Circulating CD14$^+$CD204$^+$ Cells Predict Postoperative Recurrence in Non–Small-Cell Lung Cancer Patients

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Background: The expression of CD204 on macrophages in the stroma of the primary tumor is reportedly correlated with an unfavorable prognosis for lung cancer. The purpose of this study is to investigate the correlation among the number of CD204$^+$ tumor–associated macrophages infiltrating the stroma of the primary tumor, the number of circulating CD14$^+$CD204$^+$ cells from the pulmonary vein (PV), and recurrence-free probability in non–small-cell lung cancer patients.

Methods: Human mononuclear cells were isolated from the PV of resected lungs. We examined the expressions of CD14 and CD204 on these cells by flow cytometry. Immunohistochemical staining for CD204 was performed in the resected specimens.

Results: The number of CD14$^+$CD204$^+$ cells from the PV was found to be correlated with the number of CD204$^+$ tumor–associated macrophages identified in the stroma of the tumor. Significantly more cases with high levels of CD14$^+$CD204$^+$ cells from the PV were found to have developed early recurrences. CD14$^+$CD204$^+$ cells, which were polarized to the tumor-promoting phenotype cultured in lung cancer cell line–conditioned medium, facilitated the lung metastasis of cancer cells more effectively than CD14$^+$CD204$^-$ cells in our in vivo mouse model. In multivariate analysis, only the high number of CD14$^+$CD204$^+$ cells from the PV was found to be a statistically significant independent risk factor for early recurrence.

Conclusion: Our results showed the possibility that circulating CD14$^+$CD204$^+$ cells contribute to the metastasis of cancer cells. The blockage of circulating CD14$^+$CD204$^+$ cells activity may prevent postoperative recurrence in resected non–small-cell lung cancer patients.

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Lung cancer is the leading cause of cancer death in the world.[1] Although the most effective treatment for patients with early-stage lung cancer is surgical resection, a considerable number of tumors have been observed to recur even after complete resection. Local recurrence after complete resection is in fact quite low, whereas distant recurrences occur more frequently.[2] These recurrences typically lead to postoperative cancer-related death.

Solid tumors comprised cancer cells, nonmalignant stromal cells, and migratory hematopoietic cells. Complex interactions between noncancer cells and cancer cells are crucial, and evidence has shown that stromal cells interfere with the proliferation, differentiation, and invasion of cancer cells.[3–5] One type of stromal cell which is involved in promoting tumor progression is the macrophage.[6–10] Bone marrow–derived circulating myelomonocytic CD14-positive (CD14$^+$) cells have been reported to infiltrate tumors and are commonly referred to as tumor-associated macrophages.[11] These cells receive various signals from diverse cells in the tumor tissue, and some of these cells are polarized to a tumor-promoting phenotype.[10,11] Thus far, several lines of evidence have indicated that the tumor-promoting phenotype of macrophages expresses high levels of a scavenger receptor known as CD204.[11] The expression of CD204 on macrophages in the stroma of the primary tumor is reportedly correlated with an unfavorable prognosis for cancer in several organs.[12–14] We have also previously reported that the infiltration of CD204$^+$ macrophages into the stroma of the primary tumor tissue is correlated with an unfavorable postoperative prognosis in patients with completely resected non–small-cell lung cancer (NSCLC).[15,16] However, there remains one question regarding the cause of the unfavorable postoperative prognosis of patients with a large number of CD204$^+$ macrophages infiltrating the resected primary tumor tissue: why do postoperative recurrences develop more frequently in such patients even though their cancers have been completely resected? To answer this question, we investigated the correlation among the number of CD204$^+$ macrophages infiltrating the stroma of the primary tumor, the number of circulating CD14$^+$CD204$^+$ cells, and the probability of postoperative recurrences in NSCLC patients.

MATERIALS AND METHODS

Cell Lines and Cell Cultures

A549 were obtained from the RIKEN BioResource Center (Tsukuba, Japan) and were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Sigma, St. Louis, MO). CRL-5807 was obtained from the American Type Culture Collection (ATCC, Manassas, VA) and was cultured in Roswell Park Memorial Institute medium 1640. All the cell lines were cultured in a medium containing 10% fetal bovine serum (Sigma) and 1% penicillin and streptomycin (Sigma) and were incubated at 37°C in an atmosphere containing 5% carbon dioxide.

Patient Samples

Normal fibroblasts were obtained from the surgically resected lungs of patients with lung cancer as previously reported.[17] Human mononuclear cells were isolated from the pulmonary artery (PA) or pulmonary vein (PV) of resected lungs by using BD Vacutainer Evacuated Blood Collection Tubes (BD Bioscience, Inc., San Jose, CA) according to the manufacture's instructions. In this study, a 21-gauge needle was inserted into the PA and PV that had been surgically resected from primary lung cancer patients at our hospital; the blood within the PA and PV was then collected into tubes. After centrifugation, blood mononuclear cell layers in the tubes were collected and counted. Human $CD14^+$ cells were isolated using anti-CD14 microbeads (Miltenyi Biotec, Tokyo, Japan). CD14 cells were seeded on RepCell (a temperature-responsive cell culture dish; CellSeed Inc., Tokyo, Japan) at a density of $1.5 \times 10^5/cm^2$ and cultured in DMEM or lung cancer cell line–conditioned or fibroblast-conditioned medium. After 3 to 7 days, the cells were detached by lowering the temperature of the RepCell down to 20°C for 30 minutes, and the cells were harvested.

Flow Cytometry

The expression of cell surface antigens was determined using two-color immunofluorescence staining. In brief, 100 μl of blood mononuclear cells (containing 5×10^5 cells) was incubated with 40 μl of FcR-blocking reagent (MBL, Aichi, Japan) for 10 minutes to inhibit nonspecific bindings. For double CD14 or CD204 labeling, cells were stained with 10 μg/ml of fluorescein isothiocyanate–conjugated CD14 (eBioscience, San Diego, CA) mAb, and 5 μg/ml of CD204 (R&D Systems, Wiesbaden-Nordenstadt, Germany) mAb labeled with Alexa Fluor 555 (Invitrogen, Carlsbad, CA). Fluorescein isothiocyanate–conjugated and Alexa Fluor 555 labeling isotype–matched immunoglobulin Ig-G1 (Abcam, Cambridge, United Kingdom) and IgG2a (DakoCytomation, Hamburg, Germany) antibodies were used as negative controls. A florescence activated cell sorter (FACS) analysis was performed using a FACSCalibur flow cytometer (BD Bioscience, Heidelberg, Germany). The $CD14^+CD204^-$ cells and the $CD14^+CD204^+$ cells were sorted using FACSAria (BD Biosciences).

Antibodies and Immunohistochemistry

The block containing the most extensive tumor component was selected from each specimen. Sections (4 μm each) were cut from the paraffin blocks and mounted on silanized slides. Individual slides were immunostained using mouse anti-human CD204 antibody (Scavenger Receptor class A-E5; Transgenic, Kumamoto, Japan) at a dilution of 1:100. Round cells in the stroma of the cancer tissue were counted as macrophages. Two pathologists (R.M. and G.I.) counted the CD204-positive macrophages under high-power microscopic fields (×400; 40× objective and 10× ocular; 0.196 mm^2/field), on the basis of the methodology used in a previous report.[15,16]

In Vitro Matrigel Invasion Assay

The invasive capacity of cancer cells was assessed using a matrigel invasion assay. In brief, the upper surface of a filter (pore size, 8.0 μm; BD Bioscience) was coated with basement membrane matrigel. A total of 2.5×10^4 A549 cells were added to the upper chamber and incubated for 22 hours. After incubation, the remaining cells on the upper surface of the filters were removed by wiping with cotton swabs, and the invading cells on the lower surface were fixed in 10% formalin and stained with hematoxylin and eosin. The cells that had migrated from the upper to the lower side of the filter were counted under a light microscope.

Evaluation of Lung Tumors

Six-week-old female severe combined immunodeficient mice (C.B-17 background) were purchased from CLEA JAPAN, Inc. (Tokyo, Japan) and maintained at the National Cancer Center Research Institute East (Chiba, Japan). All the animals were maintained under specific-pathogen-free, temperature-controlled environmental conditions throughout the study, in accordance with institutional guidelines. Written approval for all the animal experiments was obtained from the local Animal Experiments Committee of the National Cancer Center Research Institute.

All the injections were administered through the tail vein. We injected 5×10^4 cancer cells into the tail vein of each mouse and killed the mice 30 days later. To determine the tumor incidence and multiplicity, whole lungs were manually inflated with and fixed in 4% paraformaldehyde for at least 24 hours and then embedded in paraffin. Paraffin-embedded lungs were serially sectioned at 4 μm and were histologically examined using hematoxylin and eosin–stained sections, as previously described.[18]

Microarray Analysis

We used GeneChip Human Genome U133 Plus 2.0 arrays (Affymetrix, Santa Clara, CA), containing 54,675 probe sets, to analyze the mRNA expression levels of approximately 47,000 transcripts and variants from 38,500 well-characterized human genes. Target cRNA was generated from 100 ng of total RNA from each sample using a 3' IVT Express Kit (Affymetrix). The arrays were scanned using a GeneChip Scanner 3000 (Affymetrix), and the intensity of each feature of the array was calculated using GeneChip Operating Software, version 1.1.1 (Affymetrix). The average intensity was standardized to the target intensity, which was set equal to 1000, to reliably compare various multiple arrays. The values were log transformed and median centered. The programs GeneSpring (Agilent Technologies, Santa Clara, CA) and Excel (Microsoft, Redmond, WA) were used to perform the numerical analysis to permit gene selection.

Statistical Analysis

All the data were presented as the mean ± standard error. Continuous variables were compared using unpaired *t* tests. The length of the recurrence-free period was calculated in months from the date of resection to the date of the first recurrence or the last follow-up. To calculate recurrence-free probability, patients who died without recurrence or who were known to be recurrence-free at the date of last contact were censored. For univariate analyses, all cumulative survival rates were estimated using the Kaplan–Meier method and differences in variables were determined using the log-rank test. Multivariate analyses were performed using Cox's proportional hazard regression model. All *p* values reported were two-sided, and the significance level was set at less than 0.05. Analyses were performed using the statistical software SPSS 11.0 (Dr. SPSS II for Windows, standard version 11.0; SPSS Inc., Chicago, IL). This study was conducted as part of a National Cancer Center institutional review board–approved protocol.

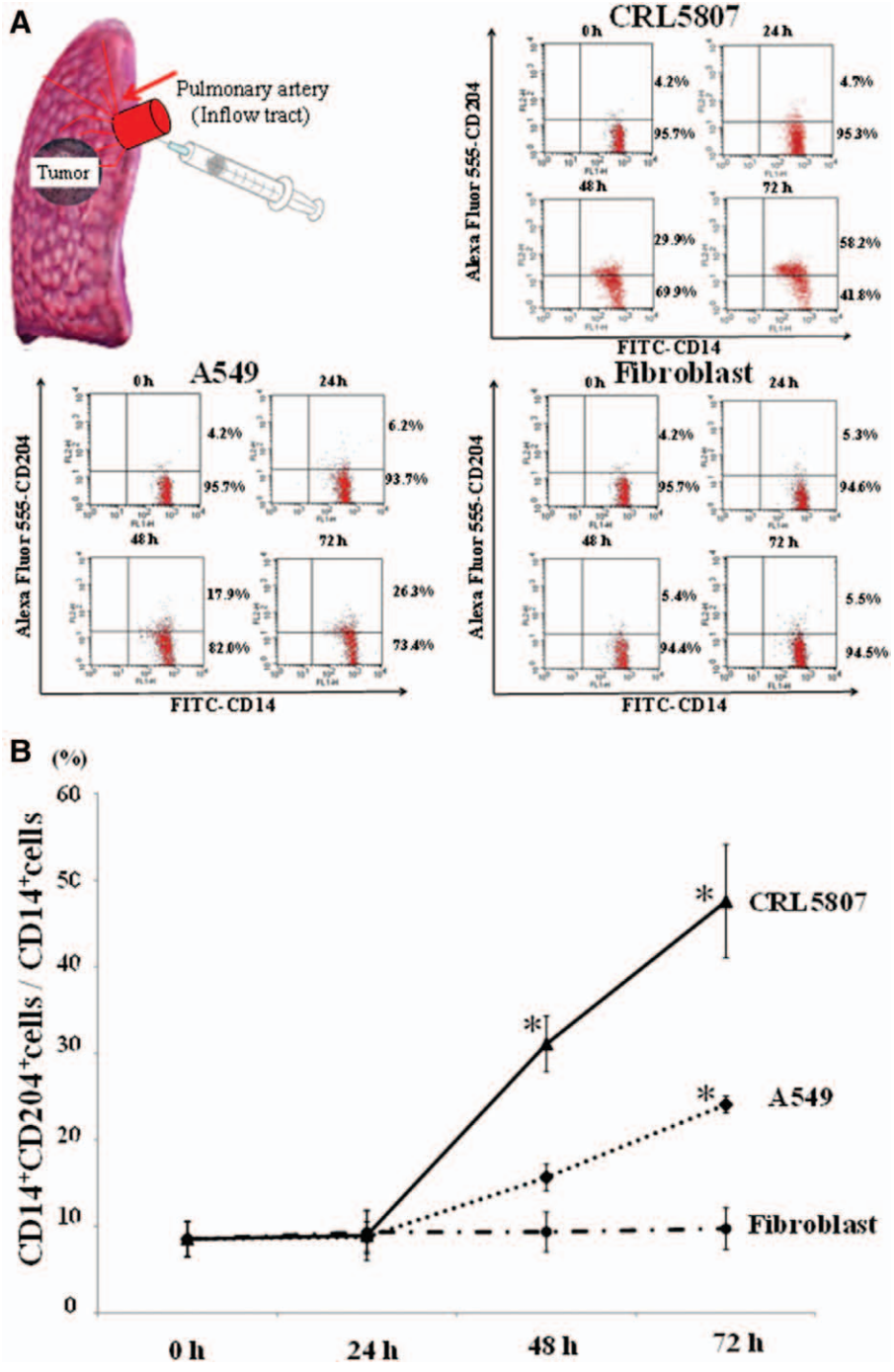

FIGURE 1. Cell surface protein expression of CD204 on CD14+ cells from PA cultured in lung cancer cell line–conditioned medium. *A*, Blood samples from ligated PA of surgically resected lungs. Change in cell surface protein expressions of CD204 on CD14+ cells as determined using flow cytometry. *B*, Blood samples from ligated PA of surgically resected lungs. Change in cell surface protein expressions of CD204 on CD14+ cells as determined using flow cytometry. *B*,Change in CD204 cells per CD14 cell ratio (n = 4 for each group). *Compared with cases cultured in fibroblast-conditioned medium (all, $p < 0.05$). PA, pulmonary artery; FITC, fluorescein isothiocyanate.

RESULTS

Cell Surface Protein Expression of CD204 on CD14+ Cells from PA Cultured in Lung Cancer Cell Line–Conditioned Medium

Blood samples were obtained from the dissected and ligated PA (an inflow tract) of surgically resected lungs (Fig. 1*A*), and CD14+ monocytes were isolated using anti-CD14 microbeads. Because CD204 is the best-known marker of the tumor-promoting phenotype of tumor-associated macrophages, we assessed the changes in the cell surface expression of CD204 occurring on the surface of CD14+ cells cultured in lung cancer cell line–conditioned medium. CD14+ cells isolated from the PA were not completely negative for CD204 expression, and

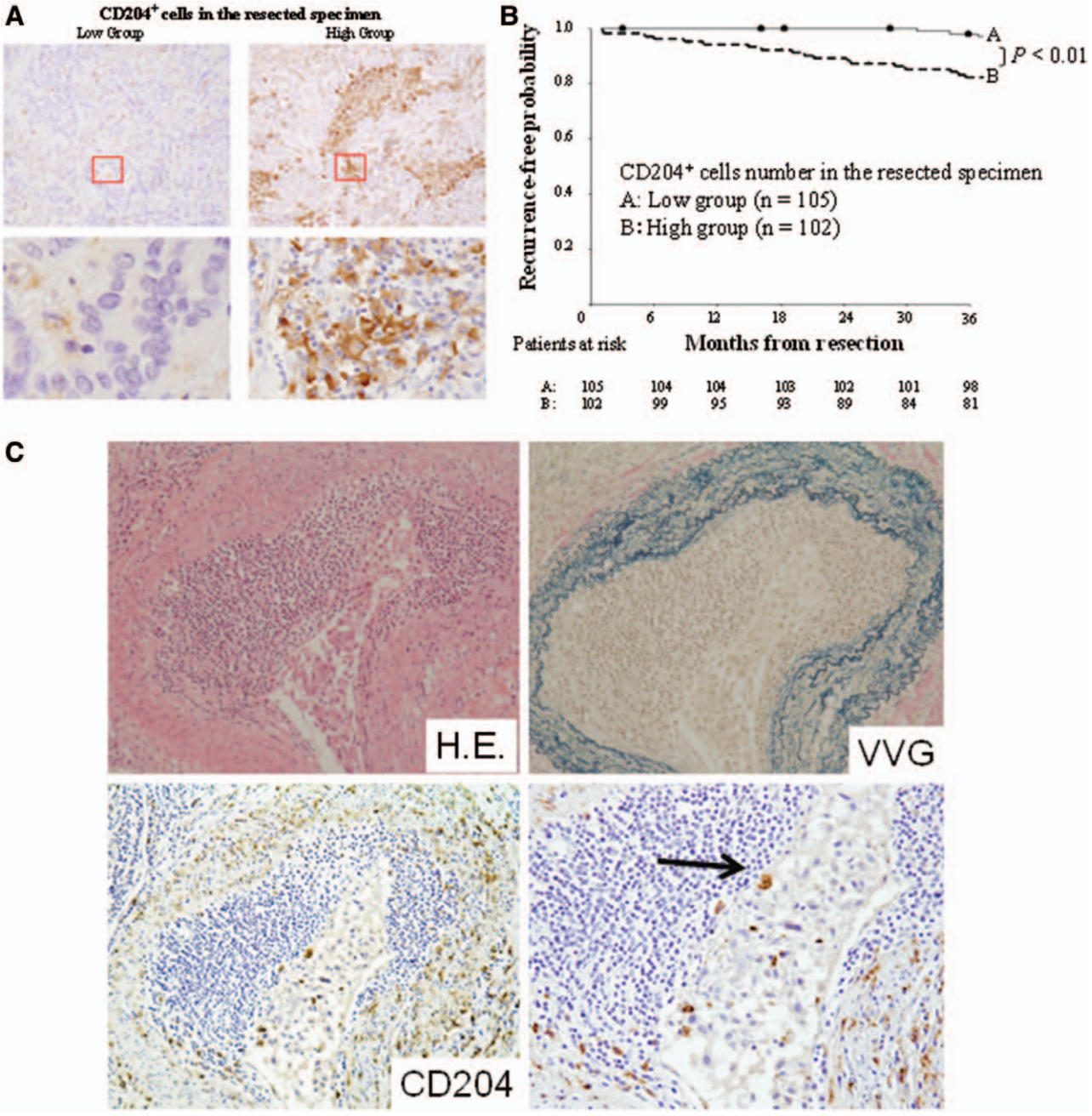

FIGURE 2. Correlation among the number of CD204+ macrophages infiltrating the stroma of the primary tumor and the recurrence-free probability in stage I adenocarcinoma patients. *A*, Immunohistochemical staining for CD204 in lung cancer specimens. The sections were obtained from tumor specimens with low or high levels of CD204+ cells. *B*, Significantly more cases with a high number of CD204+ macrophages develop early recurrence within 3 years after resection ($p < 0.01$).*C*, CD204+ macrophages are found not only in the stroma of the tumor but also in intratumoral blood vessels. H.E., hematoxylin and eosin staining (×200); VVG, Victoria blue van Gieson staining (×200); CD204, CD204 staining (×200).

the expression varied among different patients (8.5% ± 2.0%). During a 72-hour culture in CRL5807-conditioned medium, CD204 expression on the $CD14^+$ cells increased significantly (Fig. 1*A*). Although the levels of CD204 expression were observed to differ among these two media conditioned with different lung cancer cell lines, the expression on $CD14^+$ cells had increased significantly after culture in lung cancer cell line–conditioned media (Fig. 1*A*). In contrast, culturing the $CD14^+$ cells in the normal fibroblast-conditioned medium did not induce the expression of CD204 on $CD14^+$ cells during a 72-hour time course (Fig. 1*B*). From these results, we concluded that the expression of CD204 on $CD14^+$ cells is a useful marker for the detection of $CD14^+$ cells affected by cancer cells.

Correlation between the Number of $CD204^+$ Macrophages Infiltrating the Stroma of the Primary Tumor and the Recurrence-Free Probability in Resected Stage I Lung Adenocarcinoma Patients

We performed immunohistochemical staining for CD204 in 207 consecutive stage I lung adenocarcinoma patients who underwent complete resection between January 2004 and December 2006 and counted the number of $CD204^+$ macrophages in the stroma of the tumor under a high-power microscopic field (×400), as previously reported.[15,16] The median count for all 207 stage I adenocarcinoma patients was 8, with an interquartile range of 0 to 77. Representative tissue specimens are shown in Figure 2*A* for tumors with low and high levels of $CD204^+$ macrophages. Patients were classified into two groups according to whether they had high (>8) or low (≤8) levels of $CD204^+$ macrophages, based on the median number of $CD204^+$ macrophages for the entire group.

Table 1 lists the 3-year recurrence-free probabilities according to clinicopathological features. The 3-year recurrence-free probability for cases with high levels of $CD204^+$ macrophages was significantly lower than that for cases with low levels (82.1% and 98.0%, respectively; $p < 0.01$; Fig. 2*B*).

As shown in Figure 2*C*, $CD204^+$ macrophages were found not only in the stroma of the tumor but also in the intratumoral blood vessels. Because this finding may indicate that $CD204^+$ macrophages exist not only in the stroma of the primary tumor but also in bloodstream and circulate throughout the body, we next examined the number of circulating $CD14^+CD204^+$ cells from the PV (an outflow tract).

Correlation among the Number of $CD204^+$ Macrophages Infiltrating the Stroma of the Primary Tumor, the Number of $CD14^+CD204^+$ Cells from the PV, and the Recurrence-Free Probability in Resected NSCLC Patients

We examined the number of $CD14^+CD204^+$ cells from the PV using flow cytometory. Blood samples from the PV of the resected lungs were obtained from 106 NSCLC patients who underwent complete resection with a lobectomy or a more extensive surgery between December 2009 and April 2010 at our hospital. Among these patients, patients who underwent preoperative chemotherapy, radiation therapy, or both were not included. Representative data for two flow cytometry analyses are shown in Figure 3*A* for tumors with the low and high number of $CD14^+CD204^+$ blood mononuclear cells from the PV. The median count for all 106 patients was 14,500, with an interquartile range of 200 to 7,539,648 per 1 mm^3 blood from the PV. We also performed immunohistochemical staining for CD204 in these 106 tumor specimens and counted the $CD204^+$ macrophages in the stroma of the tumor. The number of $CD14^+CD204^+$ cells from the PV was found to be correlated with the number of $CD204^+$ macrophages identified in the stroma of the tumor (Fig. 3*B*). When a total of 106 lung cancer patients were classified into two groups with a high or a low number of $CD14^+CD204^+$ cells from the PV based on the median number of $CD14^+CD204^+$ cells for the entire group, a significantly greater number of $CD204^+$ macrophages in the stroma were observed among the patients with high levels of $CD14^+CD204^+$ cells from the PV ($p < 0.01$; Fig. 3*C*). In addition, significantly more cases with a high number of $CD14^+CD204^+$ cells from the PV developed early recurrence within 2 years after resection (2-year recurrence-free probability: 76.6% and 94.2%, respectively; $p < 0.01$; Fig. 3*D*). Because postoperative recurrences develop more frequently among patients with high levels of $CD14^+CD204^+$ cells from the PV, we speculated that these circulating $CD14^+CD204^+$ cells might affect postoperative recurrences.

TABLE 1. Patient Characteristics and Univariate Analysis of Risk Factors for Recurrence in Stage I Adenocarcinoma Patients

Characteristics	No. of Patients (%)	Three-Year Recurrence-Free Probability (%)	Univariate *p* Value by Log-Rank Test
Overall	207	90.1	
Age (yrs)			
≤63	105 (51)	93.2	0.106
>63	102 (49)	87.1	
Sex			
Women	113 (55)	96.4	<0.01
Men	94 (45)	82.2	
Smoking history			
Never smoker	99 (48)	94.9	0.246
Ever smoker	108 (52)	85.6	
Tumor size (cm)			
≤2.0	81 (39)	97.5	<0.01
>2.0	126 (61)	84.4	
Number of 204^+ cells in the resected specimen			
Low	105 (51)	98	<0.01
High	102 (49)	82.1	
Stage			
IA	138 (67)	96.3	<0.01
IB	69 (33)	77.8	

$CD14^+CD204^+$ Cells Promote the Metastasis of Cancer Cells In Vivo Model

We assessed the invasive capacity of cancer cells with or without the $CD14^+CD204^+$ macrophages using a matrigel

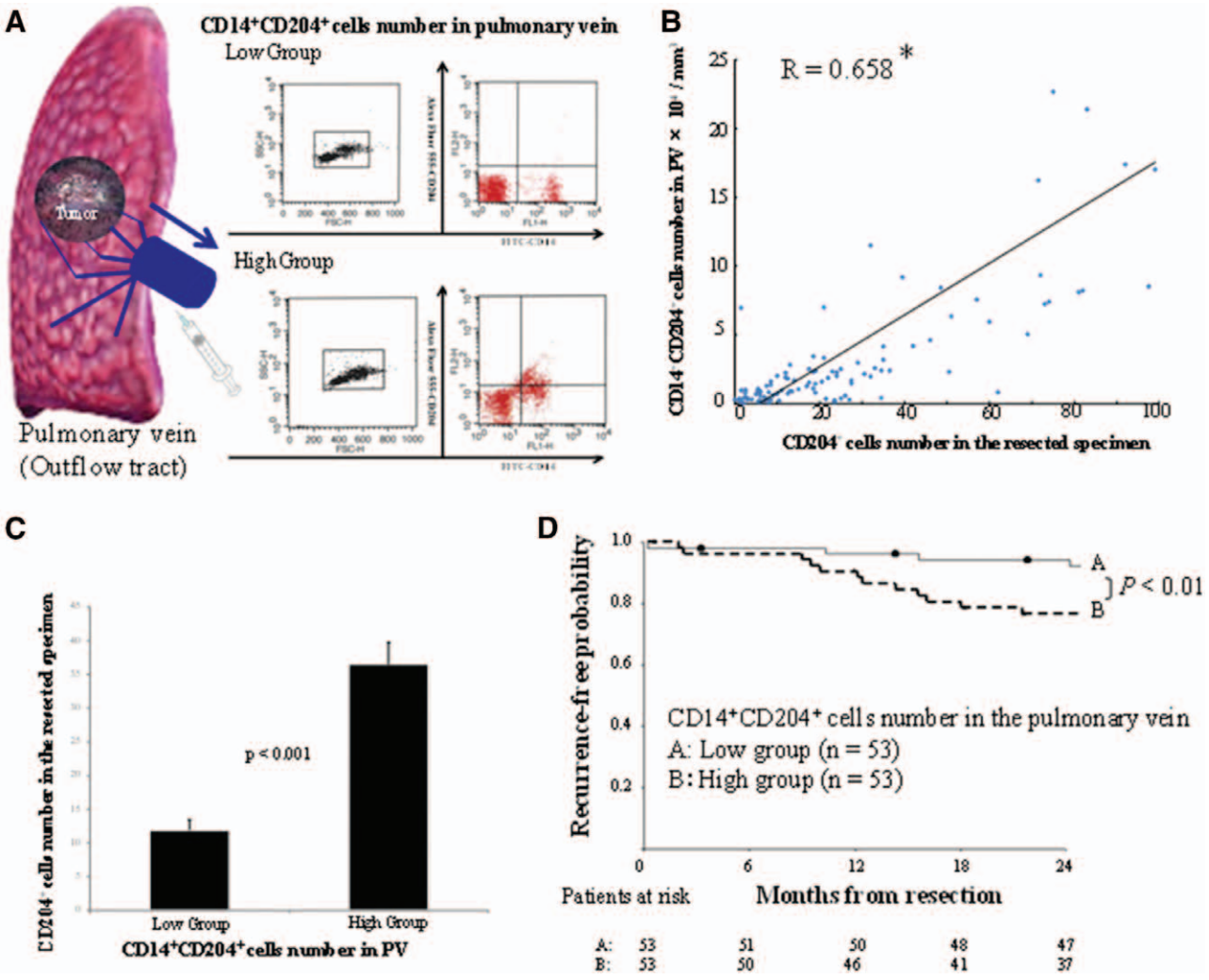

FIGURE 3. Correlation among the number of CD204+ macrophages infiltrating the stroma of the primary tumor, the number of CD14+CD204+ cells from the PV, and the probability of postoperative recurrence in lung cancer patients. *A*, Quantification of CD14+CD204+ cells among blood mononuclear cells from the PV. Representative data for two flow cytometry analyses. *B*, The number of CD14+CD204+ cells from the PV is correlated with the number of CD204+ macrophages in the stroma of the primary tumor. $R = 0.658$. *$p < 0.05$. *C*, A significantly greater number of CD204+ macrophages in the stroma are observed among the patients with high levels of CD14+CD204+ cells from the PV. *D*, Significantly more cases with a high number of CD14+CD204+ cells from the PV develop early recurrence within 2 years after resection ($p < 0.01$). PV, pulmonary vein.

invasion assay. A549 cells were seeded into the upper chamber 30 minutes after the addition of the medium alone, or in combination with the $CD14^+CD204^-$ cells, or in combination with the $CD14^+CD204^+$ cells (Figure 4*A*). After a 22-hour incubation period, a significantly increased number of invading A549 cells on the lower surface of the filters was identified when $CD14^+CD204^+$ cells were added before the addition of the A549 cells, relative to the addition of the medium alone or the addition of the $CD14^+CD204^-$ cells ($p < 0.05$, respectively; Fig. 4*A*).

We next examined whether these $CD14^+CD204^+$ cells promoted the metastasis of cancer cells. Because lung colonization of cancer cells injected through the tail vein of mice is widely used as a model for detecting metastasis, we used this experimental model to assess whether $CD14^+CD204^+$ cells promote the metastasis of cancer cells more effectively than $CD14^+CD204^-$ cells. After dividing the mice into three groups, we injected one group with the medium alone, another group with $CD14^+CD204^-$ cells, and the remaining group with $CD14^+CD204^+$ cells. Thirty minutes later, we continuously injected 5×10^4 of A549 cancer cells into each of the mice in the three groups through a different tail vein. A statistically significant increase in the total number of metastatic lesions was observed 30 days after the injections in the $CD14^+CD204^+$ cell group compared with the numbers in the other two groups ($p < 0.05$, respectively; Fig. 4*B*). Immunohistochemical staining was also performed for metastatic lesions using mouse anti-human CD204 antibody. $CD204^+$ cells were identified only in the metastatic lesions of the $CD14^+CD204^+$ cell group (Fig. 4*B*).

TABLE 2. Representative Genes Differentially Expressed in $CD14^+CD204^-$ and $CD14^+CD204^+$ Cells

	Ratio
Genes up-regulated in $CD14^+CD204^+$ cells	
Membrane receptors	
CD206	70
CD204	8
Interleukin 1 receptor	7
CD163	5
Extracellular mediator	
Vascular endothelial growth factor B	30
Fibronectin 1	9
Transforming growth factor α–induced protein	6
Platelet-derived growth factor C	6
Matrix-degrading enzymes	
MMP7	563
MMP9	388
MMP12	175
MMP2	10
MMP19	10
MMP8	5
Apoptosis-related genes	
Growth arrest and DNA-damage–inducible protein gamma	7
Cytokines and chemokines	
CCL13	1488
CCL7	793
CCL18	458
CXCL13	226
CCL2	160
CCL8	148
CCL22	140
CCL23	79
CXCL5	30
Colony-stimulating factor 1	22
CCL20	21
CXCL1	17
CCL17	11
CXCL9	9
CXCL2	8
Interleukin 10	7
Tumor necrosis factor ligand superfamily, member 2	6
Interleukin 6	5
Genes down-regulated in $CD14^+CD204^+$ cells	
Membrane receptors	
CD97	0.14
CD93	0.14
CXCR4	0.07
Extracellular mediator	
Hepatocyte growth factor	0.2
Insulin-like growth factor–binding protein 3	0.17
Apoptosis-related genes	
Growth arrest and DNA-damage–inducible protein-β	0.17
Bcl-2–associated X protein	0.11
Caspase2	0.1
Cytokines and chemokines	
Interleukin1β	0.17

Oligonucleotide microarray search for genes differently expressed in $CD14^+CD204^+$ cells compared with $CD14^+CD204^-$ cells.

MMP, matrix metalloprotease; CCL, C-C motif chemokine ligand; CXCL, C-X-C motif chemokine ligand.

Because we suspected that factors derived from the $CD14^+CD204^+$ cells may facilitate the metastasis of cancer cells, a genome-wide screening for genes with different expression patterns between $CD14^+CD204^-$ cells and $CD14^+CD204^+$ cells was performed using a microarray. Table 2 shows the representative genes that were differentially expressed in the $CD14^+CD204^-$ and $CD14^+CD204^+$ cells.

Prognostic Impact of the Number of Circulating $CD14^+CD204^+$ Cells from the PV on Resected NSCLC Patients

Table 3 shows the number of circulating $CD14^+CD204^+$ cells from the PV according to the clinicopathological features of 106 NSCLC patients. Statistically significant

TABLE 3. Correlation between Clinicopathological Characteristics and Number of $CD14^+204^+$ Cells from the PV in 106 Resected Non–Small-Cell Lung Cancer Patients

Characteristics	No. of Patients (%)	No. of $CD14^+204^+$ Cells in the PV (/ml) ± SE	*p*
Total	106	38,699 ± 7061	
Age (yrs)			
≤68	55 (52)	39,162 ± 8856	0.946
>68	51 (48)	38,199 ± 11,237	
Sex			
Women	33 (31)	22,941 ± 6010	0.04
Men	73 (69)	45,822 ± 9801	
Smoking history			
Never smoker	35 (33)	24,075 ± 6160	0.147
Ever smoker	71 (67)	45,908 ± 10,016	
Histological type			
Nonadenocarcinoma	40 (38)	50,355 ± 11,545	0.2
Adenocarcinoma	66 (62)	31,635 ± 8880	
Tumor size (cm)			
≤3.0	68 (64)	21,929 ± 3496	<0.001
>3.0	38 (36)	68,708 ± 17,809	
N status			
N0	83 (88)	30,574 ± 7366	0.028
N1-3	23 (22)	68,020 ± 17,860	
Stage			
IA	56 (53)	19,549 ± 2966	0.004
IB or higher	50 (47)	99,420 ± 14,060	

PV, pulmonary vein; SE, standard error.

TABLE 4. Univariate and Multivariate Analyses of Risk Factors for Recurrence in 106 Resected Non–Small-Cell Lung Cancer Patients

Characteristics	No. of Patients (%)	Two-Year Recurrence-Free Probability (%)	Univariate *p* Value by Log-Rank Test	Multivariate Analysis		
				HR	95% CI	*p*
Overall	106	56.1				
Age (yrs)						
≤68	55 (52)	85.0	0.881	Not included multivariable model		
>68	51 (48)	85.9				
Sex						
Women	33 (31)	87.5	0.432	Not included multivariable model		
Men	73 (69)	84.6				
Smoking history						
Never smoker	35 (33)	88.3	0.703	Not included multivariable model		
Ever smoker	71 (67)	84.1				
Histological type						
Nonadenocarcinoma	40 (38)	85.0	0.961	Not included multivariable model		
Adenocarcinoma	66 (62)	85.7				
Tumor size (cm)						
≤3.0	68 (64)	88.1	0.298	Not included multivariable model		
>3.0	38 (36)	80.6				
Number of $CD14^+204^+$ cells in the PV						
≤14,500/ml	53 (50)	94.2	0.0098	1		
>14,500/ml	53 (50)	76.6		3.193	1.025–9.944	0.045
N status						
N0	83 (88)	91.3	0.0043	1		
N1-3	23 (22)	69.6		2.205	0.658–7.387	0.200
Stage						
IA	56 (53)	92.8	0.038	1		
IB or higher	50 (47)	76.9		1.443	0.406–7.387	0.571

PV, pulmonary vein; HR, hazard ratio; CI, confidence interval.

correlations were observed between the number of circulating $CD14^+CD204^+$ cells from the PV and the patient sex, tumor size, N status, or stage (Table 3). Table 4 lists the 2-year recurrence-free probabilities according to clinicopathological features. Univariate analysis identified the following three statistically significant risk factors for recurrence: the number of $CD14^+CD204^+$ cells from the PV, lymph node involvement, and tumor stage. In multivariate analysis, only the high number of $CD14^+CD204^+$ cells from the PV was found to be a statistically significant independent risk factor for early recurrence ($p < 0.05$; Table 4).

DISCUSSION

The invasion of cancer cells into intratumoral blood vessels in the primary tumor is often observed in resected lung cancer tissue.[2] The intratumoral vessel invasion of cancer cells has been reported to be significantly correlated with the postoperative recurrence and an unfavorable prognosis.[2] This finding indicates that cancer cells mobilize into the bloodstream and circulate, resulting in postoperative distant metastasis. In this study, we observed that $CD204^+$ tumor–associated macrophages were found not only in the stroma of the tumor but also in intratumoral blood vessels (Fig. 2*C*). This finding raises the possibility that $CD204^+$ macrophages in the primary tumor mobilize into the bloodstream and circulate, in addition to the circulating cancer cells. The results of this study showed that the number of $CD14^+CD204^+$ cells from the PV was found to be significantly correlated with the number of $CD204^+$ macrophages identified in the stroma of the tumor. We considered that educated $CD14^+$ $CD204^-$ cells become polarized to $CD14^+CD204^+$ cells at the primary tumor site and are subsequently mobilized into the bloodstream and circulate. In addition, postoperative recurrences develop more frequently among patients with high levels of $CD14^+CD204^+$ cells from the PV in our clinical data. Also in our in vivo model, we demonstrated that $CD14^+CD204^+$ cells facilitated the metastasis of cancer cells more effectively than $CD14^+CD204^-$ cells. From these results, we suggest the concept that tumor-associated macrophages remote from the primary tumor and directly contribute to the metastasis of cancer cells at distant sites (Fig. 4*C*).

Although surgery effectively controls many cancers at the primary site, the development of metastatic disease after an operation signals a poor prognosis. In lung cancer patients, many patients with completely resected tumors eventually die as a result of metastatic disease occurring after resection.[2] To prevent postoperative distant metastases, adjuvant

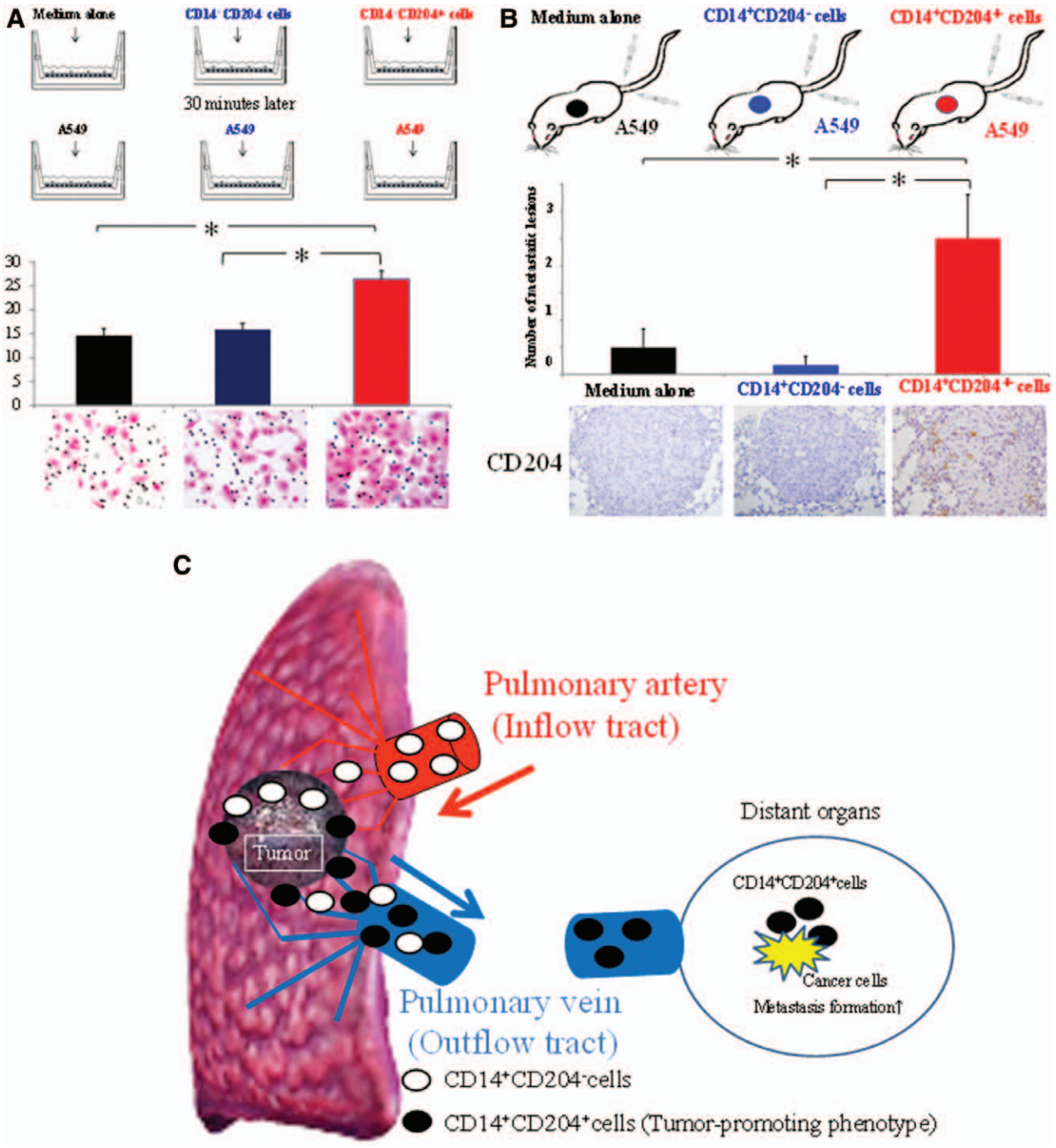

FIGURE 4. CD14+CD204+ cells facilitated the lung metastasis of cancer cells more effectively than CD14+CD204− cells. *A*, Matrigel invasion assay. A significantly increased number of invading A549 cells on the lower surface of the filters is identified when CD14+CD204+ cells are added. *$p < 0.05$. *B*, CD14+CD204+ cells promote the metastasis of A549 cancer cells. *$p < 0.05$. *C*, Our concept that CD204+ tumor–associated macrophages remote from the primary tumor directly contribute to the metastasis of cancer cells at distant sites.

chemotherapy is recommended to eliminate occult metastases[19,20] and postoperative adjuvant cisplatin-based chemotherapy has recently become the standard of care.[19,20] However, the reported reduction of the relative risk of mortality was only estimated to be 11% to 13%,[19,20] and the effect of conventional postoperative adjuvant chemotherapy targeting only the cancer cells is not satisfactory.

Tumor metastasis consists of a series of discrete biological processes which move tumor cells from the primary tumor to a distant location. Tumor cells must invade the tissue surrounding

the primary tumor, enter either the lymphatic system or the bloodstream where they survive, and eventually become arrested from circulation. Then, these cells extravasate into tissues and grow at the new site. The term "colonization" is frequently used to reflect the combined influences of tumor cell proliferation, dormancy, and angiogenesis in the formation of a progressively growing lesion at a distant site. Thus far, there have been several reports on the role of stromal cells in aiding tumor colonization of distant organs.[21,22] Hiratsuka et al.[21] provided evidence suggesting that the activation of lung macrophages by primary Lewis lung carcinoma tumors enhances lung metastasis in a model of experimental metastasis. Kaplan et al.[22] also reported that the bone marrow-derived vascular endothelial growth factor receptor 1^+ hematopoietic cells show characteristics common to physiological pathways of inflammation by providing the necessary growth conditions to create a conductive microenvironment in the lungs for the engraftment of tumor cells. These studies[21,22] have shown that the efficiency of the metastatic process may be increased by some stromal cells. Therefore, a more detailed understanding of the efficiency of the metastatic process of cancer cells, including the involvement of stromal cells in colonization, is likely to contribute to the development of better postoperative therapies and to improve patient outcomes after lung cancer resection. In this study, early recurrences were found to develop more frequently in tumors with high levels of $CD14^+CD204^+$ cells from the PV. In addition, $CD14^+CD204^+$ cells, which were polarized to the tumor-promoting phenotype cultured in lung cancer cell line–conditioned medium, facilitated the lung metastasis of cancer cells more effectively than $CD14^+CD204^-$ cells in our in vivo mouse model. These results may indicate a need for treatment that targets for circulating $CD14^+CD204^+$ cells to prevent postoperative recurrence in addition to cancer cells. Hiratsuka et al.[21] demonstrated that the primary tumors themselves induce matrix metalloprotease-9 (MMP9) expression in the macrophages of the metastatic site, thereby promoting the invasion of cancer cells. It was found that blockage of MMP9 induction could be useful for the prevention of tumor metastasis.[21] Also in the present study, the expression of MMP9 on the $CD14^+CD204^+$ cells was up-regulated compared with the expression levels in the $CD14^+CD204^-$ cells. To explore the role of $CD14^+CD204^+$ cells in facilitating the metastasis of cancer cells relative to $CD14^+CD204^-$ cells, we will focus on the increased expression of MMP9 on $CD14^+CD204^+$ cells in the future study.

CONCLUSION

Our results showed the possibility that circulating $CD14^+CD204^+$ cells contribute to the metastasis of cancer cells. The blockage of circulating $CD14^+CD204^+$ cells activity may prevent postoperative recurrence in resected NSCLC patients.

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