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Recent studies suggest that monocytes are the dominant effectors by which rituximab induces cell death in B-cell lymphoma. Because macrophage colony-stimulating factor (M-CSF) can enhance the cytotoxicity of monocytes, the authors examined whether this growth factor can enhance their ability to kill lymphoma cells *in vitro*. Monocytes derived from a healthy volunteer were cultured for 48 h in the presence or absence of M-CSF. Monocytes stimul ated with M-CSF were significantly more cytotoxic to Daudi B-cell lymphomas than unstimulated monocytes. Flow cytometry revealed that M-CSF increased monocyte expression of Fcγ receptors III and I by 1.6- and 1.5-fold, whereas the expression of Fcγ receptor II remained unchanged. These results suggest that pretreatment with M-CSF can improve the therapeutic efficacy of rituximab against intractable CD20⁺ lymphoma. (*Cancer Sci* 2007; 98: 1368–1372)

Rituximab, a chimeric anti-CD20 IgG1 monoclonal antibody, has dramatically improved the treatment of both follicular and aggressive CD20⁺ B-cell non-Hodgkin lymphomas.[1–5] However, the advantage of rituximab is not whole. A substantial number of patients suffer from relapse after rituximab containing chemotherapy or refractoriness to it.[1–5] Approximately half of patients with relapsed or refractory non-Hodgkin lymphoma do not exhibit a durable clinical response to rituximab, despite continued expression of CD20 by lymphoma cells.[1,3,6]

Although the means by which rituximab inhibits the growth of lymphoma is not fully understood, accumulating evidence indicates that it is mostly mediated by antibody-dependent cellular cytotoxicity (ADCC) rather than induction of apoptosis and complement-dependent cytotoxicity.[7–12] In addition, studies in a mouse model of rituximab immunotherapy revealed that B cells depletion via ADCC is mostly mediated by monocytes rather than T or natural killer cells.[13]

Macrophage colony-stimulating factor (M-CSF), also known as colony-stimulating factor 1, promotes the differentiation of progenitor cells into mature monocytes and macrophages and prolongs macrophage and monocyte survival.[14–20] Previous studies have demonstrated that M-CSF activates monocytes, leading to greater ADCC against target cells, including human lymphoma and leukemic cell lines.[18,19] In the present study, the authors investigated whether M-CSF can enhance the ability of monocytes to kill CD20⁺ lymphoma cells via a rituximab-dependent mechanism.

Materials and Methods

Cell lines. Daudi Burkitt's lymphoma cells, Molt-4 acute lymphoid leukemia cells, and THP-1 acute monocytic leukemia cells were purchased from the Riken Cell Bank (Tsukuba City, Japan). Daudi cells express CD20 on their surface, whereas Molt-4 cells lack cell surface CD20. Cell lines were cultured in complete medium, which contained Roswell Park Memorial Institute (RPMI) 1640 (Gibco Laboratories, Grand Island, NY, USA), 10% heat-inactivated fetal bovine serum (Gibco Laboratories), 2 mmol/L l-glutamine (Gibco Laboratories), and penicillin-streptomycin (Gibco Laboratories). Cells in the late logarithmic phase of growth were passaged regularly every 4–5 days prior to cytotoxicity assays. The cell lines used for experiments were ≥90% viable according to Trypan blue exclusion.

Monoclonal antibodies and cytokines. The chimeric anti-CD20 monoclonal antibody rituximab was purchased from Roche Pharmaceuticals (Basel, Switzerland). Mouse fluorochrome-conjugated isotype control antibodies, phycoerythrin-conjugated anti-CD32 and anti-CD14, and fluorescein isothiocyanate-conjugated anti-CD64 and anti-CD16 antibodies were purchased from BD Biosciences (San Jose, CA, USA). Anti-CD16 blocking antibody was purchased from Abcam (Cambridge, UK), anti-CD32 blocking antibody from StemCell (Vancouver, British Columbia, Canada), and unconjugated anti-CD64 antibody from BD Biosciences (San Jose, CA, USA). Recombinant human M-CSF was purchased from Peprotech (London, UK).

Flow cytometry. Flow cytometric phenotyping of target and effector cells was carried out on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA). The levels of Fcγ receptor (FcγR) I, II, and III on monocytes were quantified from the median fluorescence intensities obtained using monoclonal antibodies against CD64, CD32, and CD16, respectively. Two-parameter dot plots were generated using CellQuest software (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA), and acquired cytometric data were analyzed with FlowJo software (Tree Star Inc., Ashland, OR, USA). The mean fluorescence intensity of each marker was compared between freshly isolated monocytes and monocytes that had been cultured for 48 h with or without M-CSF.

Isolation of monocytes. Peripheral blood mononuclear cells from eight healthy volunteers were isolated using the Ficoll-Hypaque gradient (Pharmacia Biotech, Uppsala, Sweden) and used for negative selection of blood monocytes by depleting T cells, B cells, natural killer cells, and granulocytes using a StemSep Monocyte Enrichment Kit (StemCell Technologies, Vancouver, British Columbia, Canada) according to the manufacturer's instructions. The purified cell fraction contained more than 90% monocytes as determined by flow cytometry and microscopic examination of the cell morphology.

Stimulation of monocytes with M-CSF. Isolated monocytes were cultured for 48 h in the presence or absence of 66 ng/mL M-CSF at 37°C in 5% CO_2. The concentration of M-CSF for optimal stimulation of the monocytes was determined as described previously.[19]

Cytotoxicity assay. Cytotoxicity was measured using flow cytometry with a LIVE/DEAD Viability/Cytotoxicity Assay Kit

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(Molecular Probes, Eugene, OR, USA) as described previously.[21] In this assay, living cells are stained with calcein-AM (green fluorescence), and dead cells are stained with ethidium homodimer-1 (red fluorescence). Target cells were cultured with rituximab (5 μg/mL) or human IgG1 (control) for 30 min at room temperature. Effector cells were adjusted to 1×10^6/mL. Target and effector cells were cocultured in sterile polystyrene round bottom tubes (Becton Dickinson Labware, Franklin Lakes, NJ, USA) in complete medium at various effector-to-target ratios for 4 h at 37°C in 5% CO_2. At the end of incubation, cells were stained with EH-1 (100 nM). Next, 10^4 cells per sample were examined using FACScan flow cytometry without gating. Acquired cytometric data were analyzed with FlowJo software (Tree Star Inc.), and cytotoxicity was calculated according to the manufacturer's instructions. Cytotoxicity assays were repeated at least three times for each sample.

Statistical analysis. The data were expressed as means ± SD. Paired *t*-tests were performed to determine the statistical significance of differences between two groups. Values of $P < 0.05$ were considered to indicate a statistically significant difference.

Results

M-CSF enhances monocyte-mediated rituximab-induced death of CD20-positive lymphoma cells. In initial experiments, monocytes were incubated for 48 h in the presence or absence of M-CSF and then the killing of rituximab-coated lymphoma cells was measured (Figs 1,2). At an effector-to-target ratio of 5:1, monocytes treated with M-CSF were significantly more cytotoxic to Daudi CD20-positive lymphoma cells than untreated monocytes. In contrast, neither M-CSF-treated nor -untreated cultured monocytes induced the lysis of rituximab-coated, CD20-negative Molt-4 cells (Fig. 3). Also, regardless of whether the monocytes were treated with M-CSF, they did not cause substantial lysis of Daudi cells coated with isotype-matched control IgG (Fig. 2). Furthermore, in the absence of effector cells, rituximab alone did not exhibit substantial cytotoxicity against Daudi (Fig. 2) or Molt-4 cells (Fig. 3), indicating that the lysis of Daudi cells was due to rituximab-dependent, monocyte-mediated ADCC.

Analysis of FcγR on monocytes. The effect of M-CSF on the expression of FcγRI (CD64), FcγRII (CD32), and FcγR (CD16)

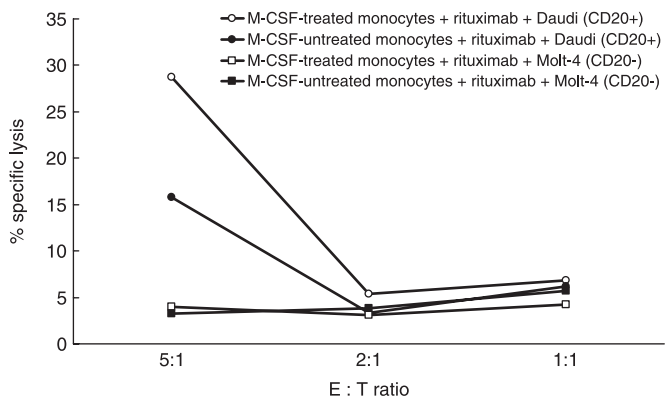

Fig. 1. Specific lysis of CD20-positive (Daudi) and CD20-negative (Molt-4) target cells at different effector-to-target (E : T) ratios in the presence of rituximab (5 μg/mL). Monocytes of healthy volunteers ($n = 8$) were isolated by negative selection and cultured in the presence or absence of macrophage colony-stimulating factor (M-CSF; 66 ng/mL) for 48 h. M-CSF-treated monocytes displayed greater cytotoxicity toward Daudi cells than M-CSF-untreated monocytes. Also, specific lysis of Daudi cells by M-CSF-treated monocytes in the presence of rituximab increased as the E : T ratio was increased. In contrast, the lysis of Molt-4 cells was not increased as the E : T ratio was increased. The results are representative of three independent experiments.

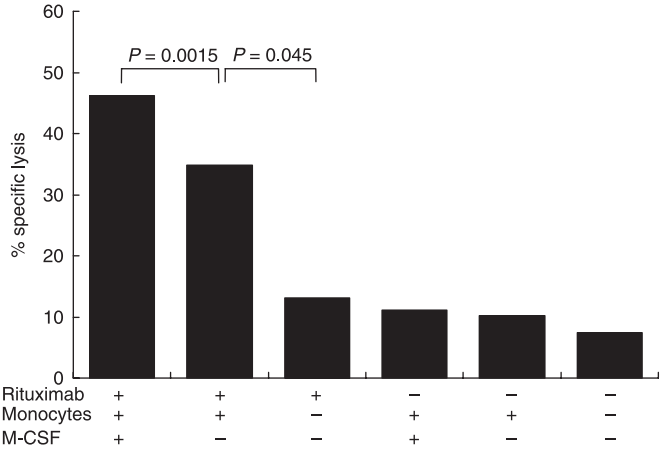

Fig. 2. Effect of macrophage colony-stimulating factor (M-CSF) on the lysis of CD20-positive cells (Daudi) by monocytes at an effector-to-target (E : T) ratio of 5:1 in the presence of rituximab (5 μg/mL). Monocytes were derived from healthy volunteers ($n = 8$) and cultured in the presence or absence of M-CSF (66 ng/mL) for 48 h. Specific lysis was significantly higher in M-CSF-treated monocytes than in monocytes that were not treated with M-CSF (mean ± SD, 46.2% ± 28.6% *vs* 34.7% ± 31.9%, respectively [$n = 8$]; $P = 0.0015$). Specific lysis by rituximab (5 μg/mL) in the absence of effector cells was 13.1% ± 8.2%. Specific lysis by monocytes in the absence of rituximab was 12.6% ± 8.1% for M-CSF-treated monocytes and 11.3% ± 7.0% for M-CSF-untreated monocytes. In the absence of rituximab, monocytes did not cause substantial cells lysis irrespective of M-CSF stimulation compared to Daudi cells' autolysis.

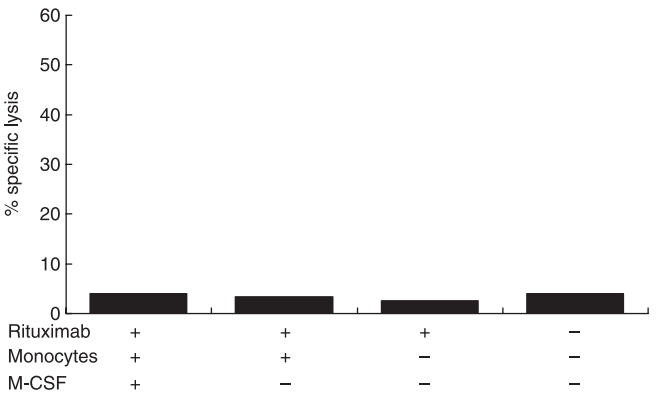

Fig. 3. Macrophage colony-stimulating factor (M-CSF)-stimulated monocytes do not cause rituximab-mediated cell death of CD20-negative cells (Molt-4). Monocytes from eight healthy volunteers were used for cytotoxicity assay. Specific lysis of target cells was 3.94% by M-CSF-stimulated monocytes in the presence of rituximab (5 μg/mL), 3.29% by M-CSF-untreated monocytes in the presence of rituximab, and 2.56% by rituximab in the absence of monocytes. The results are representative of two independent experiments.

on $CD14^+$ monocytes was examined next using flow cytometry. As shown in Fig. 4, M-CSF caused a statistically significant increase in the level of FcγRI (1.6-fold; $P = 0.00031$) and FcγRIII (1.5-fold; $P = 0.039$) on monocytes. The expression of FcγRII, however, was not affected by stimulation with M-CSF ($P = 0.25$).

Blocking of cytotoxicity by anti-FcγR antibodies. Acute monocytic leukemia cell line THP-1 cells can induce ADCC using FcγR on their cell surface, which is a good model with which to test the cytotoxic mechanisms of monocytes.[22]

Stimulation of THP-1 cells with M-CSF increased their cytotoxicity against Daudi cells coated with rituximab by 20%, and amplified their expression of FcγR I and III but not FcγR II

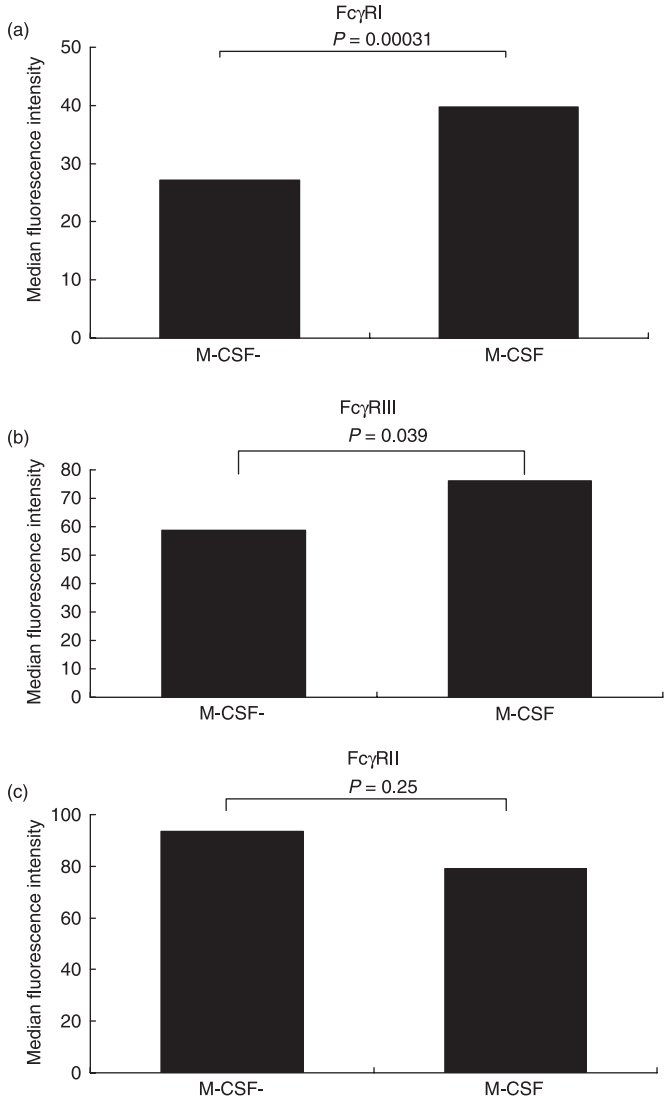

Fig. 4. Effect of M-CSF on the expression of Fcγ receptor (FcγR) by monocytes. The levels of FcγR on the surface of monocytes from eight healthy volunteers were measured using flow cytometry after a 48 h treatment in the presence or absence of macrophage colony-stimulating factor (M-CSF). The median fluorescence intensities of FcγR on monocytes from the same volunteers were compared using a paired *t*-test. (a) The expression of FcγRI on monocytes was significantly enhanced by treatment with M-CSF (60% increase; $P = 0.00031$). (b) The expression of FcγRIII on monocytes was significantly enhanced by treatment with M-CSF (50% increase; $P = 0.039$). (c) The expression of FcγRII was not significantly changed by treatment with M-CSF.

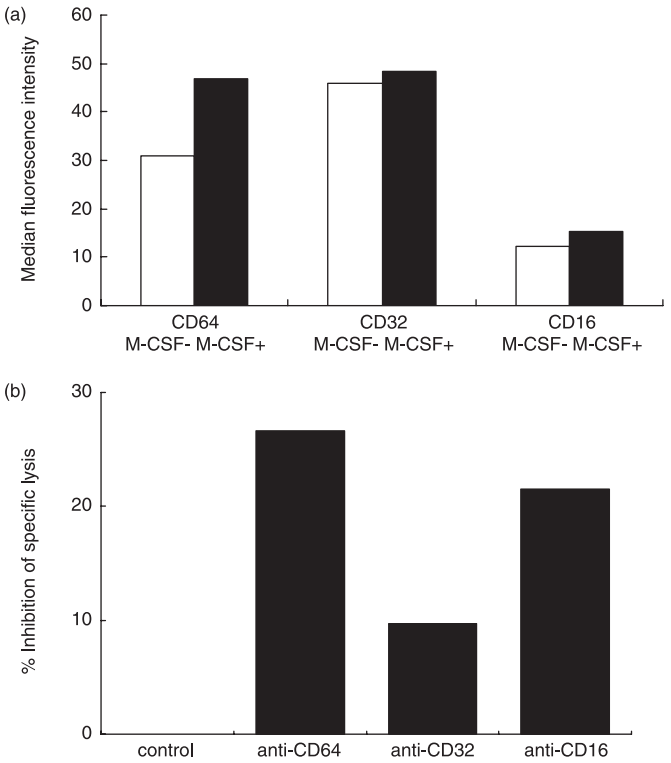

Fig. 5. (a) The expression of CD64 and CD16 on THP-1 were significantly enhanced by treatment with macrophage colony-stimulating factor (M-CSF). (b) Blocking of cytotoxicity mediated by THP-1 against Daudi cells. A total 2.5×10^5 THP-1 cells treated with M-CSF were incubated with 5×10^4 Daudi cells in the presence of 5 μg/mL rituximab in medium containing isotype control IgG (control) or indicated purified monoclonal blocking antibodies (40 μg/mL): anti-CD16, CD32, CD64. The percentage inhibition was calculated using the mean percentage of specific lysis determined from triplicate cultures after 4 h of incubation.

(Fig. 5a), as seen in freshly isolated monocytes. Blocking of FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16) inhibited the specific lysis of rituximab-coated Daudi cells by M-CSF treated THP-1 (Fig. 5b), suggesting that the FcγR expression on effector cells may be pivotal for rituximab-mediated ADCC by monocytes.

Discussion

In the present study, it was demonstrated that M-CSF enhanced monocyte-induced, rituximab-mediated ADCC of $CD20^+$ lymphoma cells. Although the mechanism by which M-CSF enhances monocyte cytotoxicity remains unknown, the present results suggest that this may partly be due to an increase in monocytes expression of FcγRI and FcγRIII. This hypothesis is supported by a previous study showing that rituximab-mediated depletion of B cells depends on monocytes expression of FcγRI and FcγRIII.[13] The importance of FcγR is also supported by the finding that follicular lymphoma patients with the FcγRIIIa 158 V allotype exhibit a higher affinity for human IgG1 and show better clinical responses to rituximab than those with the FcγRIIIa 158F allotype, who exhibit a lower affinity for human IgG1.[11,23] Monocyte enhancement of rituximab-mediated ADCC by M-CSF may also be due to prolonged activation of signal transduction pathways that promote cell survival, including the mitogen-activated protein kinase and phosphatidylinositol 3-kinase/Akt pathways,[15,16,19] although additional studies are needed to examine this possibility in detail.

Colony-stimulating factor 1^{op} mice, which are M-CSF-deficient and lack bone marrow macrophages and blood monocytes,[24] exhibit slow clearance of circulating B cells after treatment with CD20 monoclonal antibody and do not clear all of the mature spleen B cells.[13] In contrast, in mice lacking functional T cells and in perforin-deficient mice, which have defective natural killer cell function, more than 95% of blood and spleen B cells are eliminated after treatment with CD20 monoclonal antibody. Furthermore, classical or alternative pathway C activation does not contribute to B-cell depletion in the M-CSF-deficient mouse model. Based on these findings, it has been concluded that monocytes, as well as the innate monocyte network, are the major effectors mediating depletion of $CD20^+$ B cells *in vivo*. This may also be supported by the authors' findings that monocyte

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activation by M-CSF significantly improves the killing of CD20+ malignant cells via rituximab-mediated ADCC. Although the Daudi Burkitt's lymphoma cell line that was used as *in vitro* model of B-cell lymphoma is not a major target of rituximab therapy in the clinical setting, several reports[25,26] have shown Burkitt's lymphoma cell lines are acceptable as targets to evaluate rituximab-mediated ADCC.

Several studies have examined how cytokines that augment effector cell numbers and function and/or induce antigen expression on target cells affect ADCC in the context of rituximab therapy.[25,27–30] Interferon-α,[31] interleukin (IL)-2,[28] and IL-12[32] have been effectively combined with rituximab in small-group trials; however, the substantial toxicity of such proinflammatory cytokines might limit their overall utility in clinical settings.

Granulocyte colony-stimulating factor (G-CSF), which has a relatively low toxicity profile, and granulocyte-monocyte colony-stimulating factor (GM-CSF) have been reported to enhance the *in vitro* efficacy of rituximab by enhancing the ADCC of neutrophils.[25] In addition, in a severe combined immunodeficiency mouse lymphoma model, the concurrent administration of G-CSF augments the biological activity of rituximab, probably by increasing neutrophil counts.[33] Such a boost in the efficacy of rituximab does not occur with GM-CSF, although it also increased the neutrophil count. In preliminary clinical trials for relapsed or refractory B-cell non-Hodgkin lymphoma, the administration of G-CSF[30] and GM-CSF[34] appeared to enhance the effect of rituximab. Similar clinical trials using M-CSF in place of G-CSF and GM-CSF are warranted because of the lower toxicity of M-CSF.[35] Because administration of M-CSF causes a 10-fold increase in the numbers of blood monocytes and an increase in the numbers of macrophages in the liver, spleen, and peritoneal cavity,[20] M-CSF priming could be a reasonable approach for improving the therapeutic efficacy of rituximab against intractable CD20+ lymphoma, particularly in the late stages of the disease.

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