CHAPTER II

EXPANSION AND CHARACTERIZATION OF CIRCULATING $CD16^+$ MONOCYTE IN CHOLANGIOCARCINOMA PATIENTS

2.1 Introduction

Monocytes do not represent a homogenous cell population but they are different subpopulations. Depending on their state of differentiation and activation may involve in pro- or anti-inflammatory conditions (Rutherford et al., 1993). The majority of monocyte exhibit strong CD14 and CD16 negative staining ($CD14^{++}$) and are thought to represent classical monocytes that mediate inflammatory responses ("inflammatory" monocytes). $CD14^+CD16^+$ monocytes are minor subpopulation that have some characteristics patterns of cell surface molecules when compared with the $CD14^{++}$ monocytes , e.g., high HLA-DR, epidermal growth factor module-containing mucin like receptor 2 (EMR2), Ig-like transcript 4 (ILT-4), CD43, and CD45RA expression. These monocytes are thought to represent the precursors of tissue-resident macrophages and are referred to as "resident" monocytes (Allan et al., 1999; Almeida et al., 2001; Geissmann et al., 2003; Gordon, Taylor, 2005; Kwakkenbos et al., 2002; Rothe et al., 1996).

The functional significance of $CD14^+CD16^+$ monocytes is unclear. $CD14^+CD16^+$ monocytes had been documented to be potent producers of TNF but showed low IL-10 production after lipopolysaccharide stimulation (Belge et al., 2002; Frankenberger et al., 1996). In untreated asthmatics, the $CD14^+CD16^+$ cells exhibited some features of tissue macrophage activation such as increasing superoxide anion (Rivier et al., 1995). In addition, it has been shown to involve in anti-tumor response as judged by the enhanced production of pro-inflammatory cytokine (TNF-α, IL-12p40 and IL-12p70), reactive nitrogen intermediate, and increased cytotoxic and cytostatic activities (Szaflarska et al., 2004). However, these cells had shown a higher antigen-presenting capacity. At this point, there are conflicting data about the propensity of $CD14^+CD16^+$ cells to go into inflammatory tissues, and this may be dependent on the type and site of inflammation studied (Ziegler-Heitbrock, 2007).

In general, $CD14^+CD16^+$ cells represent in healthy individuals varies from 3% to 13% (Frankenberger et al., 1996; Kreutz et al., 1992; Sadeghi et al., 1999; Schmid et al., 1995; Ziegler-Heitbrock et al., 1993), but it has been shown to increase number in various acute and chronic inflammatory syndromes, such as AIDS (Thieblemont et al., 1995), bacterial sepsis (Fingerle et al., 1993), solid cancer (Saleh et al., 1995a; Saleh et al., 1995b), AIDS dementia (Pulliam et al., 1997), acute and chronic infection undergoing hemodialysis (Nockher, Scherberich, 1998) and sporadic amyotrophic lateral sclerosis (Zhang et al., 2005). CCA is a chronic inflammatory disease and high density of macrophage was accounted in CCA tissue and related to poor outcome of the patients (Krungmee et al., 2003). In the present study, the expansion of $CD14^+CD16^+$ monocytes in peripheral blood taken from CCA patients were compared with those from normal subjects and benign biliary tract disease (BBD) patients. The correlation of $CD14^+CD16^+$ monocytes level with clinicopathological features or survival of CCA patients were explored. In addition, phenotypes of $CD14^{++}$ and $CD14^+CD16^+$ monocytes were compared via expression levels of surface markers, e.g., integrins (CD11a, CD11c, CD18, CD29, CD49d, and CD54) and scavenger receptors (CD163, and CD204). The surface molecules studied involve in maturity and migration ability of these cells into tissue, of which the function are briefly described below:

CD11a or Integrin, alpha L (ITGAL), are heterodimeric integral membrane proteins composed of an alpha chain and a beta chain. This I-domain containing alpha integrin combines with the beta 2 chains (ITGB2 or CD18) to form the integrin lymphocyte function-associated antigen-1 (LFA-1), which is expressed on all leukocytes. LFA-1 plays a central role in leukocyte intercellular adhesion through interactions with its ligands, ICAMs 1-3 (intercellular adhesion molecules 1 through 3) on endothelial cells.

CD11c or Integrin, alpha X (ITGAX), are heterodimer integral membrane proteins composed of an alpha chain and a beta chain. This protein combines the beta 2 chain (ITGB2 or CD18) to form a leukocyte-specific integrin referred to as inactivated-C3b (iC3b) receptor 4 (CR4). The alpha X beta 2 (CD11c/CD18) seems to overlap the properties of alpha M beta 2 integrin in the adherence of neutrophils and monocytes to stimulated endothelial cells, and in the phagocytosis of complement

coated particles. CD11c is a type I transmembrane protein found at high levels on most human dendritic cells, but also on monocyte, macrophage, neutrophil and some B cells.

CD18 or Integrin, beta2 (ITGB2) is the beta subunit of three different structures including LFA-1 (paired with CD11a), macrophage-1 antigen (paired with CD11b) and integrin alpha X beta 2 (paired with CD11c). Intrgrins are known to participate in cell adhesion as well as cell surface mediated signaling.

CD29 or Integin, beta 1 (fibornectin, beta polypeptide), also known as ITGB1, is expressed as heterodimers that are noncovalently associated with specific alpha-chains of the CD49 family (CD49a-f). CD29 is expressed on resting and activated leukocytes and is a marker for all of the very late activation antigens (VLA) on cells (Barclay, 1993).

CD49d or Alpha-4 integrin chain, also known as very late antigen-4 alpha chain (VLA-4) that are expressed as heterodimers with CD29 (beta 1 integrin) or (beta 7 integrin) and function as adhesion receptors. CD49d/CD29 is mainly expressed on thymocytes and B cells with increased expression on activated T cells. The ligands for CD49d/CD29 include VCAM-1 and fibronectin.

CD54 or ICAM-1 (Inter-Cellular Adhesion Molecule 1) is a type of intercellular adhesion molecule continuously present in low concentration in the membranes of leukocytes andendothelial cells. Upon cytokine stimulation, the concentration is greatly increased. ICAM-1 can be induced by interleukin-1 (IL-1) and tumor necrosis factor alpha (TNFα), and is expressed by the vascular endothelium, macrophages, and lymphocytes. ICAM-1 is a ligand for LFA-1 (integrin), a receptor found on leukocytes. When activation, leukocytes bind to endothelial cells via ICAM-1/LFA-1 and then transmigrates into tissues (Yang et al., 2005).

CD163 is a member of the scavenger receptor cysteine-rich (SRCR) family of proteins exclusively expressed on the surface of monocytes/macrophages. It has been identified as the receptor for the uptake of heptoglobin-hemoglobin complex for removal and metabolism of the potent oxidant hemoglobin (Kristiansen et al., 2001). Anti-inflammatory mediators such as glucocorticoids and IL-10, stimulate macrophage surface expression of CD163, (Buechler et al., 2000; Hogger et al., 1998; Sulahian et al., 2000) whereas pro-inflmmatory mediators such as TNF-α, IFN-γ and

LPS suppress CD163 transcription and surface protein expression. This raises the potential for CD163 to exert anti-inflammatory actions.

CD204 is a class A scavenger receptor with a wide range of ligand-binding specificities; it recognizes various negatively charged macromolecules including modified low-density lipoproteins and apoptotic cells. At present, CD204 and CD163 are known as markers of tumor associated macrophage type 2 (M2 phenotype) in many cancers (Kawamura et al., 2009; Komohara et al., 2008; Shabo et al., 2008).

Since, $CD14^{+}CD16^{+}$ monocytes has not been reported in CCA and the potential migration of $CD14^{+}$ and $CD14^{+}CD16^{+}$ monocytes into lesion tissues (CCA) for developing to be macrophage is still unclear. Thus the objective of this study is to determine the level of $CD14^{+}CD16^{+}$ monocytes in peripheral blood from CCA patients and its phenotypes via expressions of its integrins and scavenger receptors.

2.2 Materials and methods

2.2.1 Blood samples

Heparinized blood were collected from three subject groups –namely healthy subjects (n = 46), benign biliary tract disease (BBD, n = 18) and CCA patients (n = 44). CCA and BBD subjects were patients who admitted at Srinagarind hospital, Faculty of Medicine, Khon Kaen University and with histological proved to be benign biliary tract disease or CCA. For CCA subjects, blood was collected twice, before and at least 2 months after surgery without any additional treatment. Healthy subjects are defined as the persons who have age and sex matched with CCA patients, normal clinically health with normal complete blood count (CBC), normal liver function test (LFT), and no appear chronic inflammatory diseases such as diabetic mellitus or hepatitis. Informed consent was obtained from each subjects and the Human Research Ethics Committee, Khon Kaen University approved our research protocol (HE471214 & HE480312).

The age, gender, tumor location, histological grading and pTNM stage were evaluated by reviewing the medical charts and pathological records. Survival of each CCA patients was recorded from the date of surgery to date of death or to June 13, 2008

2.2.2 Flow cytometry for monocyte subpopulations

All heparinized blood from CCA patients, BBD patients and healthy subjects were immediately stained (approximately 1x 10^6 cells in 100 µl/test) after they were obtained using a stain-and-then-lyse direct immunofluorescence technique with CD14 FITC, CD16 PE and CD HLA-DR PE-Cy 5 (Becton-Dickinson, San Jose, CA, USA) as manufacturer's specification. Analysis of $CD14^+CD16^+$ monocytes expansion using Coulter EPICS_MXL with CXP Software where at least 5,000 cells were counted per analysis. The experiment was held at Microbiology department, Faculty of Medicine, Khon Kaen University, under supervision of Associate Prof. Veeraphong Lulitranond

For phenotypical analyzes including surface adhesion molecules, and scavenger receptors of both$CD14^{++}$ and $CD14^+CD16^+$ monocyte was performed using peripheral blood mononuclear cells (PBMCs) from CCA patients which were separated via Ficoll hypaque separation. For four-color stainings, approximately 1x10^6 cells of PBMCs was stained with CD14 Pacific Blue, CD16 APC combination with a mix of either FITC or PE conjugated adhesion molecules (CD11a, CD11c, CD18, CD29, CD49d, and CD54) and scavenger receptors (CD163, and CD204) (Table 2.1). The expression levels of those molecules were measured and reported via mean fluorescent intensity (MFI) by LSR flow cytometer (Becton Dickinson). At least 20,000 events per samples were analyses. Nnegative staining was defined as the area of dot plots or histogram that contained over 99% of isotype-stained cells. These analyses were performed at the Division of Hematopoiesis, Center for AIDS Research, Kumamoto University, under supervision of Prof. Seiji OKADA.

Table 2.1 lists of monoclonal antibodies for studying flow cytometry

No.	Monoclonal Antibodies	Company
1	Mouse anti-human CD14-FITC	BD Pharmigen
2	Mouse anti-human CD14-Pacific Blue (clone MHCD1428)	Caltage
3	Mouse anti-human CD16- PE (Clone 3G8)	BD Pharmigen
4	Mouse anti-human CD16 -Alexa Fluor 647 (Clone 3G8)	Biolegend
5	Mouse anti-human HLA-DR- PE Cy.5	BD Pharmigen
6	Mouse anti-human CD11a – PE	Immunotech
7	Mouse anti-human CD11c – FITC	eBioscience
8	Mouse anti-human CD18 – FITC	eBioscience
9	Mouse anti-human CD29 – PE	Beckman Coulter
10	Mouse anti-human CD49d - PE (Clone 9F10)	eBioscience
11	Mouse anti-human CD54 – PE	Miltenyi Biotec (MACS)
12	Mouse anti-human CD163- FITC	Gift from Professor Seiji OKADA
13	Mouse anti-human CD204 – FITC	Gift from Professoe Seiji OKADA

2.2.3 Monocyte preparation

Heparinized blood was drawn from CCA patients or healthy subjects. Dilute heparin-treated whole blood with an equal volume of 1X PBS (Ca++, Mg++ free, sterile). Gently layered 2 volumes of the diluted blood over 1volume of Ficoli Hypaque. After centrifugation at 1,500 RPM at room temperature (Beckman table-top centrifuge, 12 cm radius swing-bucket rotor) for 20 minutes at 20°C, brake off, gently removed white colored band of cells at the plasma/Ficoll hypaque interface, and transferred to a new centrifuge tube. Cells were washed with 1XPBS, span at 2,000 RPM for 5-minutes, and then the leftover RBCs were lysed with 5 ml RBC lysis buffer for 3 min before spinning at 2,000 rpm for 5 min. Peripheral blood mononuclear cells (PBMCs) were suspended in RPMI-1640 with 10% FBS. The PBMCs ($5x10^6$) were incubated at 37°C for 3 hr in 10 cm plastic tissue culture dishes, to allow selective adherence of monocytes, followed by two successive rinses with warmed medium to remove non-adherent lymphocytes. These preparations obtained at least 90% monocytes as assessed by immunofluorescent staining with antibodies to the CD14 antigen.

2.2.4 Measurement of mRNA expression by real-time RT-PCR

Adherent cells (monocytes) were kept in TRIzol reagent and performed RNA extraction by Trizol®reagent (Invitrogen, CA) following the manufacturer's protocol. Reverse transcription reaction consisted of 500 ng total RNA in 25 µl of H_2O which was heated at 65°C for 10 min and then chilled on ice for 2 min. After that, RNA solution was transferred into a tube contained first strand reaction mixed beads (GE healthyCare, Piscataway, NJ, USA), 0.5 µM random hexamer and H_2O for getting final volume up to 33 µl. Reverse Transcription was carried on in a 37 °C water bath for 1 hr.

Transcript copy number for a specific gene of interest was measured using an adaptation of a two-step real-time reverse transcriptase–polymerase chain reaction (Real time RT-PCR) method. Real time RT-PCR for specific genes of interest *(EREG, VEGFA,CXCL3, and CXCL10)* and internal control (β-Actin) (Table 3.2) were performed using SYBR® Green PCR Master Mix assay with the ABI 7500 real time PCR system (Applied Biosystems, Foster City, CA). PCR was performed with ~25 ng cDNA sample. Amplifications included one cycle of template denaturation at 95°C for 10 minutes followed by 40 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 45 seconds. The presence of a single amplified product was confirmed by DNA melting point analysis. Threshold cycles (Ct) for each amplification reaction were determined using 7500 System SDS software version 1.4 (Applied Biosystems, Foster City, CA). The gene-specific primers for individual samples (including CCA patients and healthy subjects) were normalized to signals obtained with β-actin from the same sample. Relative change in gene expression of CCA patients and healthy subjects were analyzed by using $2^{-\Delta Ct}$ method where ΔCt = (average Ct_{target} – average Ct_{actin}). Significant differences between groups were analyzed by a Student's *t*-test.

2.2.5 Statistical analysis

First, the numbers of $CD14^{+}CD16^{+}$ monocytes from three groups of subjects were compared with One Way ANOVA (and non-parametric). Then, the numbers of $CD14^{+}CD16^{+}$ monocytes among groups and expression levels of the adhesion molecules and scavenger receptors on both $CD14^{++}$ and $CD14^{+}CD16^{+}$ monocytes were compared and analyzed with Student's *t*-test. The associations between levels of $CD14^{+}CD16^{+}$ monocytes of CCA patients and clinic-pathological features of the patients were done using cross tabulation with χ^2-test. The Kaplan-Meier survival analysis was used to estimate the overall survival and comparison between groups were done with log-rank test. Statistic analyses were determined using SPSS statistical software version16.0.1 (SPSS Inc., Chicago, IL) and STATA version 8. $P < 0.05$ was considered statistically significant.

2.3 Results

2.3.1 Level of $CD14^+CD16^+$ monocytes in blood from CCA patients and clinical significance

Stainings of whole blood samples with CD14 and CD16 antibodies allowed for detection of two monocyte populations. One population is strongly positive for CD14 and lacks CD16 ($CD14^+$). These cells are the majority of circulating monocytes. The second cell population co-expresses the CD14 antigen at a low density together with the CD16 antigen ($CD14^+CD16^+$) (Figure 2.1). In the present study, the double positive cells accounted for 13 $\pm$ 6% of all monocytes and for 48.5 $\pm$ 26.6/ml blood in the apparently healthy donors (n= 46). The significance of dramatic expansion of $CD14^+CD16^+$ monocytes was observed in the blood from both BBD and CCA patients by One Way ANOVA (and nonparametric) at $P < 0.0001$ (Figure 2.2). The level of $CD14^+CD16^+$ monocytes of patients with CCA (30 $\pm$ 12% of all monocytes or 171 $\pm$ 129/ml, n = 44) was significantly higher than those of patients with BBD (23 + 10% of all monocytes or 109$\pm$89/ml, n = 18) or healthy subjects ($P < 0.05$). In addition, three months after of tumor resection, the elevated $CD14^+CD16^+$ monocyte levels of CCA patients (29 $\pm$ 9% of all monocytes or 111 $\pm$ 28/ml, n = 5) decreased to near the normal level (16 $\pm$ 5% of all monocytes or 68 $\pm$ 24/ ml), with statistical significant at $P < 0.05$ (Figure 2.3).

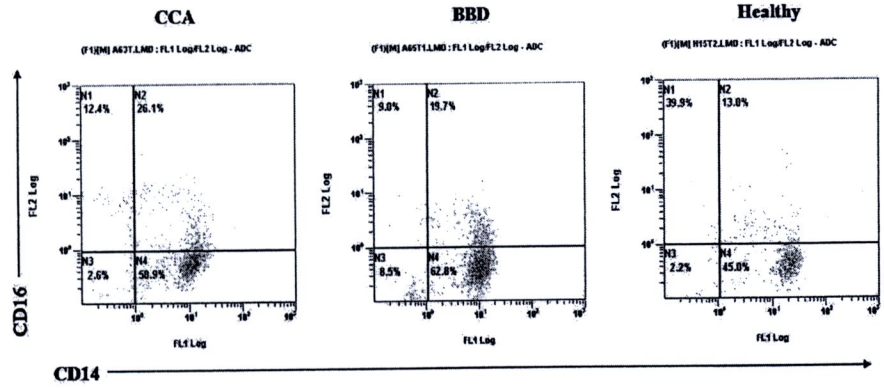

Figure 2.1 Two-color flow-cytometric analysis of monocyte subpopulation in whole blood. Monocytes were gated according to their forward and side scatter characteristics. The percentage of $CD14^+CD16^+$ circulating monocytes for representative CCA patient (A), BBD patient (B), and healthy subject (C) are shown.

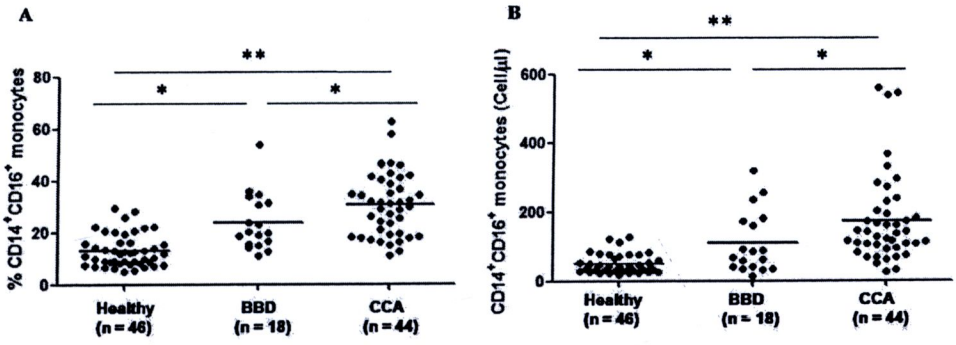

Figure 2.2 Expansions of $CD14^+CD16^+$ monocytes in peripheral blood of CCA patients, benign biliary disease patients (BBD) and healthy subjects. The expansions of $CD14^+CD16^+$ monocytes from three subject groups were compared by based on the numbers of $CD14^+CD16^+$ monocytes in percentage value (A) and absolute number (B).

*, $P < 0.05$; compared between CCA patients and BBD patients or healthy subjects by Student's *t*-test.

**, $P < 0.001$; compared between CCA patients and healthy subjects by Student's *t*-test.

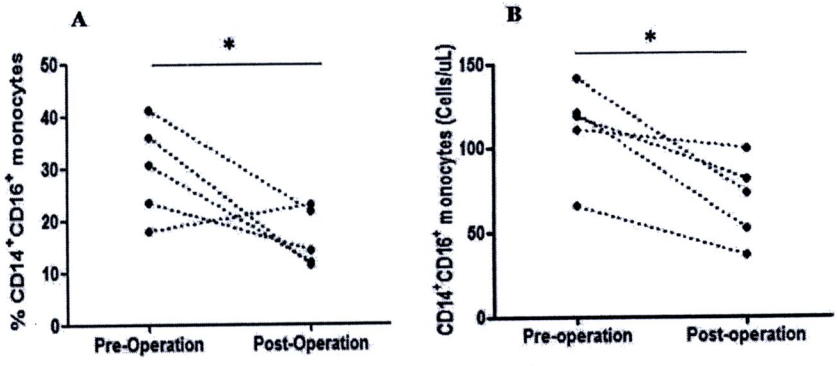

Figure 2.3 Expansions of $CD14^+CD16^+$ monocytes in peripheral blood of CCA patients between pre and post operations (n = 5) were compared by based on the numbers of $CD14^+CD16^+$ monocytes in percentage value (A) and absolute number (B).

*, $P < 0.05$; compared between pre and post operation by Pair *t*-test.

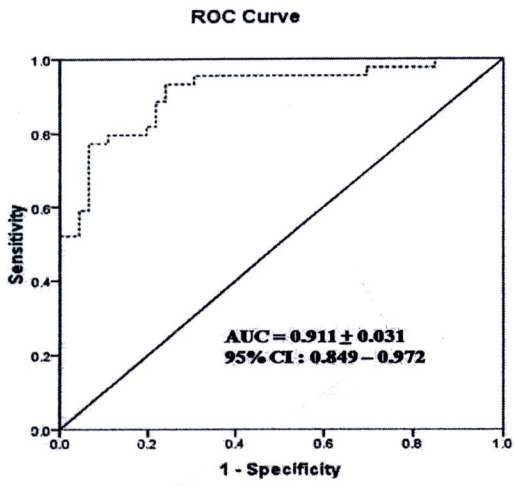

Figure 2.4 Receiver Operating Characteristic analysis. ROC curve of the expansion of $CD14^+CD16^+$ monocyte subpopulations from 44 CCA patients versus 46 healthy subjects. At cut off point value = 101.7 cell/μl (Mean + 2SD) which come from absolute levels of these cells in healthy subjects and is used to classify these sample as CCA (> 101.7) or healthy subject (< 101.7). ROC curve analysis showed area 72.2% of sensitivity and 93.5% of specificity for distinguishing CCA patients and healthy subjects.

Univariate analysis was used to investigate the correlation between the level of $CD14^+CD16^+$ monocytes and clinicopathological parameters. In the present study, only 37 of CCA patients had complete clinicopathological parameters and were included in this analysis. Based on the level of $CD14^+CD16^+$ monocytes in the blood, the patients were divided into two groups using mean $\pm$ 2SD from absolute levels of healthy subjects as cut-off point into low or high levels of $CD14^+CD16^+$ monocytes groups. ROC curve showed that at these Mean $\pm$ 2SD had 72.2% of sensitivity and 93.5% of specificity for distinguishing CCA patients and healthy subjects (Figure 2.4). Patients with high levels of $CD14^+CD16^+$ monocytes were significantly associated with non-papillary type of CCA ($P < 0.05$). There was no correlation between high levels of $CD14^+CD16^+$ monocytes with gender, age, tumor location, tumor staging and vascular invasion. Interestingly, levels of $CD14^+CD16^+$ monocytes in blood was correlated with the frequency of tumor associated macrophages (TAM) in CCA tissues ($P < 0.05$) (Table 2.2).

Table 2.2 The correlation of $CD14^+CD16^+$ monocyte levels and clinical parameters

		$CD14^+CD16^+$ (cells/ml) count				
Variable	**N**	**Low (%)**		**High (%)**		**P value**
Age: < 57	20	5	(25)	15	(75)	0.588
≥ 57	17	3	(17.6)	14	(82.4)	
Sex: Female	15	2	(13.3)	13	(86.7)	0.321
Male	22	6	(27.3)	16	(80)	
Tumor location: Intrahepatic CCA	20	4	(20)	13	(76.5)	0.795
Extrahepatic CCA	17	4	(23.5)	12	(66.7)	
Tumor type: Mass forming type	9	2	(22.2)	7	(77.8)	0.767
Periductal infiltrating type	20	5	(25)	15	(75)	
Intraductal Growth type	8	1	(12.5)	7	(87.5)	
Tumor Stage: I-II	10	3	(30)	7	(70)	0.752
III-IV	27	5	(18.5)	22	(81.5)	
Histology type: Non-Papillary	25	3	(12)	22	(88)	0.040
Papillary	12	5	(41.7)	7	(58.3)	
Vascular invasion: Absent	28	7	(25)	21	(75)	0.379
Present	9	1	(11.1)	8	(88.9)	
Tissue macrophages: low	12	5	(41.7)	7	(58.3)	0.040
high	25	3	(12)	22	(88)	

Overall survival was compared among CCA patients with low and high levels of $CD14^+CD16^+$ monocytes. Patients with survival under 30 days were labeled as 'peri-operative death' and were excluded from the analysis. The result demonstrated that there was no significant difference between the median survival times of CCA patients with low or high levels of $CD14^+CD16^+$ monocytes (Log Rank, P = 0.597) (Supplement data: Figure B1).

2.3.2 Expression of surface antigens differed between monocyte subpopulations

Adhesion molecules and scavenger receptors may be involved in localization of cells to a compartment like the marginal pool and/or may represent maturation level of monocyte subpopulations. A two-color immunofluorescence analysis of human monocyte from CCA patients defined two cell subpopulations according to the expression of CD14 and CD16 (Figure 1). To study whether these two subpopulations differ in surface antigen expression, a four-color flow cytometric analysis was performed. Among surface antigen studied, $CD14^{+}CD16^{+}$ monocytes expressed higher amount of CD11c, CD49d and CD54 than $CD14^{++}$ monocytes with statistical significance ($P < 0.05$, Student's *t*-test) (Table 2.3 and Figure 2.5). Expression of two scavenger receptors (CD163 and CD204) showed tendency of higher amount in $CD14^{+}CD16^{+}$ monocytes than $CD14^{++}$ monocytes; however, these differences were not statistically significant.

Table 2.3 Phenotypes of monocyte subpopulations in CCA

	$CD14^{++}$	$CD14^{+}CD16^{+}$	P value*
Adhesion Molecules			
CD11a	114.9 ± 56.4	137.3 ± 66.4	0.478
CD11c	276.6 ± 40.1	372.6 ± 54.11	0.001
CD18	738.5 ± 108.4	779.1 ± 136.5	0.521
CD29	601.2 ± 211.8	701.5 ± 267.2	0.420
CD49d	99.4 ± 25.9	176.6 ± 77.2	0.018
CD54	139.8 ± 34.7	159.7 ± 47.6	0.035
Scavenger Receptors			
CD163	35.3 ± 13.8	41.8 ± 15.0	0.399
CD204	16.1 ± 4.4	24.7 ± 12.6	0.091

*, P value represented comparison between subpopulation of $CD14^{++}$ and $CD14^{+}CD16^{+}$ using Student *t*-test.

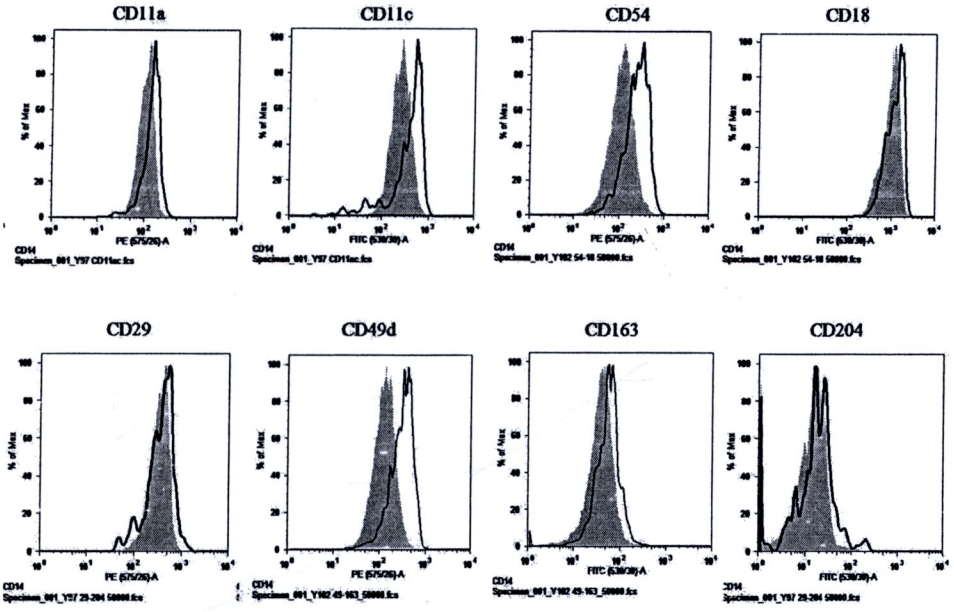

Figure 2.5 Expressions of CD11a, CD11c, CD18, CD29, CD49d, CD54, CD163 and CD204 on monocyte subpopulations. Monocyte subpopulation were defined by CD14 and CD16 labeling and expression of surface antigens was analyzed by three color immunofluorescence with $CD14^{+}CD16^{-}$ (filled histogram) and $CD14^{+}CD16^{+}$ (Open histogram)

2.3.3 Expression of growth factors and chemokines in monocyte population in CCA patients

In order to investigated the pro-tumor functions of monocyte population in CCA, we performed real-time RT-PCR of four candidate genes which were selected from our microarray data (Chapter III). These four candidate genes were growth factor (*EREG*), angiogenic (*VEGFA*, and *CXCL3*) and angiostatic (*CXCL10*) chemokine. Expression levels of these genes in monocytes from 5 CCA patients and 4 healthy subjects were compared. The results demonstrated that the EREG, and CXCL3 transcripts of monocytes from CCA patients were significantly higher whereas that of CXCL10 was lower when compared with monocytes from healthy

subjects (Figure 2.6). This evidence indicated that the monocyte population from CCA patients possessed pro-tumor function which may support CCA progression.

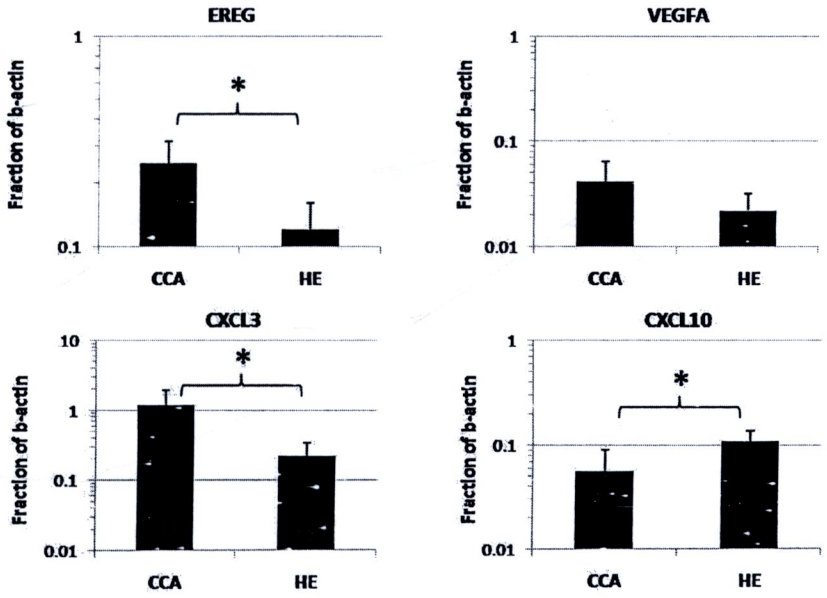

Figure2.6 Expressions of *EREG, VEGFA, CXCL3* and *CXCL10* on monocyte populations from CCA patients and healthy subjects. Monocytes from CCA patients exhibited higher expressions of EREG and *CXCL3* and lower expression of *CXCL10* significantly than those of monocytes from healthy subjects.

*, $P < 0.05$; compared between CCA patients and healthy subjects by Student's *t*-test.

2.4 Discussion

The presence of $CD14^+CD16^+$ monocytes with tissue macrophage features and the association of this subpopulation to the disease have been shown in various pathologic conditions including infection, inflammatory syndrome, sepsis, and cancers. In the present study, we demonstrated that patients with CCA displayed an increase of $CD14^+CD16^+$ monocytes in the blood. Our finding is consistent with other reports in various cancers including breast, colon, lung, melanoma and lymphoma (Saleh et al., 1995). However, the increasing levels of $CD14^+CD16^+$ monocyte subpopulation was also found in BBD patients. These observations suggest that the $CD14^+CD16^+$ monocytes level may reflect or associate with a degree of host response to significant stress. It is noteworthy that the elevation of $CD14^+CD16^+$ monocyte levels in CCA patients returned to near the level found in normal condition at least 2 months after surgical resection, suggesting a direct association of $CD14^+CD16^+$ monocytes and the tumor origin. There are many evidences reported that recombinant macrophage colony stimulating factor rhMCSF can induce elevation of $CD14^+CD16^+$ monocytes in normal subjects suggesting that patients with malignancy may have the elevated level of endogenous MCSF (Saleh et al., 1995). In addition, *in vitro* study also supported IL-10 in the presence of M-CSF and IL-4 triggers the generation of $CD14^+CD16^+$ cells from highly purified human cord blood and adult blood monocytes (Li et al., 2004; Wang et al., 2001). In general, most malignancy appears to actively immunosuppression their host via Th2 response that has produced a variety of Th2 cytokines including IL-4, IL-13, IL-10, TGF-β, etc. Thus, these evidences suggest that CCA patients may have endogenous MCSF production and/or may appear immunosuppression condition that may possibly induce elevated levels of $CD14^+CD16^+$ monocytes in CCA patients. Since the observation that high levels of $CD14^+CD16^+$ monocytes in the blood of patients was associated with poor survival type CCA-namely non-papillary type (well, moderately and poorly differentiated) is corresponded with our study reported in Chapter VI that at leading edge of tumor tissues with non-papillary type CCA had high number of tissue associated macrophages (TAM) and was associated with poor survival of patients. This observation leads us to hypothesize that $CD14^+CD16^+$ monocytes may be the monocyte subpopulation that migrates to tumor tissue and become tumor associated

macrophages. This speculation is supported by our finding that level of $CD14^+CD16^+$ monocyte in the blood was associated with frequency of TAM in CCA tissues (Table 2.2).

There are recent evidences documented that $CD14^+CD16^+$ but not $CD14^+CD16^-$ monocytes predominantly expressed Tie 2 (De Palma et al., 2005). Tie 2 is an angiopoietin receptor Tie 2/Tek found in subpopulation of human peripheral blood monocytes with marked proangiogenic activity. Angiopoietin 2 (Ang 2), a Tie 2 ligand, are found mainly in activated and angiogenic blood vessels as well as cancer cells (Venneri et al., 2007) and may induce transmigration of Tie 2/ $CD14^+CD16^+$ monocytes into the tissues (Murdoch et al., 2007; Venneri et al., 2007). These evidences support our hypothesis that $CD14^+CD16^+$ monocytes found highly in peripheral blood of CCA patients may be the subpopulation of TAM with angiogenic property.

Since monocytes obtained from CCA patients had high expression of growth factor related gene (EREG), angiogenic related genes (VEGFA, CXCL3) and low expression of angiostatic related gene (CXCL10) comparing with those of healthy monocytes (Figure 2.6), and $CD14^+CD16^+$ monocytes is highly found in CCA patients, it can be assumed that the $CD14^+CD16^+$ monocytes which expressed these markers, migrated into tumor areas and acted as a partner of tumor for promoting tumor growth and survival via their proangiogenic activity.

The data from flow cytometry revealed that the $CD14^+CD16^+$ monocytes expressed higher amount of adhesion molecules; CD11a, CD11c, CD18, CD29, CD49d, and CD54 than classical monocytes (Figure 2.5 and Table 2.3). This information implies that this monocyte subpopulation may have more ability to adhere to vascular endothelium and migrate into tumor areas. This summary is based on the fact that monocyte recruitment from the circulating blood stream to sites of inflammation involves a complex sequence of adhesion events and cytoskeletal events mediated by members of the β2-integrin family. CD11b/CD18 and CD11c/CD18 regulate the effector responses of leukocytes including adhesion (Arndt et al., 2007). The elevated expression of CD11c contributes to greater number of receptors present in clusters that are able to participate in ligand engagement and increase strength of adhesion. Thus, the increase in the level of CD11c observed in resident monocytes

that transmigrated from blood vessel to differentiate into tissue macrophages suggests a specialized role for CD11c (Georgakopoulos et al., 2008; Wu et al., 2009).

A polarization of macrophages has been delineated into M-1 and M-2 mononuclear phagocytes. The M-1(Th1 response) lineage expresses inducible nitric oxide synthase and has a high capacity to kill micro-organisms and tumor cells while the M-2 (Th2 response) macrophages scavenge debris and promote tissue repair. Tumor-associated macrophages (TAM) located in the periphery but in contact with the tumor have been described in a variety of tumors. These macrophages are of the M-2 type and express CD163, a hemoglobin scavenger receptor (Sica et al., 2006). Even it was not significantly different, the $CD14^{+}CD16^{+}$ monocytes from CCA patients in the present study showed high amount of CD163. This piece of information may initially suggest the M2 polarization occurs in CCA associated monocytes.

The existence of IL-10 in tumors has been associated with immune suppression of a Th1 response and hence increases tumorigenicity (Hori et al., 2003). IL-10, and IL-6 are cytokines which induce expression of CD163 in monocytes and macrophages (Buechler et al., 2000; Goldstein et al., 2003; Sulahian et al., 2000). Up to our search, there is no evidence of increasing IL10 in CCA patients, however, abundant amount of IL-6 has been reported in CCA patients' sera (Cheon et al., 2007; Goydos et al., 1998; Tangkijvanich et al., 2004). Therefore, it is possible that the increasing of CD163 expression detected in the resident monocytes of CCA patients may due to the activation of IL-6. However, more experiments are needed to prove this speculation.

Taken together, this study indicates that $CD14^{+}CD16^{+}$ monocytes increased in peripheral blood of CCA patients with clinical evidence of CCA. It is likely that this monocyte subpopulation was actively recruited to the inflamed or malignant tissues due to the high expression of surface adhesion molecules which are needed for interacting with endothelial cells to promote the transmigration of these cells into peripheral tissues. In addition, these $CD14^{+}CD16^{+}$ monocytes may be primed with tumor microenvironment to shift to M-2 macrophages polarization as evidenced by CD163 expression. Moreover, monocytes from CCA patients exhibited high expression of growth factor (EREG), angiogenic chemokine (CXCL3) and low

expression of angiostatic chemokine (CXCL10), which may facilitate progression of the disease. In summary, the present study suggests that the $CD14^{+}CD16^{+}$ monocytes expanded in CCA patients have pro-tumoriginic property.