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CD16$^+$ Monocyte Subsets in Patients with Total Joint Arthroplasty

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Abstract

Objective: There are two monocyte populations in human blood: CD14$^+$CD16$^-$ classical monocytes and CD14$^+$CD16$^+$ inflammatory monocytes. CD14$^+$CD16$^+$ inflammatory monocytes, account for approximately 10% of the total monocytes, may be expanded in various types of inflammatory conditions. The purpose of this study was to investigate whether the expansion of the CD14$^+$CD16$^+$ monocyte population represents a risk factor of aseptic loosening (AL). **Methods:** Peripheral monocytes subsets were measured in revision patients with AL (n = 35) and in patients with stable implants (SI, n = 56). The gene profiles of TNFα, IL-1β, CD16, CD68 and TRAP5B from collected loosening periprosthetic tissues were analyzed. **Results:** There were no significant differences in the CD14$^+$CD16$^+$ monocyte populations between the SI and AL patients. The CD14$^+$CD16$^+$ monocytes were marginally higher in revision patients with osteolysis (n = 30), compared to patients without osteolysis (n = 5) though no statistically difference was found. There was an association between the CD14$^+$CD16$^+$ monocyte subpopulation and the tissue gene profiles, including IL-1β ($p = 0.063$), CD68 ($p = 0.036$), and TRAP5B ($p = 0.073$). **Conclusion:** It was demonstrated that the expansion of CD14$^+$CD16$^+$ monocytes reflects, to some extent, the inflammatory status of the loosening periprosthetic tissues. It is unclear if some of those SI patients (no pain and negative radiograph) who have a higher frequency of CD14$^+$CD16$^+$ monocytes may be at the early stage of AL. Further evaluation of CD14$^+$CD16$^+$ monocyte population, independently or combined with other factors, will be useful to design a risk profile for AL incidence and progression.

Keywords

Osteolysis, Wear Debris, CD14$^+$CD16$^+$ Monocytes, Aseptic Loosening, AL, Flow Cytometry

1. Introduction

More than 500,000 total joint arthroplasty (TJA) operations are performed annually in the USA [1] [2]. Despite the technical advances, many patients require surgical revision for aseptic loosening (AL) and/or bone loss due to osteolysis [3] [4]. The etiology of osteolysis/AL is multi-factorial [5] [6] [7] and associated with the phagocytosis of wear debris particles by macrophages. Activated macrophages secrete pro-inflammatory cytokines that stimulate periprosthetic tissue inflammation and osteolysis [5] [8]. It is unclear why some patients develop AL/osteolysis while others do not. It is not currently possible to identify patients who are at risk of AL progression at a stage where the "end result" can be reversed [9] [10]. Therefore, the development of biomarkers that could predict or assess the risk of early implant failure and bone loss is clinically important [11]. This is especially true since all patients have some periprosthetic inflammatory changes after surgery.

Human monocytes develop in the bone marrow from common myeloid progenitor cells and are released to the peripheral circulation as non-dividing cells [12]. Newly released monocytes circulate for 1 - 3 days before entering tissues to differentiate into mature resident macrophages. Under inflammatory conditions the monocyte production within the bone marrow is increased. After being released into the circulation, the monocytes are rapidly recruited to site of injury or foreign body deposits and differentiate into inflammatory macrophages [13]. Monocytes also have a role in bone remodeling, as a putative precursor for osteoclasts [14].

There are two distinct populations of peripheral monocytes: the "typical" $CD14^{++}CD16^{-}$ cells and "activated" $CD16^{+}$ cells that consist of $CD14^{+}CD16^{++}$ and $CD14^{++}CD16^{+}$ subpopulations. $CD16^{+}$ monocytes are inflammatory. In contrast to conventional $CD14^{++}CD16^{-}$ monocytes, $CD16^{+}$ monocytes only represent for ~10% of circulating monocytes in healthy individuals [15] [16] and possess several features present in inflammatory tissue macrophages [17], notably, higher expression of major histocompatibility complex class II antigens (MHC II) [16] [18]. The $CD16^{+}$ monocytes are also a major source of a panel of proinflammatory cytokines such as tumor necrosis factor alpha (TNFα) and interleukin 1-beta (IL-1β) [17] [18]. The activated $CD16^{+}$ monocytes can rapidly migrate to a site of inflammation where they readily mature into inflammatory macrophages [15] [19]. As such, these cells are felt to be a very important part of the periprosthetic inflammation cascade.

Randolph *et al.* [20] showed that peripheral $CD16^{+}$ monocytes are increased in rheumatoid arthritis (RA) and that the increase is associated with RA disease activity. $CD16^{+}$ macrophages have been detected in RA synovial tissues, most densely in the lining layer [21], where proinflammatory cytokines are abundantly produced [22]. Peripheral monocytes from AL patients are activated before entry into the periprosthetic tissues and share phenotypic characteristics with macrophages isolated from harvested loosening periprosthetic tissue [23]. How-

ever, little is known regarding the relationship between the profiles of peripheral monocytes and the risk of developing AL/osteolysis in TJA patients. Analysis of the periprosthetic tissues from AL cases with osteolysis has demonstrated a predominance of macrophages that comprise 60% - 80% of the entire cellular population [24]. The activated macrophages play a critical role in the pathology of AL [25] through a multitude of biologic functions including production of proinflammatory cytokines (TNFα, IL-1, RANKL, etc.) [26]-[31] and differentiation of lacunar bone resorbing cells [24] [32].

With an understanding that peripheral monocytes share characteristics with macrophages isolated from the periprosthetic tissue in revision AL cases [28], the aim of this study was to determine whether the expansion of the $CD16^+$ monocyte population and/or its subpopulations represents a risk factor in predicting implant failure and AL progression.

2. Materials and Methods

2.1. Human Subjects

Between 2006 and 2010, 91 patients, 56 patients with stable implants (SI) and 35 patients undergoing revision surgery due to AL, were recruited during routine clinical follow-up and when patients were scheduled for revision surgery. The clinical study was approved by the hospital's Institutional Review Board. Informed consent was obtained from all subjects. The inclusion criteria for AL included: 1) positive physical findings of AL; 2) radiographic evidence of implant loosening with or without associated osteolysis [8]; 3) a diagnosis of osteoarthritis leading to the performance of the index joint replacement; 4) no current ingestion of NSAIDS within two weeks, and 5) Preoperative and intraoperative evaluation that did not indicate a presence of infection. We excluded patients with coexisting diseases that may interfere with analysis of the peripheral monocyte populations, including rheumatoid arthritis [33], hemodialysis [34], Crohn's disease [35], sarcoidosis [36] and/or infectious diseases [37]. Exclusion criteria also included periprosthetic infection by intra-operative observation and bacterial culture of joint fluids and periprosthetic tissues. The diagnosis of AL in all patients was confirmed at revision surgery. For patients undergoing revision (total hips 17 and total knees 18) the presence or absence of osteolysis was evaluated based on the pre-operative radiographs. Patients with SI were symptom free and had radiographs demonstrating stable implants. The demographic details of these patients are given in **Table 1**.

2.2. Flow Cytometry to Quantify Monocyte Populations Based on CD14 and CD16 Expression

Whole blood staining by two-color flow cytometry was used to quantitatively measure the fraction of monocytes expressing CD14 and/or CD16 [20]. Briefly, blood was collected in heparinized tubes (Becton Dickinson, Franklin Lakes, NJ); 100 µl of undiluted blood were labeled with 20 µl each of fluorescein isothi-

Table 1. Baseline values of recruited patients.

	Stable implant (n = 56)	Revision patient (n = 35)	*p* value
Age (mean ± SD, years)	68.5 ± 11.0	70.5 ± 10.4	0.519*
Male/Female (n/n)	20/36	15/20	0.646**

*This was calculated using the Student's *t*-test. No significant difference was observed between groups. **This was calculated using the Chi-Squared test. Using Fisher Exact (which is less stringent) there is still no significant difference between the proportion of males and females in each group ($P = 0.515$).

ocyanate (FITC)-conjugated anti-CD14 monoclonal antibody (mAb) (Leu M3; Becton Dickinson) and phycoerythrin (PE)-conjugated anti-CD16 mAb (Leu-11; Becton Dickinson) for 30 minutes at 4˚C in the dark. Red blood cells were lysed with 2.0 ml of FACS Lysing Solution (Becton Dickinson) for 10-minute incubation at 4˚C in the dark. After one wash with cold PBS, the cells were resuspended in 400 µl of PBS with 2% fetal calf serum (FCS). Cells were collected on a FACScan flow cytometer (Becton Dickinson). Analysis of the cell surface CD14 and CD16 expression was performed using Windows Multiple Document Interface flow cytometry application (version 2.8, http://facs.scripps.edu). To determine the percentage of the $CD14^{+}CD16^{+}$ cells, a monocyte gate was created from the forward scatter versus side scatter profile to limit the analysis to monocytes. However, other non-monocytic cells could have remained as part of the monocyte gate. Therefore, these cells were eliminated from the analysis by using separate gates from the side scatter versus PE CD16 profile; these gates removed $CD16^{+}$ polymorphonuclear cells, natural killer cells and eosinophils from the analysis. The fractions of $CD14^{++}CD16^{-}$, $CD14^{++}CD16^{+}$, $CD14^{+}CD16^{++}$ and total $CD16^{+}$ monocytes were calculated from the total monocyte population.

2.3. Blood Tests

The number of white blood cells, erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) were measured. ESR is a common hematology test and a non-specific measure of inflammation. CRP is a protein found in the blood and its levels rise in response to inflammation.

2.4. Gene Profiles of the Periprosthetic Tissue

At the time of the revision surgery, periprosthetic tissue was collected from patients and stored in a −80˚C freezer. The total RNA from periprosthetic tissue homogenates was reverse transcribed to cDNA as described previously [38] [39]. Real time quantitative PCR was carried out with Taqman primers and probes (Part # 4331182) according to the manufacturer's instructions (Applied Biosystems, Foster City, CA). Lot numbers used in this study were as follows: for proinflammatory cytokines, CD16 (FCGR3B): HS00275547_m1; IL-1*β*: HS01555410_m1; and TNFα: HS00174128_m1; and for bone resorbing or osteoclastic markers, CD68: HS00154355_m1; and TRAP5B (ACR5), HS00356261_m1. To standardize the target gene level with respect to variations in RNA and cDNA, the housekeeping gene GAPDH was used as an endogenous

control (lot number: HS99999905_m1). To determine the relative level of gene expression, the comparative threshold cycle (CT) method with arithmetic formula was used as we described previously [39] [40].

2.5. Statistical Analysis

Data were summarized by group and compared by Student's *t* test using the SPSS software package (version 17.0; Chicago, IL). Data were expressed as mean ± standard deviation (SD). Group mean values were compared using the Mann-Whitney U test for non-parametric data. The Spearman's test was used for correlation analysis, with *P* values less than 0.05 considered to be statistically significant. Proportions of observations were examined with the Chi-square test. *P* values less than 0.05 were considered to be statistically significant.

3. Results

3.1. Subject Characteristics

The patients' demographic data demonstrated a good match of age and sex between revision patients and patients with stable implants. As shown in Table 1, no differences were found between these two groups regarding their age or sex.

3.2. Comparison of Frequency of $CD14^+$ and/or $CD16^+$ Blood Monocytes between Stable and Revision Patients

The analysis of peripheral blood monocytes in whole blood was restricted to monocytes on the basis of forward scatter and side scatter gating. As shown in Figure 1, most of the monocytes expressed CD14 intensely and did not express CD16 ($CD14^{++}CD16^-$). For the $CD16^+$ monocytes, two distinct populations of monocytes co-express CD16 and varying intensities of CD14: $CD14^+CD16^{++}$ (R12) and $CD14^{++}CD16^+$ (R13) monocytes.

The frequencies of monocytes expressing CD14 and/or CD16 in blood samples from 35 revision patients and 33 patients with stable implants were determined by flow cytometry. As shown in Figure 2, no significant differences in $CD14^+CD16^{++}$, $CD14^{++}CD16^+$ or total $CD16^+$ cell populations were detected between stable and revision patients. We noticed that the frequency of total $CD16^+$ cells, due to differences in the $CD14^+CD16^{++}$ population, was significantly higher for female patients than for male patients in the stable implants group (Figure 3(a)). This difference was not significant in the revision group (Figure 3(b)).

3.3. Relationship between $CD16^+$ Monocytes and Patient Demographics and Blood Inflammatory Markers

As shown in Table 2, we did not find an association between the percentage of total $CD16^+$ monocytes and its subpopulations with patient age. Our results did not show a significant relationship between $CD16^+$ monocytes and the blood markers of inflammation (ESR, CRP), or white blood cell counts (Table 2).

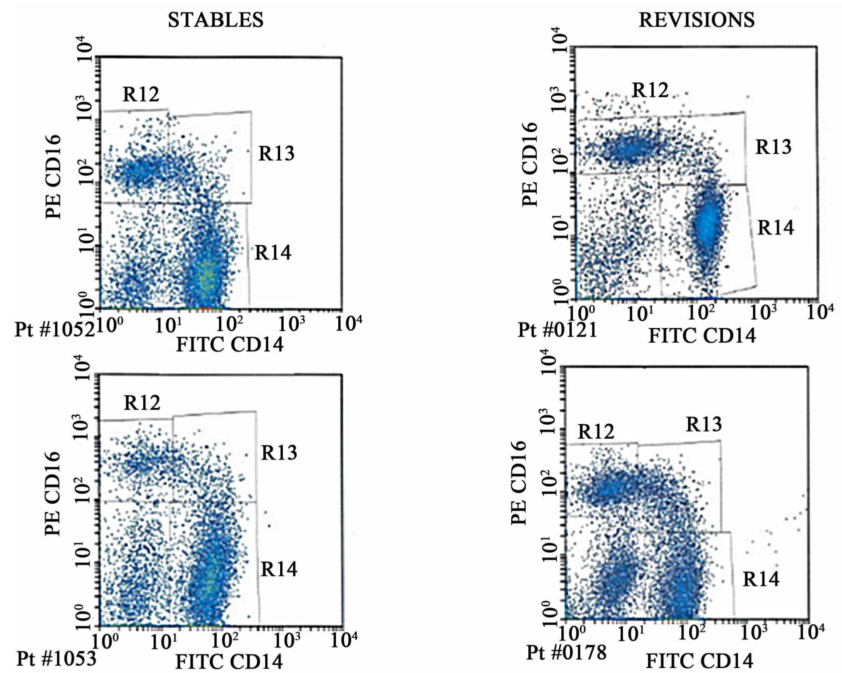

Figure 1. Flow cytometric determination of CD14^{+}CD16^{+} blood monocytes. Whole blood samples were stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD14 antibody and phycoerythrin (PE)-conjugated anti-CD16 antibody, followed by lysis of erythrocytes. Cells were collected on a Becton Dickinson FACScan flow cytometer. Flow cytometric analysis was done using the WinMDI software (version 2.8, http://facs.scripps.edu). A monocyte gate was created from the forward scatter vs. side scatter profile to limit the analysis to monocytes. However, other non-monocytic cells remained as part of the monocyte gate. Therefore, these cells were eliminated from the analysis by using separate gates from the side scatter vs. phycoerythrin CD16 profile; these gates removed CD16^{+} polymorphonuclear cells, natural killer cells and eosinophils from the analysis. This figure shows the gated regions of CD14^{+}CD16^{+}, CD14^{++}CD16^{+} and CD14^{++}CD16^{-} cell populations in 2 representative patients with stable or revised implants (*i.e.*, AL patients).

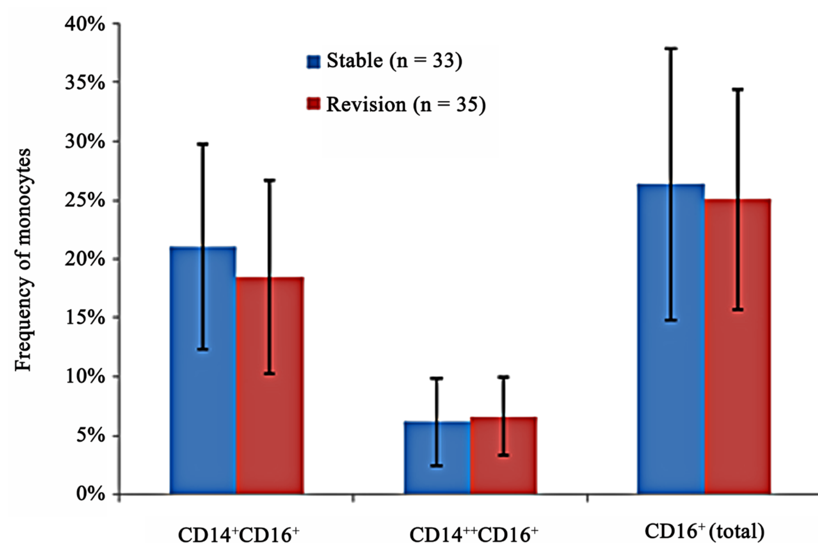

Figure 2. Comparison of frequencies of CD14^{+}CD16^{+} and CD16^{+} monocytes between stable and revision patients.

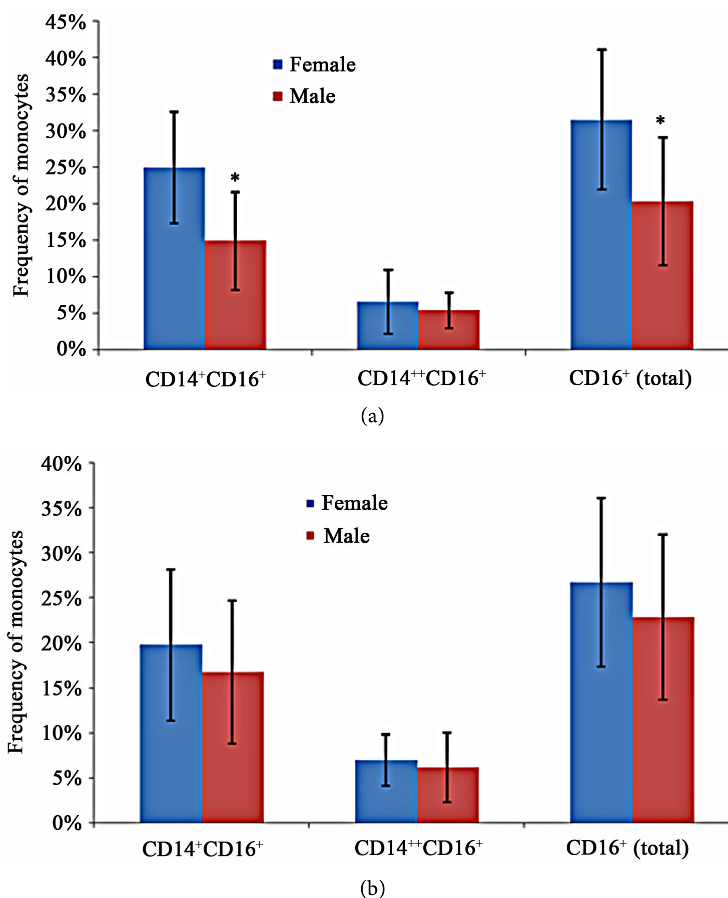

Figure 3. Female patients have a higher frequency of $CD16^+$ monocytes than male patients. (a) Stable patients, female (n = 20) vs. male (n = 13); (b) Revision patients, female (n = 20) vs. male (n = 13). $^*p < 0.01$.

Table 2. Correlation coefficient analysis between $CD16^+$ monocytes and patient demographics and blood markers. The correlation coefficient (r) analysis was done by SPSS bivariate correlation (Pearson) analysis.

	n	*r*	*P value*
With $CD14^+CD16^{++}$ monocytes			
Age	35	0.01	0.953
Blood white blood cell counts	22	0.092	0.691
Erythrocyte sedimentation rate (ESR)	20	0.03	0.900
C-reactive protein (CRP)	19	0.096	0.695
With $CD14^{++}CD16^+$ monocytes			
Age	35	0.093	0.594
Blood white blood cell counts	22	0.252	0.271
ESR	20	0.115	0.630
CRP	19	0.031	0.900
With $CD16^+$ monocytes (total)			
Age	35	0.024	0.891
Blood white blood cell counts	22	0.022	0.926
ESR	20	0.029	0.904
CRP	19	0.064	0.796

3.4. Comparison of Frequency of CD16+ Monocytes in Revision Patients with and without Osteolysis

As shown in Figure 4, the frequencies of $CD14^+CD16^{++}$ monocytes and total $CD16^+$ monocytes were slightly higher in patients with osteolysis (n = 30), compared to patients without osteolysis (n = 5), though no statistically significant difference was found. No statistically significant difference was found for the $CD14^{++}CD16^+$ monocytes between patients with and without osteolysis.

3.5. Association of Periprosthetict Issue Gene Profiles and CD16+ Monocytes

We investigated the interplay between the frequency of total $CD16^+$ monocytes and its subpopulations and the gene profiles of the periprosthetic tissues from revision patients. As shown in Table 3, the frequency of $CD14^+CD16^{++}$ monocytes had a weak correlation with gene expression levels of IL-1 ($p = 0.063$) and TRAP5B ($p = 0.073$), but a stronger correlation with expression of the macrophage gene CD68 ($p = 0.036$). When the percentages of total $CD16^+$ monocytes were examined, a weak correlation with *CD*68 ($p = 0.069$) and a stronger correlation with *IL*-1 expression levels ($p = 0.037$) were observed.

Table 3. Regression analysis of tissue gene profiles and $CD16^+$ monocytes in AL patients.

	n	*r*	*P value*
With $CD14^+CD16^+$ *monocytes*			
IL-1	17	0.377	0.063
TNF	17	0.153	0.465
CD68	17	0.42	0.036*
CD16	17	0.135	0.521
TRAP5B	17	0.365	0.073
With $CD14^{++}CD16^+$ *monocytes*			
IL-1	17	0.305	0.138
TNF	17	0.01	0.96
CD68	17	0.036	0.863
CD16	17	0.246	0.237
TRAP5B	17	0.04	0.62
With $CD16^+$ *monocytes* (*total*)			
IL-1	17	0.42	0.037*
TNF	17	0.127	0.546
CD68	17	0.369	0.069
CD16	17	0.035	0.865
TRAP5B	17	0.276	0.182

*Statistically significant.

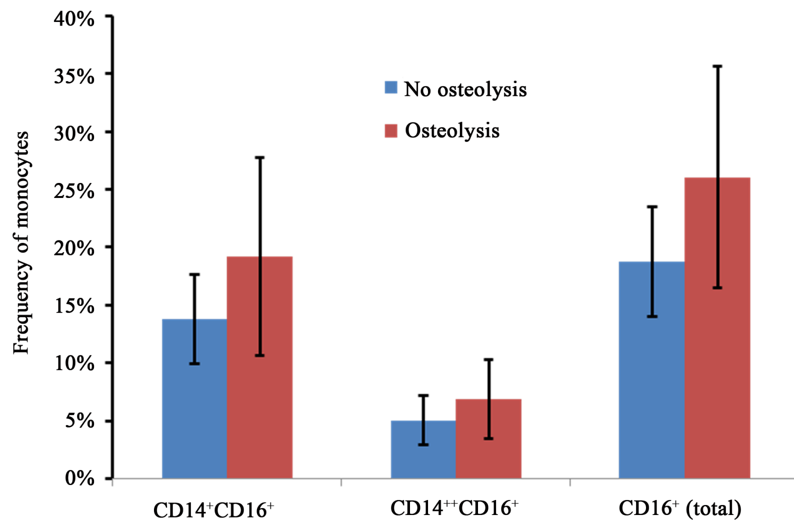

Figure 4. Comparison of frequencies of $CD14^{+}CD16^{+}$, $CD14^{++}CD16^{+}$ and $CD16^{+}$ monocytes between revision patients with (n = 30) and without (n = 5) osteolysis.

3.6. Regression Analysis of Tissue Gene Profiles and Blood Inflammatory Markers

We compared the periprosthetic gene profiles with whole blood white blood cells, ESR and CRP from revision patients. As shown in Table 4, no significant association was shown between white blood cell numbers and either ESR or CRP levels. However, while a weak association was seen between the number of white blood cells and the *CD*68 expression ($p = 0.06$), a statistically significant association was observed between the number of white blood cells and CD16 expression ($p = 0.023$).

4. Discussion

It is projected that by 2030, the number of primary TJAs in USA will grow to ~4.0 million procedures per year [3]. The public health burden of this high-volume procedure and overall societal costs are, therefore, likely to increase substantially and consistently for the foreseeable future [41]. In most cases, wear debris-induced inflammation resolves completely and without permanent injury. However, these inflammatory processes frequently become persistent, chronic, and destructive [42]. Analysis of loosening periprosthetic membranes has demonstrated an abundance of macrophages and multinucleated giant cells that comprise 60% - 80% of the entire cellular population [24] [43]. Activated macrophages play a critical role in the pathology of AL [25] through a multitude of biologic functions, including production of proinflammatory cytokines (TNFα, IL-1β, TRAP, etc.) [26]-[31] and differentiation into bone resorbing cells [24] [32] [44]. Development of simple and reliable biomarkers to identify those patients who are at high risks for developing AL is important for the early therapeutic intervention to potentially extend the longevity of the prosthesis [9]. Our data demonstrated that the population of inflammatory $CD14^{+}CD16^{+}$ mo-

Table 4. Regression analysis of tissue gene profiles and blood inflammatory markers in revision patients. The correlation coefficient (r) analysis was done by SPSS bivariate correlation (Pearson) analysis.

	n	*r*	*P value*
With white blood cells			
IL-1	12	0.028	0.921
TNF	12	0.244	0.38
CD68	12	0.497	0.06
CD16	12	0.581	0.023*
TRAP5B	12	0.171	0.542
With ESR			
IL-1	11	0.214	0.444
TNF	11	0.003	0.991
CD68	11	0.235	0.4
CD16	11	0.25	0.37
TRAP5B	11	0.18	0.522
With CRP			
IL-1	11	0.147	0.617
TNF	11	0.112	0.704
CD68	11	0.078	0.791
CD16	11	0.231	0.427
TRAP5B	11	0.049	0.867

*Statistically significant.

nocytes, to some degree, reflects the inflammatory status of the loosening periprosthetic tissues.

To minimize the diversification of periprosthetic tissue (vascular density, fibrosis, necrosis, cellular distribution) for the same patient, pooled tissue samples from different periprosthetic regions were collected for analysis of the genetic profile. IL-1β, TNFα and CD16 were used to measure tissue inflammation status [45] [46] [47] [48]. CD68 and TRAP5B were used as markers for osteoclasts [46] [49] [50] [51] [52]. We demonstrated that the frequency of the CD16$^+$ monocyte populations and subpopulations had a weak relationship with *TRAP5B* levels and a strong relationship with the tissue gene levels of IL-1β and CD68 (**Table 3**). These data suggest that the frequency of CD16$^+$ monocytes, to some extent, reflects the inflammatory status of the loosening periprosthetic tissues. The exact mechanism (s) behind this is unclear and warrants further investigation [48].

Levels of CD16$^+$ monocytes can fluctuate in both physiological (such as exercise) [53] [54] and pathological conditions [20] [36] [45] [55] [56] [57] [58] [59]. Though the frequency of CD16$^+$ monocytes was not found to be age dependent [53], lower numbers of the CD16$^+$ monocytes were found in females than that in

males [53]. Our data revealed that the frequency of CD16$^+$ monocytes was significantly higher in stable female patients than in stable male patients (**Figure 3**). The reason for the inconsistency of our findings with that reported by Heimbeck *et al.* [53] is unclear. It may be due to the fact that we used the percentage of CD16$^+$ monocytes among all mononuclear cells, rather than the absolute number of CD14$^+$CD16$^+$ monocytes. Gating strategies were different, as we included all CD14$^+$CD16^{++} and CD14^{++}CD16$^+$ cells as part of the CD16$^+$ population whereas their CD16$^+$ population included CD14$^+$CD16^{++} cells and only part of the CD14^{++}CD16$^+$ population. Since exercise increases the number of CD16$^+$ cells (63, 64), more women may have been exercising post-surgery. Another possibility is that the average age of our female patients was >68.5 years old, which was much older than the healthy volunteers reported [53]. This hypothesis is supported by a recent study [60] showing that menopause increases the absolute number of monocytes, while estrogen replacement therapy may reduce it.

Our data did not find a significant increase in the frequency of CD16$^+$ monocytesin patients with osteolysis/AL compared to that of patients with stable implants. This data is contradictory to the results reported by Wu *et al.* [45] who showed that the frequency of CD16$^+$ monocytes was significantly higher in patients with osteolysis/AL compared to patients with stable implants. In contrast to their smaller sample size (16 patients with stable implant) and shorter time of prosthesis in situ (2.2 ± 1.5 years), we had a larger number of patients with stable implants (n = 56) and longer time of prosthesis in situ (3.7 ± 3.3 years). Since the frequency of CD16$^+$ monocytes reflects the inflammatory status of the loosening periprosthetic tissues, we propose that some of the patients with stable implants (symptom free and normal radiograph) may be at an early stage of AL progression since they have an unexplained and persistent higher frequency of CD16$^+$ monocytes. Further evaluation of the CD16$^+$ monocyte frequency, independently or combined with other blood markers, may be useful to design a risk profile for AL incidence and progression. Building on these findings, a proposed clinical trial is currently under way to determine whether the frequency of CD16$^+$ monocyte reflects the periprosthetic tissue inflammation status using a molecular ^{18}F-FDG PET imaging approach.

This study does have some limitations. First, CD16 is also expressed by natural killer (NK) lymphocytes [61]. These cells may be picked up by the gating strategy in which the scatter gate for monocytes extends into the lymphocytes. Inclusion of additional HLA-DR cell surface marker could be helpful to exclude those HLA-DR$^-$ CD16$^+$ NK-cells [53]. Second, it is unclear whether the absolute numbers of the CD16$^+$ monocyte population and its subpopulations are more accurate and sensitive than current measurements of the frequency of CD16$^+$ monocytes.

Development and characterization of circulating biomarkers is expected to provide a direct means for the early diagnosis of TJA recipients who are at increased risk of the AL progress. The data presented hereinraises the possibility

that the frequency of CD16+ monocytes may represent a simple way to monitor the status of periprosthetic tissue inflammation. Further evaluation of the frequency of CD16+ monocytes, independently or combined with other blood tests or imaging approaches, may be helpful in predicting those patients who have a high risk of AL progression.

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Conflict of Interest Statement

None were declared for all authors.

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Abbreviations

AL, aseptic loosening;

TJA, total joint arthroplasty;

SI, stable implant;

TNF, tumor necrosis factor;

IL-1, interleukin 1;

TRAP, tartrate-resistant acid phosphatase.

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