Flow Cytometric Immunophenotyping of Cancer Cells in Effusion Specimens: Diagnostic and Research Applications

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Flow cytometry (FCM) immunophenotyping is frequently used as an ancillary technique for the diagnosis of hematological malignancies or for measurement of DNA content. In recent years, we applied FCM to the diagnosis of metastatic adenocarcinoma and malignant mesothelioma in effusions. We established a panel of antibodies that allows for rapid and effective differentiation between epithelial cells, mesothelial cells, and leukocytes. FCM was subsequently used for quantitative analysis of integrin subunits. Recently, we studied different parameters of the immune response, including HLA molecules and chemokine receptors, using this method. Our data suggest that FCM is an effective method for the characterization of cancer cells in clinical effusion specimens in both the diagnostic and research setting, and that this method is comparable to immunohistochemistry in terms of sensitivity and specificity, with the additional advantage of providing quantitative data. This review discusses previous work in this area and the future potential of this method in the characterization of tumor cells in serous effusions. Diagn. Cytopathol. 2007;35:568–578. © 2007 Wiley-Liss, Inc.

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The serosal cavities are frequent sites of tumor metastasis, with adenocarcinomas of the lung, breast, and ovary being the most frequent primary sites. They are also the site of origin of several tumors, including primary peritoneal carcinoma and malignant mesothelioma (MM). The cytological distinction between carcinoma cells, inflammatory cells (especially macrophages), and reactive or malignant mesothelial cells can be extremely difficult in some specimens. Immunohistochemistry (IHC) on cytopsin specimens or formalin-fixed paraffin-embedded cell block sections is currently the most widely used ancillary method in this setting and has been shown to increase the overall diagnostic accuracy in many studies.1,2 However, the use of different protocols and different antibodies or clones may lead to variable results, and some antibodies are not suitable for analysis in formalin-fixed material.

Flow cytometry (FCM) is an established method for the characterization of cells of hematological origin, and is routinely used in the diagnosis of lymphoma and leukemia.3–5 FCM immunophenotyping is rapid, reproducible, and sensitive, and is a reliable method for detecting cellular (cytoplasmic, nuclear, and surface) antigens. Multicolor FCM provides the opportunity to evaluate multiple antigens simultaneously, making it possible to characterize various cell populations in a more precise manner.

Cytological material, including effusions, is optimal for FCM analyses, since it contains single cells or small cell clusters. In effusion cytology, FCM has so far mainly been used to detect DNA-aneuploid cell populations,6–9 sometimes in combination with immunophenotyping of admixed lymphoid cells.10,11 In recent years, our group investigated the potential of FCM immunophenotyping of epithelial and mesothelial cells in effusions in the diagnostic setting, and began applying FCM to analyses of different molecules that have a role in cancer cell biology. This review presents these studies, as well as studies by other groups, and discusses the future potential of this method in research directed at cancer cells in effusions.

Studies With Focus on Effusion Diagnosis

IHC is indisputably the most widely used ancillary method in effusion diagnosis at present, and has benefited
from steady improvement in the antibody panels available.\textsuperscript{1,2} However, several diagnostic studies using FCM in cytological material have been published in recent years.

In one of the first reports in this area, Tamai et al.\textsuperscript{12} studied 26 gastric carcinomas, of which six were ascites specimens, for expression of carcinoembryonic antigen (CEA) using FCM. The authors demonstrated that detection of CEA at both the cell membrane and cytoplasm is feasible using FCM, with good correlation with IHC and the additional benefit of being able to quantitate the expression using FCM.\textsuperscript{12}

Recent studies have highlighted the role of FCM in fine-needle aspiration (FNA) cytology. Stomper et al.\textsuperscript{13} demonstrated the potential in combining analysis of DNA content and FCM immunophenotyping using antibodies against Her2/neu, transforming growth factor-\(\alpha\), and cytokeratin in the diagnosis of breast lesions. Two studies recently showed a role for FCM in diagnosing small cell carcinoma in FNA material using a panel that included CD56 [neural cell adhesion molecule (NCAM)] with or without a cytokeratin antibody.\textsuperscript{14,15} Two large series in which a total of 218 FNA specimens were analyzed showed excellent performance of FCM in differentiating lymphoid from nonlymphoid tumors in the mediastinum\textsuperscript{16} and lymph nodes.\textsuperscript{17}

These studies clearly demonstrate that FCM has a role in the diagnosis of cytological specimens.

We applied FCM for effusions diagnosis in three studies. In the first one, we used two-color FCM immunophenotyping with a limited panel of four antibodies for the detection of malignant cells in 49 effusions and peritoneal washings.\textsuperscript{18} The antibodies chosen for this study were monoclonal antibodies against Ber-EP4, CD45, CD14, and N-Cadherin. The Ber-EP4, CD45, and CD14 antibodies were preconjugated antibodies with previously observed specificity in detecting epithelial cells,\textsuperscript{19} leukocytes, and monocytes/macrophages, respectively. A non-conjugated N-cadherin antibody was chosen as marker of mesothelial cells based on studies using another N-cadherin antibody in which this protein was postulated to be specific for this cell class.\textsuperscript{20,21} FCM results were compared to those obtained based on smear and cellblock morphology, as well as IHC on paraffin-embedded cell blocks. Of the 49 specimens, 17 were cytologically diagnosed as malignant, 25 as benign, and 7 as indeterminate or suspicious for malignancy. FCM detected Ber-EP4-positive cells in 16/17 of the cytologically malignant effusions, 5/7 of the suspicious cases, and 5/25 of the specimens with benign cytology. All five Ber-EP4-positive indeterminate specimens and three of the five morphologically benign effusions showed atypical or malignant cells on reevaluation. The remaining two Ber-EP4-positive effusions contained cells shed from a benign or borderline ovarian tumor. FCM detected large numbers of CD45- and CD14-positive and relatively few N-Cadherin-positive cells in the majority of specimens compared to our impression based on morphological evaluation.

This study demonstrated the potential of FCM as an ancillary method for effusion and peritoneal washing diagnosis. FCM was able to detect carcinoma cells that have been overlooked in morphological evaluation, especially in specimens containing few cancer cells. It did, however, demonstrate that while detection of Ber-EP4-positive cells raises strong suspicion of malignancy, the evaluation must be based on morphology, in order to avoid false-positive diagnoses of malignancy in specimens containing cells shed from benign tumors, fallopian tube epithelium, or endosalpingiosis, especially in peritoneal washings.

Following our observation that macrophages constitute a larger than anticipated fraction of the cell population in effusions, we focused on this cell population in our next study.\textsuperscript{22} Ninety effusions were analyzed for the presence of CD14-positive cells using IHC in cell block sections and FCM in fresh frozen samples. Epithelial, lymphoid, and mesothelial cell populations were identified using antibodies against Ber-EP4, CD45, and N-cadherin, respectively. Thirty-nine specimens were cytologically diagnosed as nonhematological cancer, 46 were interpreted as benign, 2 as indeterminate/suspicious, and 3 as lymphoma. CD14-positive cells were detected in 94% of the effusions using FCM, and in all 90 specimens using IHC, with good concordance between these methods. Macrophages constituted up to 76% and 85% of cells using FCM and IHC, respectively. Macrophage counts were comparable in pleural and peritoneal effusions, and their levels were unrelated to the number of Ber-EP4-positive cells.

This study demonstrated that FCM and IHC have comparable sensitivity in detecting macrophages in effusions, and that high numbers of macrophages are present in both benign and malignant effusions, with a potential of being misdiagnosed as mesothelial or carcinoma cells. Beyond the diagnostic aspect, the presence of macrophages may have biological significance for cancer cell survival and tumor progression in effusions (see below).

The above two studies demonstrated that Ber-EP4 is a useful marker for the detection of carcinoma cells in effusions. However, they did not eliminate the difficulty in differentiating between benign and malignant cells of epithelial origin. Furthermore, based on the current literature, N-cadherin did not appear to be useful in the differential diagnosis between benign reactive mesothelial cells (RM) and MM. In collaboration with the Department of Pathology at Aalborg Hospital, Aalborg, Denmark, we therefore designed a new study of 92 effusions using a broader antibody panel.\textsuperscript{23} The panel including Ber-EP4, CD14, CD45, and N-cadherin, was expanded by adding a fluorochrome-conjugated antibody against epithelial membrane
antigen (EMA) and three nonconjugated antibodies against carbohydrate/glycoprotein moieties on the cell surface of tumor cells- B72.3, the AH6 antibody against the Lewisy antigen, and the HB-Tn antibody against the Tn carbohydrate antigen. EMA is a marker of both MM and carcinoma cells that we have found to be more weakly expressed in the RM surface. B72.3 was chosen because of high specificity for carcinomas in various studies by others and us.19,24–27 Other antibodies against carbohydrate epitopes have similarly been shown to be effective in staining cancer cells, in which aberrant glycosylation is frequent,28–30 including those of ovarian origin.31–34 The AH6 and HB-Tn antibodies were chosen based on IHC analysis, in which we found upregulation of their epitopes in ovarian carcinoma cells in effusion specimens.35

In cytological evaluation, 43 effusions were diagnosed as carcinoma, 42 as benign, and 7 as suspicious for nonepithelial malignancy. The use of IHC led to reclassification of 5 cytologically benign specimens as malignant and of 4 malignant epithelial specimens as benign. The 7 nonepithelial specimens were diagnosed as lymphoma (=3), MM (=3), and melanoma (=1). Using FCM, tumor cells were positive for Ber-EP4, B72.3, AH6, and HB-Tn in 64–82% of carcinomas compared to 5–29% of benign specimens, a difference that was significant for all four antibodies. Ber-EP4 and AH6 were the most sensitive markers, while Ber-EP4 was the most specific. Carcinoma cells coexpressed three of the four markers in 77% effusions compared to 7% in RM specimens, suggesting that this finding is strongly suspicious for malignancy. Cells positive for all four markers were found in 39% of carcinoma effusions and none of the reactive effusions, and this finding was therefore diagnostic for malignancy.

This study provided further evidence that FCM can be effectively used in effusion diagnosis, even when antibodies that are primarily nonconjugated are applied.

Examples of FCM immunophenotyping in the diagnostic setting are shown in Figure 1.

Our conclusion regarding these three studies is that in the diagnostic setting, FCM should probably be limited to difficult cases, where rapid and sensitive analysis is crucial, particularly when clinical suspicion of malignancy is not unequivocally supported by smear morphology and the cell block IHC findings. The use of carbohydrate antigens as additional diagnostic epitopes moderately raised the sensitivity and specificity of FCM compared to the use of Ber-EP4 alone. However, it rendered the procedure complicated due to the additional conjugation step. In practice, we used EMA and Ber-EP4 in our subsequent studies, since double-positivity for these markers is essentially diagnostic for carcinoma (MM is EMA-positive, but Ber-EP4 expression is absent or limited to few cells).

Of note, the N-cadherin antibody used in these three studies appeared to be specific for mesothelial cells. However, similarly to the carbohydrate antigens, it required an additional conjugation step and performed poorly in formalin-fixed paraffin-embedded material using IHC, making its use less attractive in routine practice. The above-mentioned Zymed N-cadherin antibody that has been described as specific for cells of mesothelial origin has shown poor specificity in differentiating MM from ovarian carcinoma and is therefore not used in the diagnostic work-up of effusions in our laboratory.

FCM in Effusion Research

Our earlier studies provided us with a reliable diagnostic panel and allowed us to study the expression of different cancer-associated molecules in subsequent work. These studies are discussed below.

Integrin Receptors

Integrins are a family of heterodimeric glycoproteins composed of α and β subunits that are involved in invasion, metastasis, angiogenesis, proliferation, and apoptosis. Intracellular signaling via integrin receptors is initiated in response to cues originating from other cells (e.g., stromal myofibroblasts) or different extracellular matrix (ECM) proteins, including laminin, fibronectin, collagen, vitronectin, entactin, tenasin, and fibrinogen, and mediates synthesis of many cancer-associated molecules.37 Altered expression of integrins (down- or upregulation) has been detected in the majority of malignant tumors, but varies considerably according to the origin of the neoplasm.38

Integrin expression and function have been investigated in a large number of studies of ovarian carcinoma.39–50 α2 and β1 integrin subunits have been shown to mediate adhesion of ovarian carcinoma cells to collagen type I.39 Three additional reports documented the role of αvβ3 and α5β1 integrins in binding to fibronectin and vitronectin, and that of the β1 subunit in binding to laminin and collagen I.40–42 The β1 integrin subunits and their ligand vitronectin has been additionally shown in normal ovarian surface cells (OSE) in two studies.40,46

Lower αvβ3 integrin protein expression was found in borderline tumors compared to invasive carcinomas,47 although not in an additional study, in which borderline tumors were grouped together with grade I invasive carcinomas and compared with grade II–III carcinomas.46 Decreased expression of the α6 and β4, and similar expression of the α2, α3, and β1 integrin subunits, was reported in a study of ascites specimens compared to solid lesions.48 Cannistra et al.42 similarly reported protein
expression of $\alpha_2$, $\alpha_3$, $\alpha_5$, $\alpha_6$, $\alpha_v$, and $\beta_1$ integrins in nine tissue and ascites specimens.

In our cohorts of ovarian carcinoma patients with effusions, $\alpha_v$ and $\beta_1$ integrin subunit protein expression was found in $>90\%$ of effusions ($n = 121$) using IHC. The $\alpha_v$ subunit was widely expressed in corresponding solid tumors, with less frequent expression of the $\beta_1$ subunit.\textsuperscript{49} In a study of solid tumors from patients with advanced-stage ovarian carcinoma with follow-up of up to 20 yr, we found more frequent $\alpha_v$ integrin subunit mRNA in carcinoma cells in tumors of short-term survivors compared to long-term survivors, with correlation between $\alpha_v$ subunit expression and poor survival in univariate and multivariate survival analyses.\textsuperscript{50}

More limited data is available regarding integrin expression in MM. Klominek et al.\textsuperscript{51} reported that integrins are expressed in MM cell lines and that they mediate migration towards ECM proteins, including collagen type IV, fibronectin, and laminin. Koukoulis et al.\textsuperscript{52} studied 22 clinical MM specimens and found that the expression of

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**Fig. 1.** Flow cytometric (FCM) immunophenotyping in effusion diagnosis. (A) A Ber-EP4-positive N-cadherin-negative ovarian carcinoma effusion. Few N-cadherin-positive mesothelial cells are additionally seen; (B,C) co-expression of Ber-EP4 with the B72.3 (B) and AH6 (C) in endometrial adenocarcinoma; (D) Co-expression of CD45 and CD14 in macrophages; (E,F) EMA-positive CD45-negative cervical carcinoma (E) and malignant mesothelioma (F).
most integrins, including the α6β4 integrin, in epithelioid MM is similar to previously reported patterns in AC of lung, and often other origin.

Although few studies have utilized FCM for studying integrin expression in effusions to date, extensive data is available regarding the potential of immunofluorescence (IF) or FCM in analyzing expression of these receptors in cancer cells in vitro and in clinical specimens. This was aided by the availability of conjugated antibodies against integrin epitopes, many of which are cluster differentiation (CD) antigens. Integrin receptor expression has been demonstrated in lung, pancreas, prostate, renal, colon, head and neck, and ovarian carcinoma using FCM or IF. We analyzed integrin expression using four-color FCM in three studies.

The first above-described study analyzed the expression of the αv and β1 integrin subunits in ovarian carcinoma. In addition to IHC, immunoblotting (IB), and mRNA in situ hybridization (ISH), we studied αv and β1 integrin subunit expression using FCM in a subset of the effusions. For FCM, the FITC-conjugated β1 and αv integrin antibodies were purchased from Dako and Santa Cruz Biotechnology, respectively. FCM showed expression of both subunits in all analyzed cases, confirming the IHC and IB results, with the advantage of providing quantitative data.

In our second study, we analyzed the expression and prognostic role of two additional ECM receptors - the 67 kd nonintegrin laminin receptor and the α6 integrin subunit, part of the α6β1 and α6β4 integrins (both laminin receptors), in ovarian carcinoma. Since laminin is a major component of basement membranes, altered expression of its receptors is involved in the metastatic process and is found in many tumors. All specimens (88 effusions, 116 solid tumors) were analyzed using ISH. Protein expression of the 67 kd receptor was studied in 24 effusions and 43 solid tumors using IHC. In the absence of a commercial antibody with proven performance in formalin-fixed paraffin-embedded tissue, we studied the expression of the α6 integrin subunit protein in 27 effusions using FCM. This analysis was executed using a PE-conjugated antibody from Santa Cruz Biotechnology. Expression of the 67 kd laminin receptor precursor mRNA and protein was frequently detected in both carcinoma and stromal cells in solid tumors, as well as in carcinoma cells in effusions, while α6 integrin subunit mRNA was less frequently detected. FCM showed protein expression of the α6 integrin subunit in 63% of the effusions compared to 41% for mRNA detection using ISH. Absence of α6 integrin subunit protein expression using FCM was associated with shorter overall survival, though not significantly.

This study showed that laminin receptor expression is generally preserved along tumor progression from primary tumor to effusion in ovarian carcinoma on both protein and mRNA level. However, tumors that show loss of α6 integrin subunit mRNA (and possibly protein) expression are associated with more aggressive disease. As with the previous study, FCM proved useful in quantification of integrin subunit expression in carcinoma cells in effusions.

The objective of our third study of integrin subunit expression was to investigate whether these receptors are differentially expressed in carcinomas of various origins, MM and RM specimens. Fresh frozen cells from 67 effusions underwent quantitative analysis of αv, α6, β1, and β3 integrin subunit expression using FCM. The material consisted of 48 carcinoma (34 ovarian, 7 breast, 7 of other or unknown origin), 7 MM, and 12 RM specimens. In addition to the 4 integrin subunit antibodies, we used antibodies against established epithelial (Ber-EP4, EMA, CD15), leukocyte (CD14, CD45) and mesothelial (EMA) cell epitopes. Frequent expression of the αv, α6, and β1 subunits was seen in carcinoma cells, as well as in MM and RM cells, with significantly higher expression of the α6 subunit in MM. Expression of the β3 integrin subunit, part of the vitronectin receptor, was not found in any of the specimens. As expected, Ber-EP4 and CD15 expression was significantly higher in carcinomas compared to RM and MM, and EMA expression was significantly higher in carcinomas and MM compared to RM specimens.

The results of this study showed that FCM is useful for comparative quantitative analyses of adhesion molecule expression in effusions containing different types of tumors. We hypothesized that the high α6 integrin subunit expression in MM may be related to preserved MM cell attachment to laminin. The frequent expression of the αv and β1 subunits and the uniform absence of the β3 integrin subunit and the uniform absence of the β3 integrin subunit expression using IHC. We hypothesized that this may have been due to the higher sensitivity of FCM in detecting low expression levels of these molecules compared to IHC, possibly as a result of antigen preservation in frozen material compared to formalin-fixed material.

Examples of FCM immunophenotyping in analyses of integrin expression are shown in Figure 2.

**Immune Response Parameters**

Recently, we studied different parameters of the immune response in effusions using FCM, in order to characterize molecules that may have a cancer-promoting effect at this anatomic site.
One of the mechanisms that cancer cells utilize in order to survive is evasion of the host immune response effectors. Cytotoxic T lymphocytes (CTL) and natural killer (NK) cells are able to identify cells that have reduced expression of major histocompatibility complex (MHC) class I molecules, receptors that are present on all normal cells, and attack them.\textsuperscript{64} However, tumor cells are able to escape the immune response by downregulating the expression of classic HLA antigens (HLA-ABC) or by expressing nonclassic antigens such as HLA-G and HLA-E.\textsuperscript{65,66}

HLA-G is present as a membrane-bound or a soluble form, and its expression in normal tissues is limited to trophoblastic cells, where it is postulated to mediate immune tolerance during pregnancy.\textsuperscript{65–68} In neoplasms, HLA-G is expressed in all trophoblastic tumors,\textsuperscript{69} and occasionally in cell lines from epithelial and nonepithelial cancers,\textsuperscript{70} as well as in clinical specimens of melanoma.\textsuperscript{71}
renal cell carcinoma, carcinoma of the lung, breast carcinoma, and lymphomas.

Elevated levels of secreted HLA-G are found in malignant compared to reactive (benign) effusions, but higher HLA-G expression in ovarian carcinoma cells in effusions using IHC predicts better survival in ovarian carcinoma.

FCM has been applied to characterization of classic HLA expression in studies of different carcinomas, including tumors of ovarian origin. It was additionally applied to analysis of HLA-G expression in cell lines of different origin, in which expression of surface HLA-G has been shown to be rare. We applied FCM to analysis of HLA-ABC expression in MM and breast carcinoma effusions.

In this study, we analyzed HLA-G expression in breast carcinoma effusions (=46) and corresponding solid tumors (=39), and in 104 MM (26 effusions, 78 solid tumors) using IHC. HLA-G protein expression was further studied in 19 effusions (10 MM, 9 breast carcinomas) using IB. RT-PCR was applied to 8 effusions (4 MM, 4 breast carcinomas) with the aim of studying HLA-G mRNA expression. HLA-ABC expression was analyzed in 15 specimens (10 breast carcinoma and 5 MM effusions) using FCM.

IHC showed predominantly focal HLA-G expression in 26% breast carcinoma effusions and 41% solid lesions. In MM, 26% solid lesions and 54% effusions were focally HLA-G-positive. Expression in MM was significantly higher in effusions. IB showed more frequent HLA-G expression in MM compared to breast carcinoma effusions, whereas RT-PCR showed HLA-G mRNA expression in all specimens from both tumor types. FCM showed conserved HLA-ABC expression in all 15 effusions analyzed.

The data from this study show that HLA-G is only focally expressed in MM and breast carcinoma, whereas HLA-ABC expression is conserved. This argues against a shift in HLA expression from the normal receptor (HLA-ABC) to the nonclassical HLA-G in these two tumors. However, the upregulated expression of HLA-G in MM effusions compared to their solid counterparts may imply a role in immune response evasion in some cases.

Our two recent studies focused on the expression of chemokine receptors in effusions. Chemokines are a family of small molecules that regulate the immune response through binding to specific receptors. Chemokines produced by cancer and stromal cells attract lymphocytes and monocytes expressing their receptors to the tumor site. However, chemokine receptors are also expressed on tumor cells, thereby creating an autocrine loop that mediates pro-growth signals (e.g., CXCL8 (Interleukin-8, IL-8) and its receptors CXCR1/2), regulates angiogenesis (CXCL8, CXCL10, CXCL12) and promotes metastasis (CXCL12 and its receptor CXCR4). Expression of the chemokines CCL2, CCL3, CCL4, CCL5, CCL8, and CCL22, and the receptors CCR1, CCR2, CCR3, CCR4, CCR5, and CCR8 was previously shown in cells isolated from ovarian carcinoma effusions. These data are supported by an additional report, in which CXCL8, CCL18, CCL-2, and CCL-3 and, to a lesser amount, CCL7 and CCL20, were detected in ascitic fluid from ovarian carcinoma patients. FCM was used in the first of these two studies.

To determine the clinical role of immune effectors and chemokine receptor expression in ovarian carcinoma effusions, we investigated the presence and prognostic role of B- and T-cells, monocytes, NK cells, and lymphocytes, as well as the expression of five chemokine receptors, in this material. Expression of leukocyte markers (CD3, CD4, CD8, CD4/CD8 ratio, CD16, CD19, and CD14) and chemokine receptors (CXCR1, CXCR4, CCR2, CCR5, and CCR7) was studied in 73 effusions using FCM. CXCR4, CCR5, and CCR7 were abundantly expressed on leukocytes, but all receptors were rarely expressed on cancer cells. The presence of NK cells and FIGO stage IV disease predicted worse overall survival. Higher percentage of CD19-positive cells and stage IV disease predicted poor survival for patients with post-chemotherapy effusions. Chemokine receptors expression on lymphocytes and monocytes had no prognostic role in our series.

Our data showed that the majority of the studied immune response parameters are weak predictors of survival in ovarian carcinoma effusions, and that the presence of B cells and NK cells in effusions correlates with worse rather than improved outcome, suggesting that the immune response is inefficient if not harmful for the patients at this anatomic site. The data additionally showed that chemokine receptors are widely expressed on leukocytes, but rarely on carcinoma cells in ovarian carcinoma effusions, arguing against an autocrine chemokine pathway for cancer cells in this malignancy.

A recent study by another group using a cytokine array system showed that the supernatant of cultured MM cells and the effusion fluid contain 25 different chemokines, including ligands of the receptors analyzed in the present study—CXCL6 (CXCR1 ligand), CXCL12 (CXCR4 ligand), CCL2, CCL7, CCL8 and CCL13 (CCR2 ligands), and CCL4 and CCL5 (CCR5 ligands). We were therefore interested in characterizing the cells that express chemokine receptors in MM effusions and analyze the potential differences in leukocyte levels and chemokine receptor expression in MM versus RM effusions. Expression of the above-detailed leukocyte markers and chemokine receptors was studied in 11 MM and 16 RM specimens using FCM. MM specimens showed significantly lower lymphocyte counts and higher monocytes numbers compared to RM effusions. CXCR1 and CXCR4 expression was significantly higher in MM monocytes.
Chemokine receptors were infrequently expressed in MM, while RM cells were uniformly negative. Similar findings for both leukocytes and tumor cells were seen in metastatic adenocarcinomas of uterine cervical and endometrial origin. These findings showed that as in ovarian carcinoma, chemokine receptors are widely expressed on leukocytes in MM and RM effusions, but are infrequently found on cells of mesothelial origin, supporting the presence of an autocrine chemokine pathway in leukocytes, but not in MM cells. We hypothesized that the increased monocyte infiltration and their higher chemokine receptor expression in MM effusions may have a tumor-promoting rather than inhibiting effect. The latter conclusion is in agreement with recent literature detailing the pro-tumor effect of these cells that is largely mediated through production of a myriad of molecules that promote tumor growth, invasion and metastasis.88,89

Examples of FCM immunophenotyping in the analysis of immune response parameters are shown in Figure 3.

**Conclusion and Future Directions**

Our growing experience with FCM immunophenotyping suggests that this procedure is reliable as an ancillary method for effusions diagnosis. Not less significantly, our
studies demonstrate that cancer-associated molecules and different parameters related to tumor biology can be studied in effusion material using this method. This is not surprising in view of the extensive use of FCM in in vitro experiments. Although other researchers have been applying FCM to effusion analysis in recent years in both the diagnostic and research setting, the potential of this tool is still unfulfilled. Our group currently focuses on additional parameters of the so-called hallmarks of cancer (e.g., proliferation and inhibition of apoptosis) using FCM, with the hope of better understanding the mechanisms that allow cancer cells to survive at this anatomic site. Since metastatic cells are the ones responsible for patient morbidity and mortality in the majority of tumors, we believe that any effort to counteract the lethal effects of cancer needs to focus on this cell population. Effusions provide ideal material for this purpose.

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