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CD16⁺ Monocytes in Patients With Cancer: Spontaneous Elevation and Pharmacologic Induction by Recombinant Human Macrophage Colony-Stimulating Factor

By Mansoor N. Saleh, Samuel J. Goldman, Albert F. LoBuglio, Arthur C. Beall, Hernan Sabio, Melissa C. McCord, Lori Minasian, R. Katherine Alpaugh, Louis M. Weiner, and David H. Munn

The small subset of circulating monocytes that express the maturation-associated CD16 antigen has recently been reported to be elevated in patients with bacterial sepsis. We now show that this novel CD16⁺ monocyte population is also spontaneously expanded in patients with cancer. We studied 14 patients with metastatic gastrointestinal carcinoma enrolled in a clinical trial of recombinant human macrophage colony-stimulating factor (rhMCSF) plus monoclonal antibody D612. We found that before any cytokine treatment, 12 of 14 patients constitutively displayed significant elevations in both the percentage and the absolute number of CD16⁺ monocytes, as compared with both normal subjects and ill patients with elevated monocyte counts but without malignancy. CD16⁺ monocytes accounted for 46% \pm 22% of total monocytes in the patients with cancer versus 5% \pm 3% for controls ($P < .01$). The increase was not attributable to infection or intercurrent illness and appeared to be associated with the underlying malignancy itself. A similar

spontaneous elevation of CD16⁺ monocytes was observed in 35 of 44 additional patients diagnosed with a variety of other solid tumors. When patients with gastrointestinal carcinoma were treated with rhMCSF, there was a marked further increase in the percentage of CD16⁺ monocytes (to 83% \pm 11%), as well as in the absolute number of CD16⁺ cells and the level of CD16 antigen expression. In every case, the patients with cancer showed a greater CD16⁺ monocyte response than the maximal response obtained in normal volunteer subjects treated with a similar regimen of rhMCSF ($n = 5$, $P < .001$), suggesting that the presence of malignancy primed patients for enhanced responsiveness to rhMCSF. We hypothesize that spontaneous expansion of the CD16⁺ monocyte population may represent a novel biologic marker for a widespread and previously unsuspected host immune response to malignancy.

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THE CD16 ANTIGEN is the low-affinity IgG receptor, Fc γ RIII, originally described on natural killer (NK) cells and granulocytes.^{1,3} In the monocytic lineage, CD16 was initially believed to be restricted to a subset of mature macrophages,^{2,4} where it apparently plays an important role in phagocytosis.⁵⁻⁷ Recently, however, CD16 has also been found on a small subset of circulating monocytes, representing less than 10% of total monocytes, or approximately 1% of white blood cells.^{8,9} The functional significance of this small CD16⁺ population is unknown. The only clinical setting in which CD16⁺ monocytes have been shown to be elevated is that of bacterial sepsis,¹⁰ and even under these conditions, the elevation was found to be transient.

We have previously reported that administration of recombinant human macrophage colony-stimulating factor (rhMCSF) to cynomolgus monkeys causes a marked in-

crease in the number of CD16⁺ monocytes, to the point that this phenotype may come to constitute the majority of monocytes in circulation.¹¹ In this report, we extend these observations to human subjects. We examined the expression of CD16 on monocytes taken from normal subjects and from patients with cancer, and compared the constitutive level of CD16 expression in these two groups and the response of each to treatment with rhMCSF.

MATERIALS AND METHODS

Patients and rhMCSF regimens. Five healthy adult subjects were treated with rhMCSF (Genetics Institute, Cambridge, MA), 100 μ g/kg/d continuous intravenous (iv) infusion for 14 days, under an Institutional Review Board-approved protocol after appropriate informed consent. Fourteen patients with metastatic gastric, colon, and rectal carcinoma were enrolled in an Institutional Review Board-approved phase II clinical trial of rhMCSF (80 μ g/kg/d continuous iv infusion for 14 days) plus monoclonal antibody (MoAb) D612¹² administered on days 4, 7, and 11 of rhMCSF treatment.¹³ All patients had a Karnofsky performance status greater than 70, were free of infection or other intercurrent illness, and were greater than 30 days past their last exposure to chemotherapy, radiation, or immunosuppressive drugs. All patients had baseline monocyte immunophenotype analysis performed; 12 of 14 had repeat analysis on day 11 of rhMCSF treatment (two patients were not assessable due to technical difficulties).

The doses of rhMCSF used in these two studies produced similar hematologic changes and were treated as comparable for purposes of this report. In phase I trials, D612 has not been observed to display any hematologic toxicities,¹⁴ and the on-therapy immunophenotyping sample was obtained 72 hours after the preceding dose of antibody, so we considered the observed hematologic changes to be a result of rhMCSF rather than MoAb.

Forty-four patients were studied at two additional centers (14 at the Medical College of Georgia, Augusta, GA, and 30 drawn from a study of rhMCSF plus γ -interferon conducted at Fox Chase Cancer Center, Philadelphia, PA¹⁵ to assess the incidence of spontaneous CD16 expression in patients with malignancy. These patients had a

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variety of solid tumors including breast, lung, colon, and renal cell carcinomas, lymphoma, melanoma, and soft-tissue sarcomas. All were studied before initiation of treatment (either with newly diagnosed disease or relapsed off therapy for greater than 1 month).

Normal values for CD16 expression were established based on 14 samples with normal complete blood counts (CBCs) and differential cell counts and without known malignancy. Mean CD16 expression in this group was 5% \pm 3% of total monocytes; the upper limit of normal was considered 15% (+3 SD), which agrees with published values.⁸ For comparison, samples were also obtained from patients with monocytosis (greater than 15% monocytes on CBC or an absolute monocyte count greater than 1,000/ μ L) due to systemic illnesses other than cancer (infection, rheumatologic disorders, multiple trauma, and others).

Flow cytometry. Monocytes were identified based on the criteria of forward and right-angle light scatter as previously described.¹¹ This is an accepted approach that has been used in other investigations to study CD16⁺ monocytes.^{8,10} The reliability of these gates in our study was shown by the fact that the cells falling in the putative monocyte gate expressed FcRI (87% \pm 13%), CD11b (95% \pm 6%), CD14 (81% \pm 12%), and HLA-DR (78% \pm 11%), unambiguously identifying them as monocytes (data from 22 patient samples). Cells were always analyzed before any adherence step, as we found that adherence could cause unpredictable downmodulation of CD16 and other antigens (R.K.A., L.M.W., and D.H.M., unpublished observations, November 1994).

In the normal volunteer study, two-color direct immunofluorescent staining was performed by standard whole-blood lysis technique (FACS-Lyse, Becton-Dickinson, Mountain View, CA). CD14 was used in one color to identify monocytes,¹⁶ and in the second color, antibodies against CD16 (Leu11c, clone B73.1,¹⁷), CD11a/LFA-1, CD11b/Mac1, CD11c, HLA-DR, Fc γ RI (Medarex, West Lebanon, NH), Fc γ RII (Medarex), CD2, CD19, CD56, and isotype-matched controls were used. All antibodies were from Becton Dickinson unless otherwise noted.

To directly confirm the identity of the CD16⁺ cells as monocytes, other samples were analyzed by three-color staining using CD14 versus CD16 plus the panel of antibodies listed above in the third color. Based on our experience with these techniques, in the gastrointestinal carcinoma trial, we used a simplified approach using single-color staining with antibodies against CD16 and HLA-DR. Monocytes were identified based on light-scatter criteria as above, and HLA-DR expression was used to confirm that CD16⁺ cells in the monocyte gate were not contaminating granulocytes.

We emphasized the percentage of CD16⁺ monocytes as an outcome measure rather than the absolute number of CD16⁺ cells. Although we present data to show that rhMCSF affected both of these variables, derivation of absolute values requires that the number of monocytes in peripheral blood be accurately known. We have recently found that routine CBCs (such as those used in our studies) may not identify up to 75% of monocytes in patients treated with rhMCSF, as compared with detection by flow cytometry.^{17a}

Cytotoxicity and tumor cell-binding studies. Antibody-dependent cytotoxicity (ADCC) was measured by indium release assay. Monocytes were purified by adherence to gelatin/plasma-coated plastic flasks¹⁸ and tested at an effector:target ratio of 5:1 against the colon carcinoma cell line SW948 [American Type Culture Collection (ATCC), Rockville, MD] with and without opsonizing antibody (17-1A, 5 μ g/mL), essentially as described.¹⁹ Antibody-independent cytotoxicity was measured by thymidine release assay. The entire mononuclear cell fraction was prepared by Ficoll-Hypaque centrifugation²⁰ and tested against the NK cell-resistant leukemia line ARH-77 (ATCC) as described.²¹ For tumor cell-binding studies, monolayers of SW948 were prepared in tissue culture plates. Adherence-

isolated monocytes were labeled with [⁵¹Cr]-chromate (Amersham, Arlington Heights, IL) and allowed to adhere to the monolayers for 1 hour at 37°C, and nonadherent monocytes were removed by washing. The percentage of monocytes adhering to tumor cells was calculated as the fraction of total radioactivity bound to the monolayer after washing.

Monocyte culture. Human monocytes were isolated by Percoll gradient centrifugation and plastic adherence and then cultured in 200 U/mL rhMCSF as previously described.²¹ Differentiating macrophages (M ϕ s) were tested at intervals for development of cytotoxicity (ADCC against SK-MEL-31 in the presence of MoAb 3F8) by enzyme-linked immunoassay, as described.²² Mature cells (day 7) were harvested with 2 mmol/L EDTA and stained for expression of Fc γ R as described above.

Statistical analysis. Means of the two groups were compared by *t*-test. Because we did not know whether CD16 expression was normally distributed, we also compared these data nonparametrically by Wilcoxon paired-rank test (for pre- *v* post-rhMCSF samples) or Wilcoxon rank-sum tests with a Bonferroni adjustment for multiple comparisons (patient groups *v* control groups).

RESULTS

Preferential expansion of the CD16⁺ monocyte population by rhMCSF in normal subjects. Treatment of normal subjects with rhMCSF induced a significant increase in the overall number of circulating monocytes, from 542 \pm 313/ μ L on day 0 to 2,370 \pm 1,160/ μ L on day 14 (*P* < .01). Most of this increase was attributable to a greater than 20-fold expansion of the CD16⁺ monocyte population, from 45 \pm 42/ μ L to 1,100 \pm 770/ μ L (*P* < .001), as shown in Fig 1. By day 14, CD16⁺ monocytes comprised 29% to 57% of total monocytes. Most CD16⁺ monocytes also expressed reduced levels of the CD14 antigen (CD14^{dim}), a feature that has been identified as characteristic of these cells.⁸

To define the immunophenotype of the CD16⁺ monocytes, we examined a panel of additional antigens using three-color flow cytometry. Monocytes were identified by light-scatter gates as described and then further gated into the CD16^{pos}/CD14^{bright} (conventional monocyte) and CD16⁺/CD14^{dim} populations shown in Fig 1. As shown in Fig 2, both subpopulations expressed each of the monocyte markers tested (thus confirming their monocytic origin), but the CD16⁺ cells showed a reproducible pattern of quantitative differences compared with conventional monocytes. This included increased expression of HLA-DR, CD11a, and CD11c, with decreased levels of CD11b and FcRI. (In addition to the markers shown, both populations were negative for CD2, CD19, and CD56.)

CD16⁺ monocytes are spontaneously elevated in patients with metastatic carcinoma. We next examined samples from 14 patients with metastatic gastrointestinal carcinoma enrolled in a phase II trial of rhMCSF plus MoAb D612. Unexpectedly, we found that the majority (12 of 14) of these patients had significantly elevated levels of circulating CD16⁺ monocytes present at baseline. As shown in Fig 3, the percentage of CD16⁺ monocytes expressed spontaneously by the patients with cancer was comparable with the maximum levels induced by prolonged rhMCSF administration in normal subjects. Although patients with cancer and normal subjects had similar numbers of circulating monocytes at base-

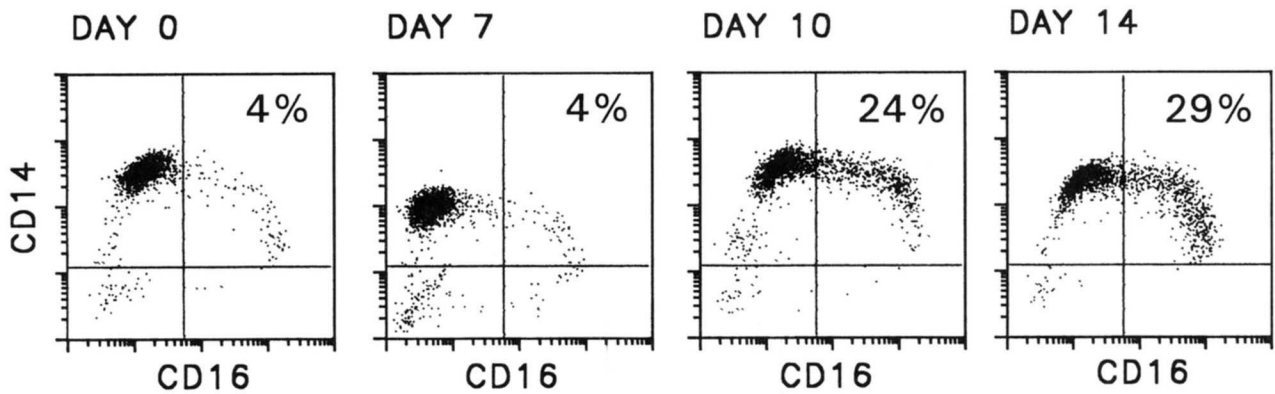


Fig 1. Progressive emergence of the CD16⁺ monocyte phenotype in subjects treated with rhMCSF. Normal volunteer subjects were treated with rhMCSF as described in Materials and Methods, and peripheral blood was obtained for immunophenotyping on the days indicated. The dot plots show monocytes, identified by light-scatter criteria, stained for expression of the CD14 and CD16 antigens. Representative of five subjects.

line ($418 \pm 129/\mu\text{L}$ v $540 \pm 420/\mu\text{L}$, respectively), the patient group had four times as many CD16⁺ monocytes ($240 \pm 137/\mu\text{L}$ v $45 \pm 42/\mu\text{L}$, $P < .01$).

The CD16⁺ monocyte population is further increased by administration of rhMCSF. Figure 3 also shows that the administration of rhMCSF to the patients with cancer induced a marked further increase in the percentage of CD16⁺ monocytes, from $45\% \pm 23\%$ to $83\% \pm 11\%$, which was significantly higher than the on-therapy mean for the normal subjects ($43\% \pm 11\%$, $P < .001$). Even the two patients

whose baseline CD16⁺ percentages were initially in the normal range showed dramatic increases, from 9% to 81% and 14% to 70%, respectively. In addition to the percentage of CD16⁺ monocytes, the absolute number of CD16⁺ cells also increased, from a mean $240 \pm 137/\mu\text{L}$ (range, $107/\mu\text{L}$ to $630/\mu\text{L}$) to $582 \pm 346/\mu\text{L}$ (range, $150/\mu\text{L}$ to $1,357/\mu\text{L}$, $P < .01$).

We next compared the level of CD16 expression on individual monocytes before and after rhMCSF treatment, assessed as changes in the relative proportion of CD16^{dim} and

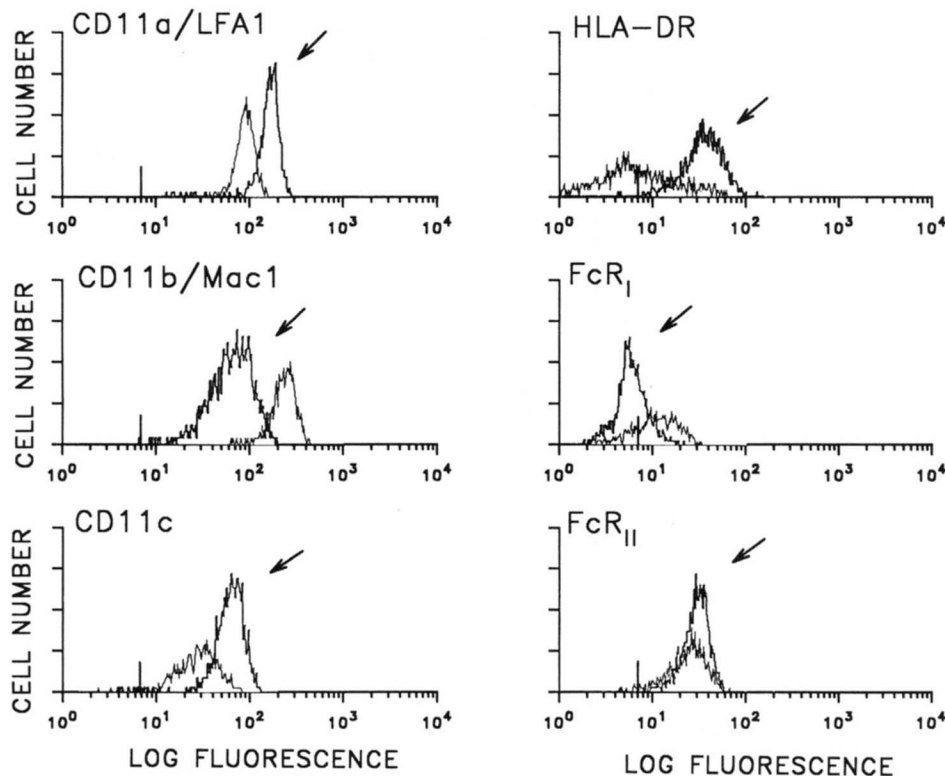


Fig 2. Immunophenotype of the CD16⁺ monocyte population. The rhMCSF-induced CD16⁺ monocytes were analyzed by three-color flow cytometry using CD14, CD16, and a panel of three color antibodies. The histograms compare the third color staining of the CD14⁺/CD16^{neg} (conventional monocyte) subpopulation and the CD14^{dim}/CD16⁺ novel population (marked with an arrow) from the same sample. Negative control antibodies have been omitted for clarity; the upper limit (98%) of negative is shown as a vertical tic on the horizontal axis. Identical phenotypes were obtained in eight of eight subjects tested on days 8 through 14 of rhMCSF administration.

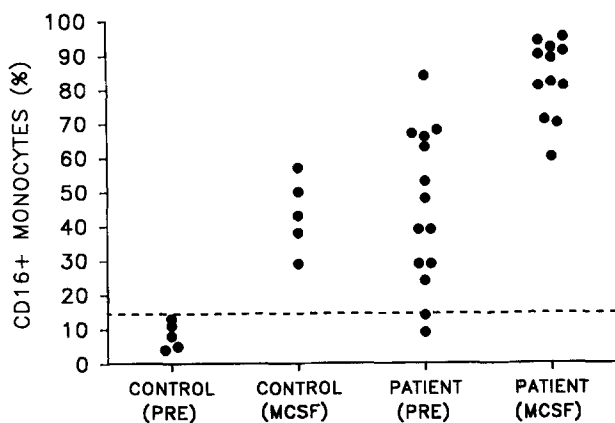


Fig 3. Spontaneous elevation of the CD16⁺ monocyte population and enhanced responsiveness to rhMCSF in patients with gastrointestinal carcinoma. Monocytes were identified by light-scatter gates. The level of CD16⁺ monocytes, expressed as the percentage of total monocytes, was assessed by single-color flow cytometry before and after 11 days of rhMCSF treatment. For comparison, data from the normal subjects studied in Fig 1 (labeled control) are also shown before and after 14 days of rhMCSF treatment. The upper limit of normal for spontaneous expression of CD16⁺ monocytes in our laboratory is shown as a dashed line. Both groups showed a significant increase in CD16⁺ monocytes after rhMCSF treatment compared with baseline values ($P < .01$ by Wilcoxon paired rank test), and the patient group was significantly higher than the control group in both pre-treatment and on-therapy samples ($P < .01$ by Wilcoxon rank sum test).

CD16^{bright} cells. We found that at baseline all patients displayed a mixture of CD16 negative, dim, and bright monocytes. After treatment, the proportion of CD16^{bright} cells was increased in all assessable patients and fell into one of two patterns: in 4 of 12 samples, there were both dim and bright cells present after treatment, while in 8 of 12 samples, the monocytes became almost exclusively CD16^{bright} (Fig 4). This striking predominance of CD16^{bright} cells was not observed in any of the subjects in the normal volunteer study ($P < .05$ by Mann-Whitney test).

CD16 expression in cancer and in nonmalignant disorders. We prospectively examined monocyte CD16 expression in studies at two additional institutions, studying 44 patients with neoplasms of various types (breast, lung, colon, and renal cell carcinomas; lymphoma; melanoma; and soft-tissue sarcomas). Patients were studied before initiation of therapy, and all were ambulatory and free of intercurrent illness. As a comparison population, we analyzed patients who were systemically ill (infection, trauma, etc) and had elevated monocyte counts, but who had disorders other than cancer. As shown in Fig 5, in both studies the patients with cancer had a significantly elevated percentage of CD16⁺ monocytes compared with controls.

Functional effects of rhMCSF treatment. We wanted to determine whether the presence of a spontaneous CD16⁺ monocyte population was associated with enhanced antitumor activity. As we have previously shown, circulating monocytes display little cytotoxicity unless allowed to differentiate into monocyte-derived M ϕ s.²¹ We obtained samples

($n = 33$) from patients with malignancy at various times before therapy, during treatment, and in remission. Mononuclear cells from these samples were simultaneously assayed for monocyte CD16 expression and for cytotoxicity by thymidine release assay against the NK cell-resistant cell line ARH-77, as described in Materials and Methods. (In these experiments, the entire mononuclear cell fraction was used to ensure detection of cytotoxicity, whether in the adherent or nonadherent population.) As expected, we found little detectable cytotoxicity by fresh monocytes (specific release, $9\% \pm 7\%$), and there was no correlation between cytotoxicity and the number of CD16⁺ monocytes [$r = .20$; P value not significant (NS)]. Similar negative results were obtained in a second study, this time using adherence-purified monocytes and indium-release assays to measure ADCC (SW948 colon carcinoma, 17-1A antibody; ADCC, $-2\% \pm 7\%$). As both of these target cells are susceptible to killing by monocyte-derived M ϕ s after they have differentiated in vitro in response to rhMCSF (unpublished results, November 1994), the failure of fresh monocytes to mediate effective cytotoxicity appeared to be attributable to their immaturity.

We next asked whether there was an effect of rhMCSF treatment on monocyte functional properties. As shown in Fig 6, monocytes isolated from patients after treatment with rhMCSF showed a significantly enhanced ability to bind to monolayers of SW948 carcinoma cells compared with pretreatment values, and this effect was independent of antibody opsonization. However, despite the increased binding, fresh monocytes again showed no detectable ability to kill the tumor cells (nine patients studied on day 8 of rhMCSF treatment by indium release assays; ADCC, $-1\% \pm 4\%$).

Finally, we asked whether CD16 expression on circulating monocytes might represent a more mature, rhMCSF-induced monocyte phenotype. As shown in Fig 7A, normal monocytes differentiating in vitro under the influence of rhMCSF acquired a pattern of Fc γ R expression similar to that seen on rhMCSF-induced CD16⁺ circulating monocytes (increased Fc γ RIII/CD16, stable Fc γ RII, reduced Fc γ RI; eg, the CD16⁺ population shown in Fig 2). However, monocytes differentiating in vitro also underwent morphologic maturation into M ϕ -like cells²¹ and progressively acquired antitumor cytotoxicity (Fig 7B). Neither change was apparent in circulating CD16⁺ monocytes induced by rhMCSF in vivo.

DISCUSSION

In this report we describe the selective expansion of a specific cell population, CD16⁺ monocytes, in individuals with cancer. This uncommon phenotype is known to be elevated in patients with bacterial sepsis,¹⁰ and we have observed it in some patients with systemic illnesses accompanied by monocytosis (Fig 5). Both observations suggest that the phenotype is associated with a host response to significant stress. We now report the unexpected finding that patients with cancer frequently display a similar increase in CD16⁺ monocytes, comparable in magnitude to that induced by overwhelming sepsis,¹⁰ at a time when they are clinically stable, are free of intercurrent illness, and have normal CBCs.

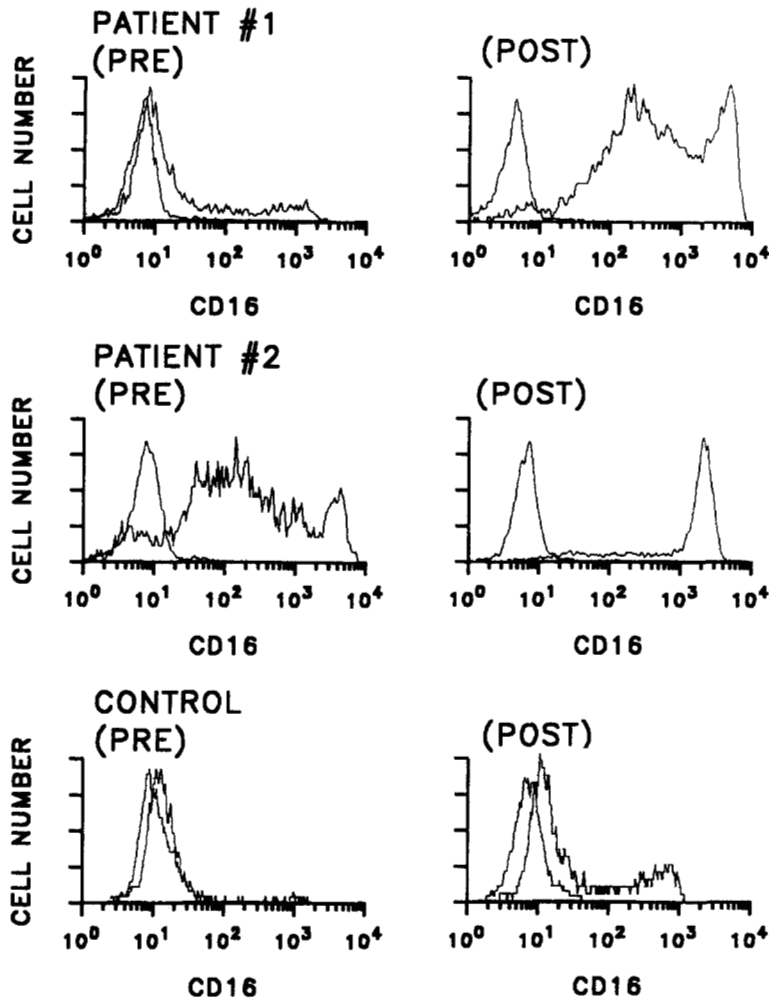


Fig 4. Two patterns of CD16 induction by rhMCSF. Treatment with rhMCSF produced an increase in CD16 expression in all patients studied, resulting in either a pattern of mixed dim and bright cells (patient 1, representative of 4 of 12 patients) or a pattern of essentially exclusively bright cells (patient 2, representative of 8 of 12 patients). The latter pattern was unique to patients with cancer and was not seen in the normal subjects (control, representative of five of five subjects). HLA-DR expression was greater than 90% in all samples shown, confirming that the CD16 bright cells were monocytes.

Some malignancies (eg, those arising from the immune system) may produce symptoms suggestive of low-grade inflammation. In general, however, most tumors elicit little immunologic reaction²³ and often appear to actively immunosuppress their host.²⁴ Thus, it was unexpected to find evidence of a constitutive immune response occurring in a large majority of patients with cancer, particularly a response previously associated only with conditions such as overwhelming sepsis. Furthermore, the occurrence of this phenotype in a variety of patients with many types of tumors suggests that it may represent a fundamental reaction to the presence of malignancy per se. To our knowledge, evidence of this type supporting a widespread and apparently fundamental host response to malignancy has not been previously reported.

We show that a phenotypically similar CD16⁺ monocyte population can be pharmacologically induced in normal subjects by administration of exogenous rhMCSF. This raises the question of whether patients with malignancy might have elevated levels of endogenous rhMCSF. Such increases have been reported in pregnancy and idiopathic thrombocytopenic purpura,²⁵ and a recent study in mice suggests that tumors may also trigger host MCSF production.²⁶ However, while

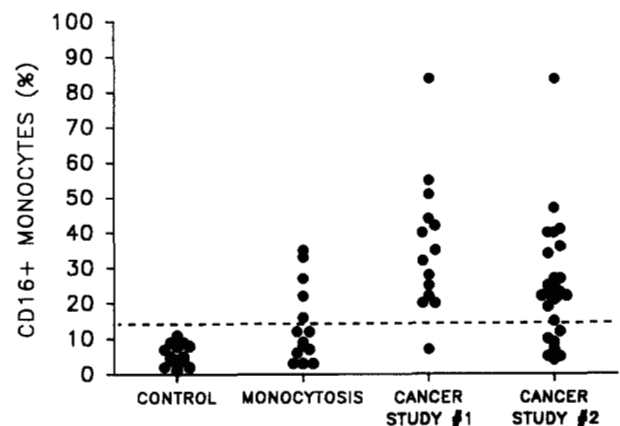


Fig 5. Spontaneous elevation of CD16⁺ monocytes in patients with malignancy. Additional patients with a variety of neoplasms were tested prospectively for CD16 expression (14 in study 1, 30 in study 2). Fourteen normal subjects (control) and 14 patients with systemic illness and elevated monocyte counts but without malignancy (monocytosis) are also shown. The control population was significantly different from the two patient populations by pairwise Wilcoxon rank sum tests ($P < .01$).

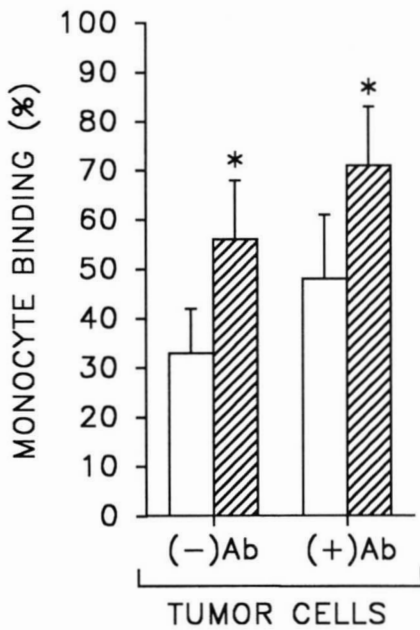


Fig 6. Enhanced binding of monocytes to tumor cells after treatment with rhMCSF in vivo. Adherence-purified fresh monocytes were tested for binding to SW948 monolayers in the presence and absence of 17-1A antibody. Bars show mean values for eight patients before (□) and after (▨) 7 days of rhMCSF administration (100 μg/kg/d). **P* < .01 compared with pretreatment value.

we have begun prospective studies to measure MCSF levels in patients with cancer, the actual relationship (if any) between such serum levels and the hematologically relevant concentration of MCSF in the bone marrow remains contro-

versial.^{27,28} We speculate that one mechanistically relevant observation may be that of Chen:²⁸ systemic cytokines such as interleukin-1 can trigger local production of MCSF in the bone marrow microenvironment.²⁸ Because such local MCSF production cannot be accurately quantitated by serum measurements, and (more importantly) because the biologically active forms of MCSF may not be secreted at all,^{29,30} a biologic marker such as CD16 expression may provide an important functional insight into the local hematopoietic microenvironment.

A second question raised by our data is whether the effect of rhMCSF on CD16 expression was truly greater in the patients with cancer than in the normal subjects, or was merely an additive effect superimposed on the preexisting CD16⁺ population. We note, however, that the majority of patients with cancer treated with rhMCSF achieved a high percentage of CD16⁺ monocytes, significantly greater than that induced in normal subjects, and that this occurred even in patients without a large preexisting CD16⁺ population. Moreover, the majority of patients with cancer converted to an exclusively CD16^{bright} phenotype, which was not seen in any of the normal volunteers. Thus, it appeared that the patients were in some way primed by their disease to respond to rhMCSF more vigorously than normal subjects, suggesting a form of immunologic preactivation.

Although CD16 is revealing as a marker, the functional properties of the CD16⁺ monocyte population are not yet known. CD16 has been proposed as a developmental marker for Mφ maturation,⁴ and Ziegler-Heitbrock et al, have shown that circulating CD16⁺ monocytes display a phenotype suggestive of maturation toward tissue Mφs,³¹ which would be consistent with our in vitro model of MCSF-induced differentiation (Fig 7). We speculate that the appearance of CD16

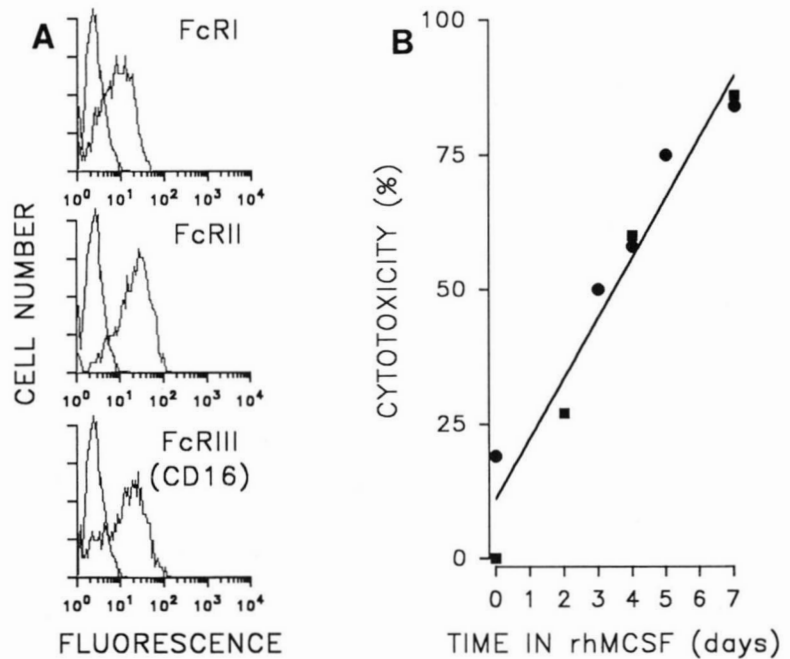


Fig 7. In vitro differentiation of monocytes in rhMCSF. Monocytes from normal donors were allowed to differentiate in vitro for 7 days in the presence of 200 U/mL rhMCSF, as described in Materials and Methods. (A) Pattern of FcγR expression after maturation (representative of four experiments). (B) Progressive acquisition of antitumor cytotoxicity (ADCC measured against SK-MEL-1) during differentiation [pooled values for two identical experiments (● and ■), representative of a total of five experiments].

on circulating monocytes may reflect accelerated maturation in the bone marrow. However, the fact that the circulating CD16⁺ monocytes do not yet display the efficient antitumor activity associated with mature MCSF-activated M ϕ s suggests that the maturation process was still in an early or transitional stage. CD16⁺ M ϕ s have recently been identified as the predominant immunologic cell type infiltrating a variety of solid tumors,³² and we have shown that CD16 is functionally important in mediating M ϕ antitumor ADCC.⁶ However, whether circulating CD16⁺ monocytes subsequently differentiate into cytotoxic M ϕ s in vivo can only be determined by future studies of tumor-infiltrating M ϕ s.

The significance of the present study is that it documents for the first time the existence of a novel biologic marker, a marker that reveals a widespread and previously unsuspected host response to malignancy. We propose that this CD16⁺ monocyte population offers a readily accessible, quantifiable means by which to identify and follow this formerly unmeasurable host response.

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