acute Myeloid Leukemia (AML) as much as 4 (22.22%) and 1 case of Neuroblastoma (5.56%). Malignancy

Table 1. Gender, malignancy diagnosis of positive and negative blood culture case, and control groups characteristics

Characteristics	Group		
	Case (FN) (n=30)		Control (n=15)
	Positive culture (n=8)	Negative culture (n=22)	
Gender [n(%)]			
Male	3 (37.5%)	9 (40.9%)	9 (60%)
Female	5 (62.5%)	13 (59.1%)	6 (40%)
Diagnosis [n(%)]			
ALL	6 (75%)	14 (63.6%)	12 (80%)
AML	2 (25%)	7 (31.8%)	2 (13,3%)
Neuroblastoma	0 (0%)	1 (4.5%)	0 (0%)
Retinoblastoma	0 (0%)	0 (0%)	1 (6.7%)

diagnosis of the control group was also dominated by ALL, as many as 12 (80%). The rest were 2 cases of AML (13.33%) and 1 case of Retinoblastoma (6.67%). There was not one episode of febrile neutropenia in the case group that was diagnosed clinically or laboratory as septic shock.

The characteristics such as gender, group malignancy diagnosis of positive and negative blood culture cases, and control groups are summarized in Table 1. The distribution of age, ANC, and temperature in Table 2, is normally distributed with $p > 0.05$ and not normally distributed with $p < 0.05$.

Blood culture results had eight positive results (26.66%) from 30 FN episodes. The types of bacteria in the positive blood cultures can be seen in Table 3.

The results and differential test results for TNF-α levels in the positive and negative blood culture case and control groups are presented in Tables 4 and Figure 1. Data distribution for TNF-α levels in each group is presented in Table 4, normally distributed with $p > 0.05$ and not normally distributed with $p < 0.05$. The Mann-Whitney U test showed no significant difference in TNF-α levels between the positive and negative blood culture case groups ($p > 0.05$). However, the T-square test for two independent samples and the Mann-Whitney U test show a statistically significant difference in the case groups with positive and negative blood cultures towards the control group ($p < 0.05$).

Malignancy is one of the leading causes of central death following accidents in children and teens worldwide.[12] Leukemia is the number one malignancy, with ALL as the most common type, followed by AML and Acute Monocytic Leukemia (Amol).[13] Malignancy patients usually experience neutropenia due to chemotherapy and the

Table 2. Age, ANC, temperature, TNF-α positive and negative case; and control groups characteristics

Group	Age (y.o)			ANC (cell/µL)			Temp (°C)			TNF-α Levels (pg/mL)		
	Mean±SD	Median (min–max)	p	Mean±SD	Median (min–max)	p	Mean±SD	Median (min–max)	p	Mean±SD	Median (min–max)	p
Case												
Positive culture (n=8)	7.5±3.928	6 (4–16)	00.04	116.25±90.859	100 (10–290)	00.51	38.10±0.193	38 (38–38.5)	0.001	14.72±5.773	0,5458333 (8.2–24.2)	00.23
Negative culture (n=22)	6.45±3.474	5.5 (2–14)	00.02	186.82±127.928	165 (30–480)	00.15	38.45±0.475	38.35 (38-40)	0.0005	13.33±5.559	12.19 (7–25.7)	0.008
Control (n=15)	7.07±4.431	6 (1–16)	00.49	256±109.009	260 (80–430)	00.57	36.76±0.417	36.8 (36.1-37.5)	00.53	9.785±2.743	9 (6.8–14.9)	00.08

Table 3. Types of bacteria for positive blood cultures

Gram (+) Bacteria	Total (%)	Gram (-) Bacteria	Total (%)
Staphylococcus hominis	2 (25%)	*Enterobacter cloacae*	1 (12.5%)
Streptococcus agalactiae	1 (12.5%)	*Salmonella species*	1 (12.5%)
		*Escherichia coli**	1 (12.5%)
		Klebsiella pneumoniae	1 (12.5%)
		Morganella morganii	1 (12.5%)

*[1] with *Escherichia coli* ESBL

Table 4. Differential tests for the characteristics of age, ANC, temperature, and TNF-α levels in the case group with positive and negative blood culture in the control group

Group	n	p-value			
		Age (y.o)	ANC (cell/µL)	Temperature (C)	TNF-α Level (pg/mL)
Positive culture Negative culture	8 22	0.23*[1]	0.08*[2]	0.01*[1]	0.527*[1]
Positive culture Control	8 15	0.43*[1]	0.002*[2]	0.00006*[1]	0.049*[2]
Negative culture Control	22 15	0.35*[1]	0.04*[2]	<0.00001*[1]	0.027*[1]

*[1]: Mann-Whitney U test *[2]: T2 test for two independent samples

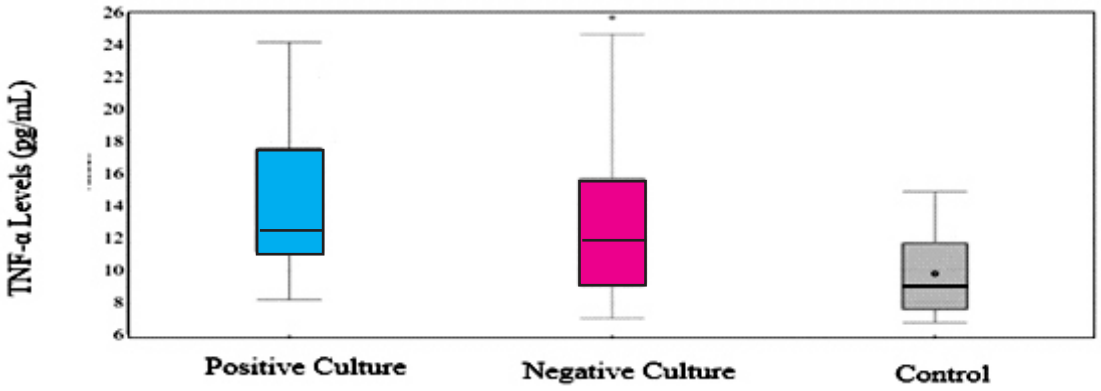

Figure 1. The difference between TNF-α levels in the case group positive and negative blood culture toward control (p=0.049 and p=0.027)

malignancy itself, causing a decrease in one's capability to fight infection.[1] The release of endogenic cytokines such as TNF-α by the epithelial cells during bacterial invasion can cause fever during neutropenia.[14] The fever can also be a side effect of chemotherapy, viral or fungal infection, blood transfusion, or malignancy.[15]

The TNF-α levels in the positive blood culture case group in this study showed an increase in the minimal detection limit of an instrument, which was 7.81 pg/mL. Macrophages that are activated by bacterial or endotoxin (Lipopolysaccharide/LPS) exposure will release TNF-α into the circulation is the main factor in the increase of TNF-α levels in the positive blood culture group, besides other factors such as neutropenia and the malignancy itself.[16] Neutropenia can increase the levels of TNF-α in the circulation due to the absence of neutrophil extravasation that could bind TNF-α to the site of infection.[17] Macrophage activation by a malignancy that triggers the release of TNF-α can happen to the host's macrophages and the tumor-associated macrophages (TAMs) of the malignancy cell itself. The malignancy can activate the host's macrophage as a target cell necrosis and apoptosis mechanism (malignancy) to suppress growth. Malignancy cells' TAMs have a role in the growth and progressivity of the tumor through several mechanisms such as angiogenesis, lymph angiogenesis, migration, invasion, and immunosuppression that make the tumor cells survive from the host's immune system and therapy.[18,19]

The cut-off of TNF-α levels to predict bacterial infection may vary between institutions. Rebecca and Srinivas reported that the cut-off for bacterial infection in neonatal sepsis was 17 pg/mL, while Rukmono *et al*. reported 28.3 pg/mL.[20,21] The mean TNF-α levels in this study's positive blood culture case group are lower than the cut-off set by that research. There are a couple of reasons that cause suboptimal TNF-α production. No found cases with severe toxic symptoms or septic shock complications in this study caused an insignificant increase of TNF-α or possibly only a momentarily increase that could not be detected during sampling.[21,22] Several studies reported that TNF-α was quickly secreted into the circulation but rapidly decreased.[23] TNF-α is also known to not show a consistent gene expression pattern and variates among individuals. The expression can be affected (downregulated) by IL-6 and IL-10.[4] The production of suboptimal TNF-α can also happen due to the LPS tolerance phenomenon. Macrophages become insensitive or tolerant toward repeated LPS exposure, affecting the output of TNF-α.[24] The presence of a TNF-α inhibitor such as IL-10, IL-4, transforming growth factor-β (TGF-β), prostaglandin E2 (PGE2), and glucocorticoid can cause low levels of TNF-α.[25,26]

The insignificant increase of TNF-α of the positive blood culture case group in this study may indicate its origin from the growth of contaminating bacteria such as *Staphylococcus hominis*. These contaminating bacteria are known not to be able to activate the host's defense mechanism directly. Proinflammatory cytokines such as TNF-α and bacterial infection markers such as procalcitonin do not increase significantly compared to an actual pathogenic bacterial infection, and vice versa when a contaminating bacteria becomes a new pathogen. These tests are commonly used as an alternative to differentiate an actual pathogen bacteria and a contaminating bacteria.[27]

TNF-α levels of the negative blood culture case group in this study generally increase compared to the minimum detection level of the instrument, even though they are not as high as the positive blood culture case group. Several conditions in the negative blood culture case group activate

macrophages to release TNF-α in the circulation, such as nonbacterial infection (viral or fungal), blood transfusion effect, chemotherapy, or the malignancy itself, in addition to neutropenia.[17,19,28] Blood transfusion can activate macrophages due to inflammation from biological matter that resembles therapeutic components such as infectious pathogens, pathogen antibodies, unwanted antigens, or allergens.[28] Tumor-Associated Macrophages (TAMs) can be activated by the administration of chemotherapy, causing the secretion of cytokines, including TNF-α, as an immunosuppression mechanism to create resistance to chemotherapy.[19]

Negative blood cultures do not guarantee that the patient is free of bacterial infection. Only 30% of child patients with FN are proven to have a bacterial infection, including in this study, so there is a possibility that the culture results are false negative. Bacterial detection in the blood is complicated, depending on whether the patient has received prophylactic antibiotics, the volume and timing of blood sampling, and the number of culture bottles collected.[6,29] The relatively small amount of bacteria in specific periods and the periodic release of bacteria from the target organ to the bloodstream can decrease the possibility of bacterial detection in cultures.[30] False negative cultures can also be caused by the slow growth of bacteria in culture bottles that do not produce enough carbon dioxide to fulfill the automatic blood culture instrument detection limit.[31] Bacterial infections that do not show growth on blood cultures but still trigger the activation of macrophages to release TNF-α into the circulation, causing high cytokine levels in a negative culture condition.

The mean TNF-α level in the control group increased from the instrument detection limit but was still lower than the positive and negative blood culture case groups. Neutropenia, chemotherapy effect, and malignancy itself can cause a release of TNF-α into the circulation, causing an increase of TNF-α in the control group.[19]

Mann-Whitney U test shows no significant difference in TNF-α in positive and negative blood culture case groups with p=0.527 (p>0.05). An increase in TNF-α in the negative blood culture followed by the fact that there was no significant increase in TNF-α in the positive blood culture case group caused no significant difference between the both of them, so TNF-α cannot be a marker of bacterial infection in that population. A significant difference between TNF-α levels of the positive and negative blood culture case groups and the control group was found in this research. Fever in the case groups is usually caused by a bacterial or nonbacterial (viral or fungal) infection or by non-infection can explain why there is a difference in TNF-α levels in the case and control groups.[15,29] These various conditions activate and trigger the macrophages to release TNF-α, causing the increase of TNF-α levels in the circulation.[23]

This study has a couple of limitations, namely: the uniformity of the duration of sickness before the patient was hospitalized, the length and number of chemotherapy cycles, the distance between febrile neutropenia episodes are variated, culture validity is hard to control and is done in one institution (single center study) with a relatively small number of samples in the positive and negative blood culture case and the control groups. This limitation may cause a relatively large bias towards each group's measurement of TNF-α levels.

CONCLUSIONS AND SUGGESTIONS

There was an increase in TNF-α levels in the group with positive and negative blood culture cases and the control groups. There was no difference in TNF-α levels in the positive and negative blood culture case groups, but there was a difference in both groups with the control group. No difference between the positive and negative groups followed by the limitations of this study could cause a bias, which deems TNF-α not yet able to be a bacterial infection marker in the case group. The author suggests further research with the uniform length of disease before the patient is admitted to the hospital, length and total chemotherapy cycles, the same sampling time between FN episodes, and culture validity that was controlled and involved various institutions with a larger sample.

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