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Original Article

Enumeration of monocytes subsets using different gating methods by flow cytometry

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Abstract: Objective: To compare the efficiency of different gating methods for the enumeration of monocytes subsets using flow cytometry. Methods: Forty-eight healthy individuals received physical examination in our hospital was randomly included in this study. Peripheral blood monocytes subsets were analyzed by flow cytometry with FSC-SSC, CD45 and CD14 gating methods. Besides, the effects of different storage times on the test results were analyzed. Results: Statistical difference was noticed in the enumeration of Mon1 and Mon3 obtained using three different gating methods ($P<0.001$). The detection results of Mon2 by gating with FSC-SSC showed no significant difference compared with those obtained by CD45 or CD14 gating ($P>0.05$). The percentage of Mon2 determined using CD45 gating showed remarkable difference compared with that of CD14 gating. Correlation test showed that the three gating methods for Mon1, Mon2, Mon3 detection showed a significant positive correlation ($P<0.05$). For the effects of storage duration on the test results, significant increase was noticed in the percentage of Mon2 determined by FSC-SSC gating at 8 h compared with that of CD14 gating at 1 h. Nevertheless, remarkable decrease was identified in the Mon3 by FSC-SSC gating at 8 h compared with that of CD14 gating at 1 h ($P<0.05$). Significant difference was observed in the Mon1, Mon2 and Mon3 proportion in SSC^{low} cell populations at 8 h compared with those obtained at 4 h ($P<0.05$). For the samples stored at room temperature for 4 h, significant differences were identified in the Mon1 and Mon3 proportion in SSC^{low} compared with those of the SSC^{hi}. For the samples stored at room temperature for 8 h, significant differences were observed in the Mon1, Mon2 and Mon3 proportion in SSC^{low} compared with those of SSC^{hi}. Conclusion: FSC-SSC gating or CD45 gating was effective for the enumeration of Mon2. CD14 gating is more suitable for the enumeration of Mon1. As the extension of storage duration may affect the analysis of monocytes subsets, clinical samples for analysis should be tested as timely as possible.

Keywords: Flow cytometry, monocytes, CD14, CD45

Introduction

Monocytes, one of the major sources of the macrophages and dendritic cells in tissue, play important roles in the regulation of inflammation and inflammatory reactions [1, 2]. Circulating monocytes are divided into three subtypes according to the levels of CD14 and CD16 expression, including CD14++/CD16- (Mon1), CD14++/CD16+ (Mon2), and CD14+/CD16++ (Mon3) cells [3]. The expression of these subsets has been considered as an important evaluation criteria for the pathogenesis in various inflammatory reactions.

To date, accumulating evidence reveals circulating monocyte subsets as surrogate cellular biomarkers are closely related to the cardiovascular and cancer diseases. However, no well-acknowledged standards for the quantification of monocyte have been established [4-9], which precluded a routine monitoring and comparative interpretation of the clinical studies. In a previous study, Hristov et al [10] used flow cytometric protocol for enumeration of monocyte subsets in human blood, which proved the possibility of such technique for the quantification of circulating monocytes. Ever since that study, rare studies have been carried out to investigate the efficiency of enumeration of monocyte subsets using flow cytometric protocols. In this study, different gating methods were used to analyze mononuclear cells in peripheral blood of healthy human. Besides, a compara-

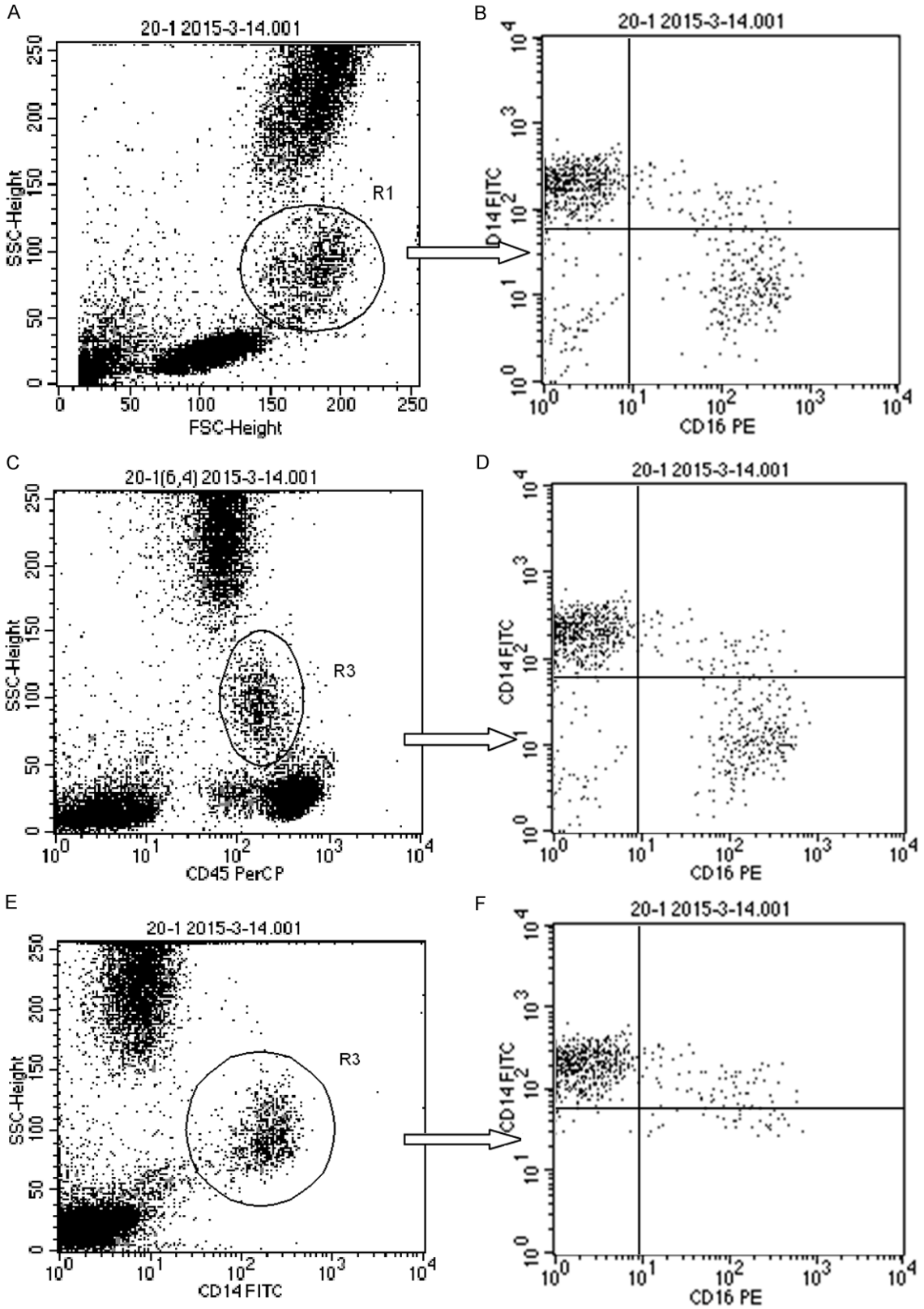

Figure 1. Analysis of different gating methods for the detection of monocytes subsets (A and B: FSC-SSC gating. C and D: CD45 gating. E and F: CD14 gating).

Table 1. Comparison of different gating methods of monocytes subsets in the literatures

Authors	Gating methods	Mon1 (%)	Mon2 (%)	Mon3 (%)
Hristov M [4]	FSC-SSC	85.4 (83.2-88.1)	4.1 (2.5-4.9)	7.5 (6.4-8.3)
Katarina E [5]	FSC-SSC	67 (59-72)	7.1 (5.1-9.8)	3.5 (2.6-4.9)
Krychtiuk KA [6]	CD45	82.1 ± 6.7	5.6 ± 3.3	12.3 ± 5.9
Chelombitko MA [7]	CD14 gating	74.8 ± 7.6	12.8 ± 3.4	
Tiziano Tallone [8]	HLA-DR gating	87.0 (78.5-95.9)	3.3 (1.1-7.5%)	5.8 (1.7-10.2)
Van Craenenbroeck AH [9]	FSC-SSC and CD86	88.09 ± 4.73	4.51 ± 2.05	7.39 ± 3.17

Table 2. Comparison of results by different gating methods of monocytes subsets

	Mon1 (%)	Mon2 (%)	Mon3 (%)
① FSC-SSC gating	69.92 ± 5.72	5.26 ± 3.29	12.22 ± 3.83
② CD45 gating	80.10 ± 5.28	4.19 ± 2.76	7.83 ± 3.58
③ CD14 gating	86.64 ± 4.66	6.62 ± 3.51	3.87 ± 2.10
Comparison	F=116.501 P<0.001	F=6.523 P=0.002	F=73.955 P<0.001
①&②	t=9.220 P<0.001	t=1.591 P=0.114	t=6.392 P<0.001
①&③	t=15.146 P<0.001	t=2.012 P=0.090	t=12.156 P<0.001
②&③	t=5.925 P<0.001	t=3.604 P=0.001	t=5.764 P<0.001

tive study was performed to the results of different monocytes subsets, based on which to identify the potential the factors affecting the test results.

Materials and methods

Subjects and sample collection

Forty-eight healthy subjects received physical examination in our hospital (male: 25, female: 23, mean age: 35.4 ± 8.3 years) were randomly included in this study. Written informed consent was obtained from each subject. The study protocols were approved by the Ethical Committee of Taizhou People's Hospital. After inclusion, fasting peripheral venous blood (2 ml) was collected from each subject using EDTA-K2 as anticoagulant.

Staining of samples

The samples were divided into two groups including: Group A, which was added with CD45-PerCP (BD Bioscience Company, #347464) and CD14-FITC (BD Bioscience Company, #347493), PE Mouse Anti-Human CD16 (BD Bioscience Company, #347617); and Group B (isotype control), which was added with CD45-PerCP and Mouse IgG2a (BD Bioscience Company, #349051), as well as Mouse IgG1-PE (BD Bioscience Company, #349043). Subsequently, the mixture abovementioned was added with 50 μl blood, and was kept in the dark conditions after votexing for 15 minutes. Afterwards, 2 ml hemolysin was added followed by keeping in the dark for 10 min after votexing. The mixture was centrifugated at 1,500 rpm for 5 min, and then the supernatant was discarded. After washing with 2 ml PBS, the cell was finally resuspended in 100 μl PBS for the further analysis.

Flow cytometry

SSC was set as the y-coordinate, and FSC, CD45 and CD14 were set as the x-coordinate, respectively. Data of monocyte subsets were obtained on three types of charts (**Figure 1**), and 2000 mononuclear cells were obtained by CELL Quest software. On this basis, the proportion of the Mon1, Mon2 and Mon3 was analyzed.

Statistical analysis

Data were expressed as mean ± standard deviation. Student's t-test or One-Way ANOVA test was used for the inter-group comparison. The potential correlation between two variants was analyzed using Pearson's correlation coefficient. All the statistical analysis in this study was performed using the Sigma Plot 12.0 software. P<0.05 was considered statistically significant.

Results

Comparison of different gating methods of monocytes subsets in the literatures

To date, few gating methods are available for the flow cytometric methods, including FSC-

Table 3. The correlation analysis of different gating methods to detect different monocytes subsets

	Mon1		Mon2		Mon3	
	CD45 gating	CD14 gating	CD45 gating	CD14 gating	CD45 gating	CD14 gating
FSC-SSC gating	r=0.447	r=0.589	r=0.902	r=0.976	r=0.537	r=0.520
	P=0.00210	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001
CD45 gating		r=0.632		r=0.876		r=0.428
		P<0.001		P<0.001		P<0.001

SSC gating, CD45 gating and CD14 gating, respectively. As revealed in **Table 1**, large variances were noticed in these methods.

Comparison of monocytes subsets percentage using different gating methods

Among the three gating methods used in this study, statistical difference was observed in the percentage of Mon1 and Mon3 (P<0.001, **Table 2**), respectively. The percentage of Mon2 determined using CD45 gating showed remarkable difference compared with that of CD14 gating. However, no significant difference was noticed in the percentage of Mon2 determined by FSC-SSC gating compared with those obtained using CD45 gating or CD14 gating (P>0.05).

Correlation analysis of different gating methods

In this study, correlation analysis was performed to analyze the potential correlation between the gating methods. As revealed in **Table 3**, a strong positive correlation was observed in the three gating methods for Mon1, Mon2, and Mon3, respectively (P<0.05).

Effects of storage times on the detection of monocytes subsets

Blood samples from 10 cases were randomly selected, and placed at room temperature for 1 h, 4 h and 8 h, respectively. Significant increase was noticed in the percentage of Mon2 determined by FSC-SSC gating at 8 h compared with that of CD14 gating at 1 h. Nevertheless, remarkable decrease was identified in the Mon3 by FSC-SSC gating at 8 h compared with that of CD14 gating at 1 h (P<0.05, **Figure 2**).

Percentage of monocytes subsets at different time points by FSC-SSC gating

The monocytes' signals on SSC direction decreased with the extension of storage time at room temperature (**Figure 3**). With the extension of storage duration, the monocytes were gradually clustered into higher SSC detection signal group (SSC^{hi}) and lower SSC detection signal group (SSC^{low}). Significant difference was observed in the Mon1, Mon2 and Mon3 proportion in SSC^{low} cell populations at 8 h compared with those obtained at 4 h (P<0.05). Nevertheless, no statistical difference was noted in the proportion in SSC^{hi} cell populations at 8 h compared with those obtained at 4 h (P>0.05) at room temperature. For the samples stored at room temperature for 4 h, significant differences were identified in the Mon1 and Mon3 proportion in SSC^{low} compared with those of the SSC^{hi} (t=17.946, P<0.001; t=12.177, P<0.001). For the samples stored at room temperature for 8 h, significant differences were observed in the Mon1, Mon2 and Mon3 proportion in SSC^{low} compared with those of SSC^{hi} (t=8.278, P<0.001; t=2.864, P=0.019; t=7.323, P<0.001, **Table 4**).

Monocytes subsets at different storage time by CD45 gating

Monocytes on SSC direction weakened with the extension of storage time at room temperature by CD45 gating (**Figure 4**). The boundaries of lymphocytes and monocytes on SSC direction were inconspicuous for samples stored for 8 h.

Discussions

The quantification of circulating monocyte is still a challenge. To date, rare flow cytometric protocols have been established for the enumeration of monocyte subsets in peripheral blood in human. In this study, a flow cytometry-based method was established to determine the percentage of the monocyte subsets in the peripheral blood. Besides, we identified the potential the factors affecting the determination of monocyte percentage.

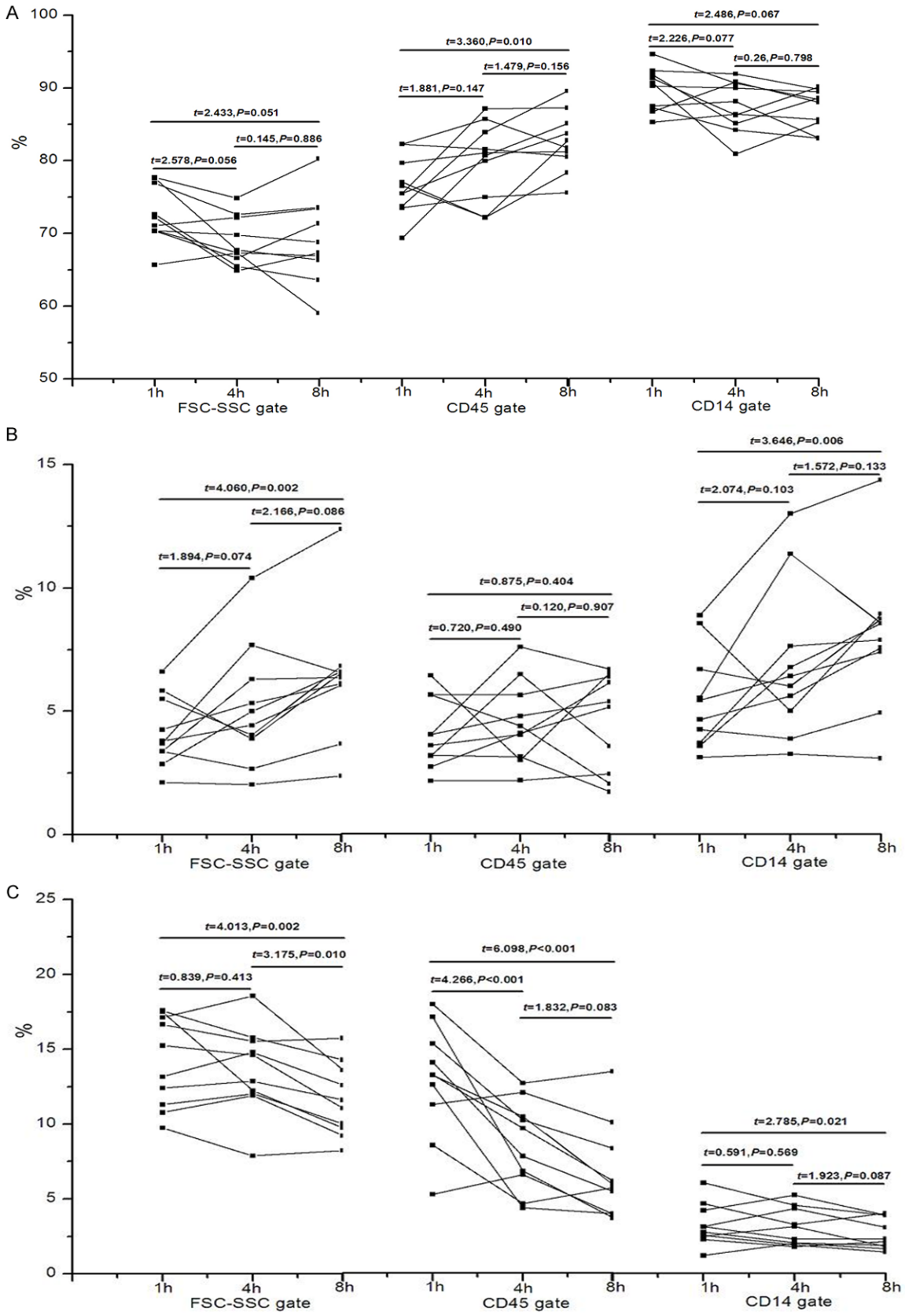

Figure 2. Effect of different storage times on the detection of Mon1 by different gating methods. A: Mon1. B: Mon2. C: Mon3.

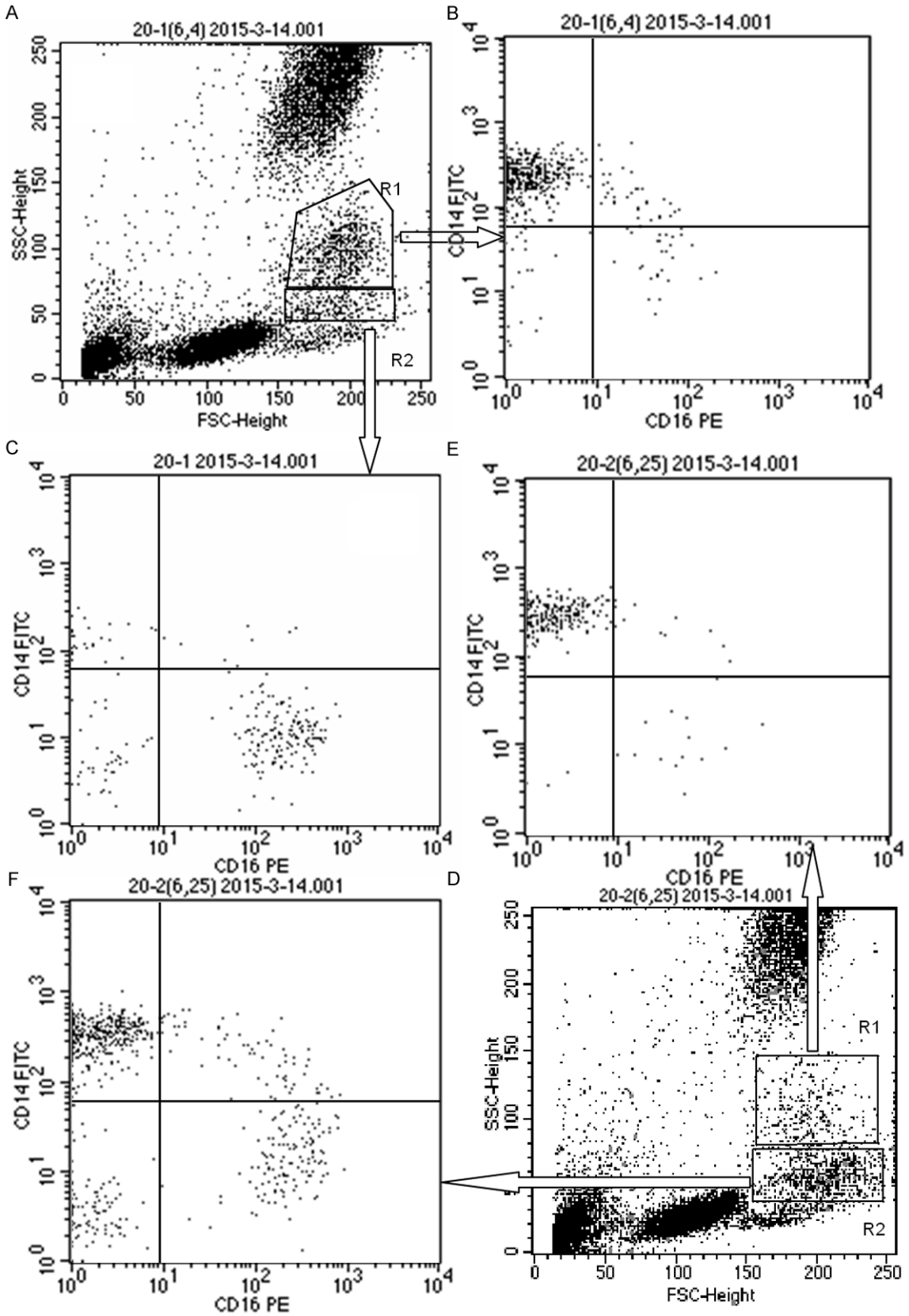

Figure 3. The expression of monocytes subsets of samples stored for different time (R1: SSC^{hi}, R2: SSC^{dim}) (A-C: The expression of monocytes subsets of samples stored for 3 hours; D-F: The expression of monocytes subsets of samples stored for 6 hours).

Table 4. The detection of SSC^{hi}, SSC^{low} for different storage time of samples

		4 h	8 h	*t*	*P*
SSC^{hi}/SSC^{low}		2.52 ± 0.83	1.55 ± 1.07	3.428	0.008
SSC^{hi}	Mon1 (%)	86.79 ± 3.81	87.17 ± 3.92	0.354	0.731
	Mon2 (%)	4.11 ± 1.57	3.54 ± 1.42	0.949	0.368
	Mon3 (%)	5.66 ± 2.95	5.79 ± 3.25	0.324	0.753
SSC^{low}	Mon1 (%)	24.19 ± 12.76	41.96 ± 18.49	3.566	0.006
	Mon2 (%)	3.88 ± 2.69	6.73 ± 2.70	8.427	<0.001
	Mon3 (%)	32.74 ± 7.57	22.08 ± 10.18	3.312	0.009

Mon1 cells, expressing CD163+, CCR2+, CX3CR1+, CD62L+, CD115+, CCR5+, and CD11b++, are responsible for phagocytosis and proteolysis, as well as the secretion of pro-inflammatory cytokines through differentiating into M1 macrophages [10, 11]. Mon2 cells, expressing CD163++, CCR2+, CX3CR1++, CD62L-, CD115+, CCR5+, CD11b++, are closely involved in the generation of IL-10, haem oxygenase-1 (HO-1) and other immunoregulatory factors [12, 13]. In addition, these cells can differentiate into the anti-inflammatory M2 macrophages and Mon1 cells. Upon the stimulation by inflammation, Mon2 cells can convert to Mon1 cells [14]. Mon3 is mainly localized at the surface of endothelial cells in normal tissues and express CD163+, CCR2-, CX3CR1+++, CD11b+, CD62L-, $VCAM^{high}$, and $CD64^{low}$.

In recent years, extensive studies have been conducted to determine the monocyte subsets using gating methods such as FSC-SSC gating [4], CD45 gating [6] and CD14 gating [15] methods. According to the previous studies, the proportion of Mon1 was 67% (59-72%) by FSC-SSC gating [5], and 82.1 ± 6.7(%) by CD45 gating [6]. The proportion of Mon2 was 4.1% (2.5-4.9%) by FSC-SSC gating [4], and 12.8 ± 3.4 (%) by CD14 gating [7]. The proportion of Mon3 was 3.5% (2.6-4.9%) by FSC-SSC gating [5], and 12.3 ± 5.9 (%) by CD45 gating [6]. However, no consensus has achieved on these studies. For example, the incidence of coronary events was associated with the remarkable increase of Mon1 by FSC-SSC gating method [5]. Whereas, using CD14 gating method, Mon1 cells were significantly lower in patients with coronary atherosclerosis than those of the healthy controls [7]. Our results revealed no significant difference for the determination of Mon2 by FSC-SSC gating compared with CD45 gating or CD14 gating, and the correlation coefficient was 0.902 (P<0.001) and 0.976 (P<0.001), respectively. Nevertheless, the percentages of Mon1 and Mon3 obtained by FSC-SSC gating were positively correlated with those of CD45 and CD14 gating (P<0.05). This raises concerns for the selection of appropriate method for the percentage evaluation of Mon1 or Mon3. As the cellular debris was effectively removed by CD14 gating, it may be effective for Mon1. In our study, the results of Mon1 were similar to the previous reports [4, 8, 9]. However, the CD14dim cells may be neglected by CD14 gating. Thus, it is recommended to use FSC-SSC gating or CD45 gating rather than the CD14 gating method for the determination of Mon3.

In this study, we also detected the effects of sample storage duration on the percentage of monocytes subsets obtained using different gating methods. The percentage of Mon2 was elevated and that of the Mon3 was decreased at 8 h using FSC-SSC gating or CD14 gating method at room temperature compared with those at 1 h. Besides, elevation of Mon1 percentage and decrease of Mon3 percentage was observed by CD45 gating with the extension of storage duration. For the reason, monocytes subsets represent different developmental stages of differentiation [16]. Therefore, with the extension of storage duration, part of Mon1 transformed into Mon2, and part of Mon2 transformed into Mon3, and part of Mon3 subject to necrosis gradually. Finally, the proportion of Mon2 was increased, while the proportion of Mon3 was declined. Furthermore, we also observed changes in cell morphology. Decrease of SSC signals and increase of FSC signals were identified for the samples stored at room temperature for 4 h. At 8 h, SSC^{hi}/SSC^{low} dropped significantly. Part of the cells in SSC^{loww} overlapped with lymphocyte on SSC direction (**Figure 4**). The results indicated the Mon3 cell count in SSC^{hi} was significantly lower than that in SSC^{low}. This may result in partial monocytes transporting to lymphocyte population by CD45 gating. Finally, the proportion of Mon3 was declined as vast majority of Mon3 cells were localized in the monocytes (**Figure**

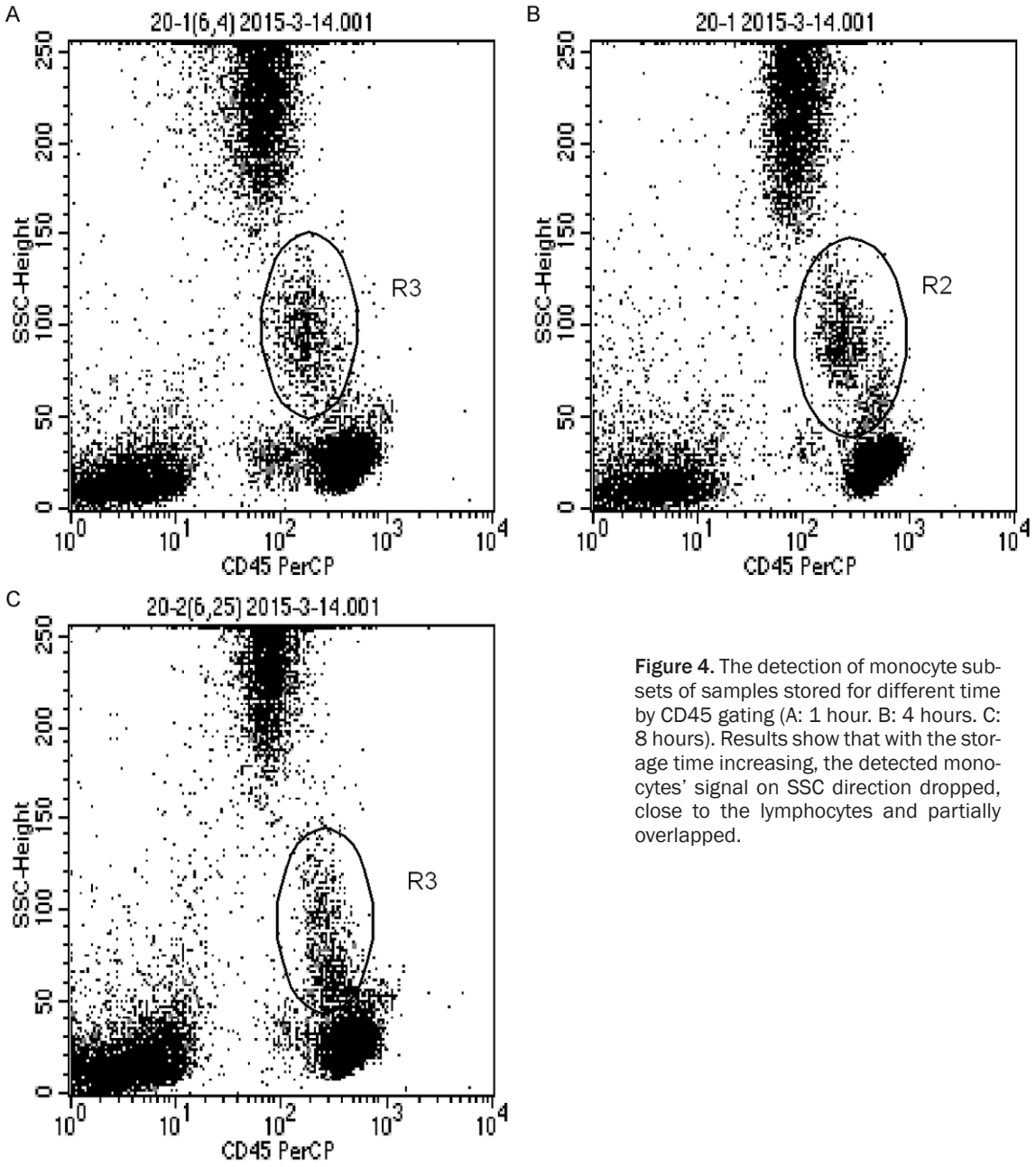

Figure 4. The detection of monocyte subsets of samples stored for different time by CD45 gating (A: 1 hour. B: 4 hours. C: 8 hours). Results show that with the storage time increasing, the detected monocytes' signal on SSC direction dropped, close to the lymphocytes and partially overlapped.

3). Apparently, the storage duration of samples after collection affected the detection of monocytes subsets. Therefore, clinical samples for analysis should be tested as timely as possible.

In conclusion, FSC-SSC gating or CD45 gating was effective for the enumeration of Mon2. CD14 gating is more suitable for the enumeration of Mon1. Extension of storage duration may affect the analysis of monocytes subsets. Therefore, clinical samples for analysis should be tested as timely as possible.

Disclosure of conflict of interest

None.

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