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Original Article

IDO enzyme and IL-10 to TNF-α ratios as immune response silicone granuloma: Predictors factors

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Abstract

Background Silicones are still being commonly used in medicine, whether in filling materials or their mixture. Some may penetrate human body tissue through injections or application with advanced medical technology, particularly transdermal administration. The study was aimed to determine predictor factors to assess the immune response to silicone filler injection.

Methods An analytical cross-sectional study was conducted to evaluate immune response based on blood in patients with granuloma following silicone injection compared to normal patients; The materials included blood cultures with RPMI media, which were categorized into three groups, i.e. RPMI alone, with PHA and with 3% silicone groups. Evaluation on immune response was performed by analyzing IDO enzyme using ELISA and detecting TNF-α, IFN-γ, IL-10 cytokines using the Luminex technology. The study was conducted at Eijkman Foundation Laboratory, Faculty of Medicine, the University of Indonesia, between 2012 and 2015.

Results Patients with granuloma had higher levels of proinflammatory cytokines in their blood, and the ratios of TNF-α/IL-10 and TNF-α/IDO in blood were significantly different between patients with granuloma and standard control.

Conclusion IL-10 and IDO enzyme play critical roles in immune tolerance caused by silicone injection and can be utilized as predictors to assess immune response induced by silicone injection.

Key words
Granulomas, silicone, immune response, tolerance.

Introduction

Silicones are still commonly used in medicine, whether in filling materials or a mixture for filling material, implant reconstruction or cream for daily use. Some may penetrate human body tissue through filler injections or cream application in line with advanced medical technology, particularly transdermal administration. A survey performed by the "Indonesian Association of Plastic Surgeons between 2004 and 2007 found that there were 249 cases of complications associated with silicone injection".[1] "Epidemiological data in other countries were not precise because silicone injection had been banned. In 1990, more than 100,000 patients in the United States had received silicone injection in their face.[2] There have been numerous case reports documenting the consequences of silicone filler, both domestic and overseas reports".[2-5] The use of "silicone filler injection for cosmetic treatment was banned by the Federal Food, Drug & Cosmetic America (FDA) since 1992".[2] "Liquid silicone injected into the skin can migrate and cause morphological changes and uncontrolled inflammatory response. Liquid silicone in the

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tissue is persistent, leading to chronic inflammation and granulomas formation. In severe cases, it could be followed by infection, necrosis, and abscess".[3,5,6]

Pathogenesis of silicone-induced granuloma has not been confirmed; however, pathogenesis for granuloma caused by infection can be classified into two types, which are type 1 and type 2, depending on the type of T helper (Th) cells which play a role in the immune response. Th-1 cells secrete proinflammatory cytokines, which are TNF-α, IFN-γ, IL-2, while Th-2 cells produce anti-inflammatory cytokines, IL-10, TGF-B, IL-4.[7,8] Some studies on granuloma have correlated them with slow-type hypersensitivity reaction characterized by increased IL-2 and IFN-γ levels.[7,8] Although the pathogenesis of silicone-induced granuloma has not been known, some studies have reported it may be associated with increased TNF-α level.[9-11] Nevertheless, previous studies on the immune response to granuloma due to the rupture of silicone breast implants have provided contradictory results.[10,11] Liquid silicone will induce foreign body reaction (FBR) only in some individuals.

Since the mechanism of the development of granuloma is still not precise, the changes in body tissue due to liquid silicone are hardly predictable. Immune response to a foreign body (including silicone) is different for each individual. It is assumed that the development of silicone-induced granuloma after silicone injection is similar to the immune response of the human body to chronic infection, in which Th-1 cells play the role through proinflammatory cytokines and Th-2 cells through anti-inflammatory cytokines. The type of chronic granuloma developed depends on which type of T helper cells plays the role.[6,7] Recent advances in "immunology have revealed that the subpopulation of T cells, which are T regulator cells ($CD4^+CD25^+$) with their IL-10 and TGF-B cytokines, play essential roles in controlling the activity of both Th-1 and Th-2 cells. T regulator cells and indoleamine 2, 3-dioxygenase (IDO) enzyme secreted by an antigen-presenting cell (APC) have been recognized to have an essential role in immune balance, homeostasis, and immune tolerance well as it can also determine the occurring immune response".[12-15] It has been assumed that chronic granuloma in the tissue may affect immune response changes in the blood. The "changes can be reflected by altered levels of various cytokines that play a role in the inflammatory response, i.e. the TNF-α, IFN-ɣ, IL-10, and IDO enzyme".[7,13,-15]

The materials for evaluating cytokine levels were taken from blood culture using Roswell Park Memorial Institute (RPMI) medium and RPMI medium, which were stimulated with phytohemagglutinin (PHA) and 3% industrial silicone. It is expected that the results of the present study on immune response may provide a better understanding of the pathogenesis of granuloma caused by silicone injection, and it may become the basis for clinical prediction and management. The present study is a continuation of a study about the foreign body reaction classification due to industrial silicone injection, which has been published in Dermatologic Surgery Journal, volume 44, number 9, September 2018.[16]

Methods

The Control group consisted of 37 normal skins which had facelift surgery of the same age and sex. All tissues were examined histopathologically (HE staining) for evaluating the degree of foreign body reaction (FBR) (it had been reported in the Dermatologic Surgery Journal, volume 44. Number 9, September 2018). Laboratory experimental study was

performed to assess blood cytokine levels by (a) culturing whole blood cells from granuloma patients and normal individuals, using Roswell Park Memorial Institute (RPMI) medium. The RPMI was stimulated by phytohemagglutinin (PHA) and 3% of industrial-grade silicone; (b) examing cytokine levels from cell culture on day 3, which included TNF-α, IFN-γ, and IL-10. All of the cytokines were analyzed using Luminex and IDO with ELISA. The study was conducted at Eijkman Foundation Laboratory, Faculty of Medicine, the University of Indonesia, between 2012 and 2015. Following the study's aim, a statistical test was carried out using SPSS software program version 16.0. For numerical data, univariate and bivariate analysis was performed, in which a normality test was performed to determine to use either parametric or non-parametric test. Categorization of numerical data was made based on cut-off point, which was analyzed using the receiver operator curve (ROC) test.

Result

The present study is a continuation of our previous study, i.e. "the classification of foreign body reaction due to Industrial silicone injection; therefore, it has the same subjects as the previous study".[16] The silicone levels in submental skin were significantly higher than those in granuloma tissue [688mcg/g (4.1-10430) vs. 944 mcg/g (0-6065)]. Another interesting finding was that silicone is also found in normal skin tissue with an approximate level of 44.07mcg/g.

Hitherto there has been no finding or study on average silicone level in skin tissues of female individuals. The results of the present study were new findings. It can be used as a reference; when the silicone levels are higher than the reference, health problems may occur, presumably caused by the intake of silicone substance in the cosmetic product.[17]

The characteristics of immune response in the blood of standard and granuloma group - The action of injecting silicone filler (foreign body) into body tissue will induce an immune response, both cellular and humoral responses. Large-sized silicone cannot be destroyed by the phagocytes, such as the macrophages; therefore, the foreign body will remain and cause chronic inflammation. In some individuals, the reaction induces the development of granuloma. The pathogenesis of granuloma is categorized into two types, which are type 1 and type 2, depending on the type of T-helper cells (Th) that have roles in immune response.[12,24] "Th1 releases proinflammatory cytokines, i.e. TNF-α, IFN-γ, IL-2, and Th2 releases anti-inflammatory cytokines, i.e. IL-10, TGF-β, IL-4".[8]

Table 1 Clinical examination, onset of granuloma symptoms, and silicone concentration for subjects of different age categories.

Variables	*Normal Control Group (n = 37)*	*Chin Granuloma Group (n = 31)*	*Submental Skin Group (n = 31)*
Age (yr), X + SD	47 (28–55)	40.1 + 8.78	40.1 + 8.78
Clinical examination, %			
Mild		6 (19.4%)	
Moderate		18 (58.1%)	
Severe		7 (22.6%)	
The onset of granuloma symptom (yr.)		12.5 + 5.5	12.5 + 5.5
Silicone concentration (mg/g), median (minimum–maximum)	0 (0–253)	688 (4.1–10,430)	944 (0–6,065)

Table 2 Correlation of TNF-α, IFN-γ, IL-10, IDO cytokine levels in the blood of PHA, RPMI and silicone between the standard and granuloma groups.

Types of cytokines / *Types of culture*		*Cytokines levels in the normal group n=31*	*Cytokines levels in granuloma group n=31*	*P-value*
TNF-α pg/mL	PHA	757.51 (24.6-13274.3)	2689.61 (29.5-7573.6)	p= 0.056[m]
	RPMI	91.62 (21.9-1253.8)	185.10 (23.5-1469.4)	**p= 0.021[m*]**
	Silicone	73.99 (22.8-3710)	127.06 (18.1-2910.45)	p= 0.100[m]
IFN-γ pg/mL	PHA	372.36 (27.1-52503.7)	6868.31 (12.5-81372.5)	p= 0.075[m]
	RPMI	84.45 (6.6-864.9)	175.38 (9-5329.45)	p= 0.304[m]
	Silicone	37.83 (5.7-12736.8)	169.40 (21.3-1021.5)	p= 0.310[m]
IL-10 pg/mL	PHA	191.6 (10.2-927)	192.94 (13.6-890.4)	p= 0.392[m]
	RPMI	23.1 (8.6-112.6)	17.6 (6.5-418.5)	p= 0.207[m]
	Silicone	22.4 (8.6-417.4)	17.13 (6.1-339.95)	p= 0.281[m]
IDO ng/mL	PHA	1112.7 (92.1-7146.7)	979.39 (106.7-7366.7)	p= 0.449[m]
	RPMI	784.2 (92.1-6280)	458.2 (97-6440)	**p= 0.049[m*]**
	Silicone	773.1 (66.7-5106.7)	471.51 (100.6-5600)	**p= 0.050[m*]**
TNF-α/ IL-10 ratio	PHA	7.6 (0.3-32)	10.7 (1-60.9)	p= 0.055[m]
	RPMI	3.8 (0.3-27.9)	10.6 (0.2-63)	**p= 0.002[m*]**
	Silicone	4.1 (0.7-25.4)	6.66 (0.3-51.1)	**p= 0.011[m*]**
TNF-α/ IDO ratio	PHA	0.7 (0-5.9)	3.2 (0.0-14.5)	**p= 0.008[m*]**
	RPMI	0.1 (0-5.2)	0.33 (0-12.0)	**p= 0.008[m*]**
	Silicone	0.11 (0-7.0)	0.17 (0-12.2)	**p= 0.026[m*]**

Note:
[m]Mann Whitney test, ng/mL:nanogram/mililiter, pg:pikogram/ mililiter.

In normal patients and those with granuloma, there was no significant difference regarding the levels of TNF-α, IFN-γ, IL-10 cytokines and IDO in blood, which was stimulated by 3% silicone compared with the negative control (RPMI blood). A significant difference was only found between the negative and positive controls (PHA), ($p<0.001$).

Table 2 The level of TNF-α cytokine in patients with silicone granuloma was significantly different from normal patients (p=0.021). The level of IDO enzyme in patients with silicone granuloma was significantly different from normal patients. (RPMI: p=0.049; silicone p=0.05).

Patients in the silicone granuloma group had higher TNF-α inflammatory cytokine level than the average group; however, their anti-inflammatory or immune tolerance and IDO enzyme levels were lower than average **(Table 2)**.

From those data results, it is assumed that the development of granuloma in the case group was associated with excessive inflammatory response (TNF-α) when there was silicone-induced stimulation, which had not been balanced out by adequate immune tolerance.

Predicting the development of granuloma based on cytokine levels in the blood

The TNF-α level in blood was correlated inversely with the development of granuloma disorder (p= 0.003, r=-0.516). It is concluded that TNF-α has a systemic effect. The level of TNF-α in the blood can predict immune response that may lead to the development of granuloma. The development of granuloma is affected by proinflammatory cytokines (TNF-α), and body tolerance and the role is played by IL-10 and IDO enzyme. A ratio of proinflammatory to anti-inflammatory or tolerance should be

Table 3 Correlation of TNF-α/IL-10 ratio and TNF-α/IDO ratio with duration of developing symptoms.

Types of blood culture	*TNF-α/IL-10*			*TNF-α/IDO*		
	PHA	*RPMI*	*Silicone*	*PHA*	*RPMI*	*Silicone*
Duration of developing symptoms (years)	p=0.043[s*] r=-0.367 R^2=0.135	p=0.038[s*] r=-0.374 R^2=0.140	p=0.118[s*] r=-0.286	p=0.136[s] [*] r=-0.274	p=0.028[s*] r=-0.395 R^2=0.156	p=0.034[s*] r=-0.381 R^2=0.145

Note:
[s] Spearman test

made to evaluate a more accurate prediction. Results on TNF-α/IL-10 ratio, TNF-α/IDO ratio and the duration of developing symptoms can be seen in **Table 3**.

In **Table 3,** the RPMI TNF-α/IL-10 ratio inversely correlated with the duration of developing granuloma symptoms. (p=0.038[s*], r=-0.374). RPMI TNF-α/IL-10 ratio can be explained by the duration of developing granuloma symptoms as many as 14% (R^2=0.140).

Discussion

The immune response of silicone granuloma in blood

Silicone is a non-biodegradable molecule; therefore, it continues to exist in body tissue when it is injected. Silicone in the tissue has hydrophobic interaction with blood protein, which will initiate clotting, fibrinolytic and kinin systems and complement activation. "It contains various cytokines, chemokines and growth factors on matrix protein of silicone or biomaterial surface, and it causes activation of numerous immune cells. Those series of reactions are called the Vroman effect".[18] Another literature suggests that the immune response against silicone occurs indirectly, but it still works through matrix protein. Hydrophobic materials, such as silicone breast implant, is wrapped by the host's protein within hours after attachment, with at least 70% will have been wrapped in 1 hour. In general, inflammatory cells never have any direct contact with silicone biopolymer, but they do have contact through plasma protein, such as IgG, albumin, fibronectin and complement components.[11,18]

Another study has reported that inflammatory cells that have penetrated the tissue do not respond to the silicone material itself but with a plasma protein matrix with specific receptors for neutrophils and macrophage. In an *in vitro* study using blood culture with silicone stimulation that aimed to evaluate the mechanism of action of lymphocytes, the results showed no significant difference with RPMI (negative control); it seems that silicone requires protein to induce the development of immune response. These facts are consistent with the results of A Harlim study that reported silicone has non-immunogenic properties.[17]

Results of examination have provided evidence that there was no difference of IDO enzyme between those in RPMI blood culture and those with PHA-stimulated lymphocytes (positive control) or silicone in the normal control group granuloma. It could be understood since IDO does not act on lymphocytes but APC or dendritic cells.[19] TNF-α level in RPMI blood culture was significantly different in the normal group from those in the granuloma group (**Table 2**). Although the other cytokines were not significantly different, the levels of proinflammatory cytokines, TNF-α and IFN-γ, were consistently more significant in the granuloma group than the average group; therefore, the granuloma group with silicone antigen always have an inflammatory process.

With further notice, we found that regarding the levels of TNF-α, IFN-γ and IL-10 cytokines, there was a higher increase in the granuloma group than the standard control group. The increase in the regular group was only approximately eight folds for TNF-α, IL-10 PHA; but in contrast, in the granuloma group, the increase of TNF-α was up to 14 folds, for IL-10, it was approximately ten folds. IFN-γ in the normal group only had an increase of approximately four folds; however, it increased up to 39 folds in the granuloma group. It can be understood since PHA will stimulate lymphocytes and IFN-γ cytokines, notably produced by lymphocytes.[20,21] In silicone blood culture, the levels of IDO enzyme in the granuloma group, were different significantly from the normal group, i.e. the level of IDO enzyme in the normal group was higher than the enzyme in the granuloma group **(Table 2)**. It indicates that in patients with granuloma on their skin due to silicone injections, there are increased proinflammatory cytokines, which is not followed by an increase of anti-inflammatory cytokines.

The development of granuloma is affected by proinflammatory cytokines, i.e. TNF-α and body compensation (tolerance), carried out by IDO enzyme and IL-10 (an anti-inflammatory agent). The TNF-α/IL-10 and TNF-α/IDO ratios may show the balance of cytokines in granuloma and the function of Treg cells (in the blood specimens, they were not evaluated). It can be proven by comparing the levels of proinflammatory cytokines and anti-inflammatory cytokines, i.e. TNF-α/IL-10 and TNF-α/IDO ratios, which showed a significant difference between the standard and granuloma groups. The granuloma group had a higher level of ratio than the normal group **(Table 2).**

Although they were not significant, other cytokines could increase in the granuloma group but no increase in the normal group. It could be understood since there is a memory on lymphocytes of patients with granuloma who had received silicone injection. The developed memory does not occur on the silicone, but the protein tangled on the silicone. The issue has been discussed before, and it is consistent with the findings in the Anderson study.[22]

The potency of immune tolerance in the present study was evaluated based on anti-inflammatory mediators, i.e. IL-10 and IDO enzyme. The greater the ratio, the stronger the behaviour of immune cells producing inflammatory mediators or the weaker the capacity of immune cells that mediate immune tolerance. Results of the analysis are listed in **Table 1**, which shows that the TNF-α/IL10 ratio and TNF-α/IDO ratio, both in RPMI-, PHA- and silicone-stimulated cultures, nearly significantly higher in all subjects of the granuloma group compared to the control group. Such a result has answered the assumption and confirmed that individuals with granuloma due to silicone injections are subjects with immune cells expressing high proinflammatory cytokine levels; however, it is not followed by the adequate capacity to produce anti-inflammatory drugs cytokines when being stimulated by silicone.

Prediction analysis on the development of granuloma based on cytokines levels in the blood

By evaluating the occurring inflammation and comparing body capability to fight in the form of developing anti-inflammatory agent or tolerance capacity, which are represented by IL-10 and IDO, we can use TNF-α/IL-10 and TNF-α/ IDO ratios to predict the development of granuloma. The correlation of TNF-α/IL-10 and TNF-α/IDO with the duration of developing symptoms can be seen in **Table 3**. Results of calculation that were found could be used as prediction showed that of the blood specimen, they were TNF-α/IL-10 and TNF-α/IDO ratios

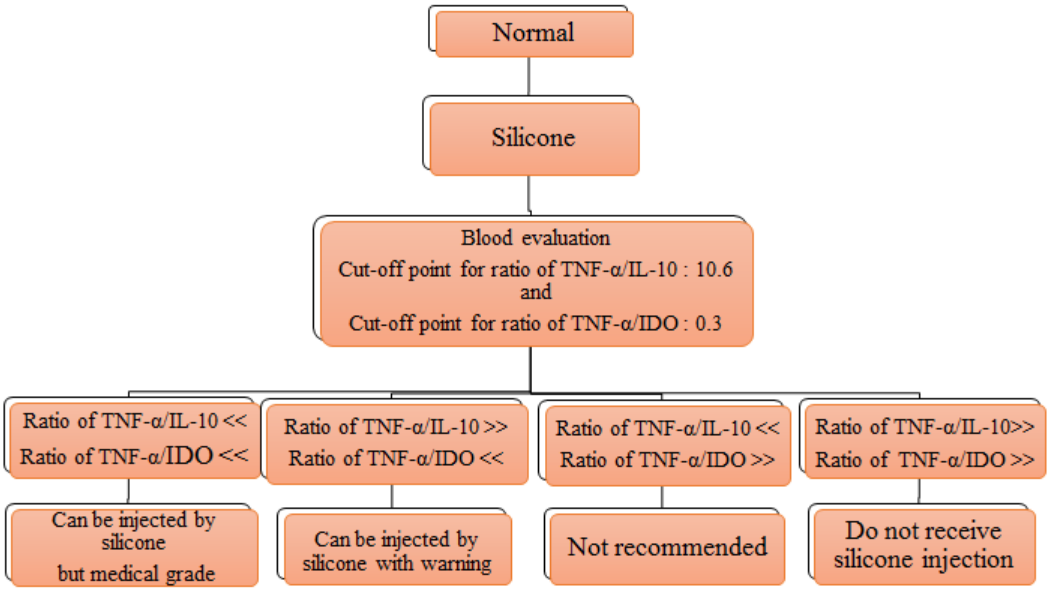

Figure 1 Procedural management for silicone injection

in the RPMI blood culture. All results showed inverse correlations, which means that the lower the ratio, the patients have more delayed or later visit medical service or the slower the development of granuloma symptoms. In **Table 2**, it is evident that there was a significant difference in silicone levels between the standard and granuloma groups. The level of TNF-α/IL10 of blood in the normal group was significantly different from those in the granuloma group. TNF-α/IDO of blood culture in the normal group was significantly different from those in the granuloma group. Based on those data, a cut-off could be made about the development of granuloma. The median cytokine level in RPMI blood culture in the granuloma group could be made as to the cut-off limit for predicting granuloma development. The cut-off of the TNF-α/IL10 ratio was 10.6, while for TNF-α/IDO was 0.3.

Based on the cut-off, if an average individual has low IDO secretion or a high level of TNF-α/ IL-10 and TNF-α/IDO ratios in his/ her blood, then the development of granuloma symptoms will occur faster when he/ she received silicone (Algorithm, **Figure 1**). Hence, even in the normal group, when they received silicone injection, granuloma will develop if they do not have a high capacity of anti-inflammatory properties or high TNF-α/IL-10 and TNF-α/IDO ratios. An algorithm of the plan of silicone injection for regular patients.

Conclusion

The level of proinflammatory cytokines tends to be higher in patients who experience granuloma due to silicone injection than patients without granuloma, while anti-inflammatory cytokine levels in the blood tend to be lower than in regular patients. The levels of TNF-α in the blood can be used as a predictor to assess the immune response to silicone injection. The ratios of TNF-α/IL-10 and TNF-α/IDO in the blood can be used as predictors for granuloma formation. The present study provides essential information on the immune response to the development of silicone granuloma, which certainly needs further studies on granuloma tissue.

Notes

Patients who want to receive silicone injection then recommended to do blood cultured test to observe TNF-α, IL-10 and IDO. Assessment is based on the ratio of TNF-α/IL-10 and TNF-α/IDO. If the result below the cut-off ratio, patients can receive silicone injection for medical therapy. If ratio of TNF-α/IL-10 and TNF-α/IDO are above the cut-off points, patients can not receive silicone injections. If ratio of TNF-α/IL-10 above the cut-off and TNF-α/IDO below the cut-off points, patients can receive silicone injection, but with precaution about granuloma formation. Patients with ratio of TNF-α /IL-10 under the cut-off point and TNF-α /IDO above the cut-off point, are not recommended to receive silicone injectio.

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