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M2 Macrophages Phagocytose Rituximab-Opsonized Leukemic Targets More Efficiently than M1 Cells In Vitro[1]

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Because macrophages have been implicated as major players in the mechanism of action of rituximab, we have investigated the factors that modulate their tumor cell killing potential. Human macrophages, differentiated in vitro from peripheral blood monocytes, were used in binding and phagocytosis assays using B-chronic lymphocytic leukemia or lymphoma target cells opsonized with rituximab. Phagocytosis was maximal at 0.1 μg/ml rituximab and was not significantly affected by CD20 expression levels or by CD16A polymorphism at position 158 (Val/Phe). The role of FcγRs was demonstrated by complete inhibition of phagocytosis by excess human Igs. Because macrophages can be differentiated to M1- or M2-type cells with either GM-CSF or M-CSF, respectively, and can be classically activated by proinflammatory stimuli (IFN-γ/LPS) or undergo alternative activation with cytokines such as IL-4 or IL-10, we have analyzed the effect of these different polarization programs on the phagocytosis mediated by rituximab. Macrophages differentiated in presence of M-CSF showed a 2- to 3-fold greater phagocytic capacity compared with GM-CSF-induced cells. Furthermore, addition of IL-10 significantly increased, whereas IL-4 decreased phagocytosis by both M-CSF- and GM-CSF-differentiated macrophages. LPS/IFN-γ had little effect. Expression of CD16, CD32, and CD64 in different macrophage populations correlated with phagocytic activity. Interestingly, several B lymphoma cell lines were observed to secrete 400-1300 pg/ml IL-10 in vitro, and coculture of human macrophages with lymphoma conditioned medium increased significantly their phagocytic capacity. Our data suggest that cytokines secreted by lymphoma cells can favor alternate activation of macrophages with a high phagocytic capacity toward rituximab-opsonized targets. *The Journal of Immunology,* **2009, 182: 4415–4422.**

Rituximab (Mabthera) is a chimeric unconjugated IgG1 mAb with therapeutic activity in low- or high-grade non-Hodgkin B lymphomas, B-chronic lymphocytic leukemia (B-CLL),[3] and Ab-mediated autoimmune diseases, such as rheumatoid arthritis and ITP (1, 2). Neoplastic or normal B cell depletion by rituximab in these different diseases is thought to be mostly through immune mediated mechanisms: these include complement-dependent cytotoxicity (CDC), which is triggered by classical pathway activation and is followed by cell lysis through the membrane attack complex; Ab-dependent cell-mediated cytotoxicity (ADCC), which leads to lysis of Ab-opsonized target cells by effector cells such as NK cells; and phagocytosis, which involves the ingestion of opsonized targets by phagocytes such as macrophages (3, 4). Indeed, in vivo studies in mice have suggested a role for complement activation, NK cells, neutrophils, as well as macrophages in different model systems in which the therapeutic activity against different B cell tumors or normal B cells was investigated (5–8). Furthermore, in mice, several FcγRs have been implicated in the B cell-depleting activity of anti-CD20 Abs, suggesting a role for macrophages in addition to NK cells and granulocytes (8, 9). Phagocytosis by macrophages has also been implicated in the mechanism of action of other therapeutic mAbs (10).

Although factors affecting the efficacy of rituximab, mediated by complement activation and ADCC, have been quite extensively investigated in vitro, less is known about the phagocytosis of rituximab-opsonized target cells by macrophage and the factors regulating them. Macrophages are critical mediators of inflammation during the immune response to pathogens, but can also display anti-inflammatory properties, presumably as a mechanism to avoid excessive tissue damage during infection. Indeed, cells from the monocyte-macrophage lineage show a great deal of plasticity (11–16). Cells with different cell surface phenotypes and functions can be generated in vitro and in vivo by different growth factors (GM-CSF, M-CSF) and/or cytokines. Thus, macrophages can be broadly divided into two broad groups, as follows: 1) classically activated or type I macrophages (M1) that are typically induced in vitro by GM-CSF and IFN-γ and/or LPS, are proinflammatory effectors, and express mediators of inflammation such as IL-12, TNF-α, CC chemokines, and NO synthase, and 2) alternatively activated or type II macrophages (M2) induced by M-CSFs that secrete IL-10, but not IL-12, and have anti-inflammatory properties and immunosuppressive properties. Further polarization of M-CSF-induced macrophages with IL-4/IL-13, immune complexes, or IL-10 allows their full differentiation into distinct M2 populations, called

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[3] Abbreviations used in this paper: B-CLL, B-chronic lymphocytic leukemia; ADCC, Ab-dependent cell-mediated cytotoxicity; CDC, complement-dependent cytotoxicity; M1, type I macrophage; M2, type II macrophage; MCL, mantle cell lymphoma; MFI, mean fluorescence intensity; TAM, tumor-associated macrophage.

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M2a, M2b, and M2c, which differ in some cell surface properties and capacity to secrete immune mediators. M2-type macrophages express higher levels of scavenger receptors and proangiogenic factors (11, 12, 15, 17). They favor tissue repair and remodeling. Tumor-associated macrophages (TAM) have generally been shown to display a M2-like phenotype and are thought to favor tumor growth by down-modulating immunity and favoring angiogenesis (11–13, 18).

In view of the important role of macrophages in the mechanism of action of rituximab, we have studied their capacity to phagocytose rituximab-opsonized leukemic target cells and have investigated the factors that regulate this process.

Materials and Methods

Target cells and cell culture

Peripheral blood was obtained from B-CLL or mantle cell lymphoma (MCL) patients after informed consent. Mononuclear cells were isolated by standard Ficoll-Hypaque gradient centrifugation (Lympholyte), and aliquots were stored frozen in liquid nitrogen. The BJAB human B lymphoma, CEM T lymphoma cell line stably transfected with GFP alone (CEM-GFP$^+$), or both GFP and CD20 (CEM-GFP$^+$CD20$^+$), Raji, DOHH-2, DHL-4, Jeko1, and Karpas 422 B lymphoma cell lines have been described previously (19, 20). They were grown in RPMI 1640 medium (Cambrex), supplemented with 10% heat-inactivated FBS (Euroclone), 0.1 mM gentamicin (Mayne Pharma), and 2 mM glutamine (Euroclone) (thereafter called complete RPMI 1640 medium).

Generation M1 and M2 macrophages

CD14$^+$ monocytes were purified from healthy donors' mononuclear cells by anti-CD14 microbeads magnetic cell sorting, according to the manufacturer's instructions (Miltenyi Biotec). Purity of the cells was 90–98%. They were cultured in 48-well plates (Corning Glass) or 8-well chamber slides (LabTek; Nunc) at 5×10^5/well or 2×10^5/well, respectively, for 6–7 days in RPMI 1640 medium supplemented with either 20 ng/ml human rM-CSF (R&D Systems) or 100 ng/ml human rGM-CSF (Mielogen; Schering-Plough). For full M1 polarization, 100 U/ml IFN-γ (Boehringer Ingelheim) and 1 ng/ml LPS (Cambrex) were then added during the last 24 h of culture. For full M2 polarization, either 20 ng/ml IL-4 or 10 ng/ml IL-10 (R&D Systems) was added during the last 48 h of culture. In all experiments comparing M1 and M2 macrophages, these cells were generated in parallel from the same donor monocytes.

To generate lymphoma conditioned medium, cells were plated at 1×10^6/ml in fresh medium, and supernatant was collected after 24 h and frozen in aliquots. In some experiments, macrophages were cultured in GM-CSF and lymphoma conditioned medium was added at a 1/4 dilution during culture. In the experiments with conditioned medium, human rIL-10 was added as control in separate wells at either 10 ng/ml or 100 pg/ml.

Immunofluorescence analyses

Macrophages were detached in cold PBS and stained with CD16 FITC, CD32 FITC, CD64 FITC, CD14 FITC, CD11b PE mAbs (BD Biosciences), or isotype-matched FITC- or PE-conjugated control Abs (BD Biosciences), according to standard procedures. For the analysis of intracellular IL-10 and IL-12 expression, cells were fixed and permeabilized by Fix & Perm reagents (BD Biosciences), according to the manufacturer's instructions, and labeled with anti-IL-10 PE and IL-12 PE or control Abs (BD Biosciences). B-CLL cells or cell lines in suspension were stained with CD20 FITC or control FITC-labeled IgG1 Abs (BD Biosciences). In some experiments, levels of expression of CD20 were quantified by staining cells with CD20 PE Abs (BD Biosciences) and quantification of the number of CD20 molecules using calibrated bead (Quantibrite), according to the manufacturer's instructions (BD Biosciences). All stained cells were analyzed using a FACScan Instrument, collecting at least 5000 events/sample using the CellQuest software (BD Biosciences).

Secreted cytokines were analyzed using the Flex Set kit (BD Biosciences), according to the manufacturer's instructions. Samples were analyzed on a FACSCanto instrument (BD Biosciences).

Cell-binding assays

CD20$^+$ or CD20$^-$ control target cells were labeled with 0.1 μM CFSE (Molecular Probes-Invitrogen) for 10 min at 37°C and washed with RPMI 1640 containing 10% FBS. Labeled cells were incubated with 0.001–10 μg/ml rituximab or negative control IgG1k mAbs (anti-Erb2 trastuzumab or anti-EGFR cetuximab) for 30 min at 4°C and then added to the macrophage effector cells at a 1:1 ratio and incubated for 5 min at 4°C. Cells were then detached in cold PBS and labeled with PE-conjugated anti-CD11b (BD Biosciences) for 30 min at 4°C, washed, and analyzed on the FACS by double immunofluorescence. The capacity of macrophages to bind target was calculated as the percentage of double-positive macrophages with respect to total macrophages (all CD11b-positive cells).

Phagocytosis assays

Unless otherwise indicated, for phagocytosis assays, unlabeled target cells were incubated with 0.001–10 μg/ml rituximab or control Abs, as above, and then added to macrophages in 24-well plates at a 1:1 ratio in RPMI 1640/10% FBS. At the end of the 2-h incubation at 37°C, cells were detached in cold PBS, washed, and centrifuged onto cytospin slides using a Shandon 2 Cytofuge (Thermo Scientific). Cytospins were fixed and stained with May-Grünwald Giemsa. Wherever indicated, phagocytosis was performed in 8-well chamber slides (LabTek). Briefly, CD14$^+$ monocytes were plated at 200,000 cells/well and allowed to differentiate as above. A total of 2×10^5 B-CLL targets was then added in each well in presence or absence of 0.01 or 0.1 μg/ml rituximab. After 2 h at 37°C, slides were gently rinsed in PBS, fixed, and stained with May-Grünwald Giemsa. In some experiments, both cytospin and chamber slide methods were used and gave equivalent results (data not shown). Phagocytosis was evaluated by a double-blind method, with a separate operator counting under the microscope at least 200 cells for each experimental condition, using the ImageJ 1.38 image processing and analysis software, and calculating the percentage of macrophages that engulfed at least one tumor target cell with respect to total macrophages.

ADCC assays

The target cell line BJAB was incubated at 37°C for 30 min with 3.5 μM/ml calcein-AM (Sigma-Aldrich), washed, and then stained with 1–10 μg/ml rituximab Ab. Increasing amounts of macrophages (E:T ratio ranging from 1:1 to 90:1) were added. After 4-h incubation at 37°C, the released fluorescence in the supernatant was read at 485 nm excitation and 535 nm emission in a fluorometer (Genios; Tecan).

CDC assays

Complement lysis was performed by incubating 10^5 B-CLL in presence or absence of 10 μg/ml rituximab and 20% human serum. After 24-h incubation at 37°C, cytotoxicity was measured by propidium iodide staining and FACS analysis.

Analysis of CD16A polymorphism

DNA was isolated from donors' mononuclear cells using the Genomic DNA Purification kit, according to the manufacturer's instructions (Gentra Systems). CD16A polymorphism was analyzed by standard PCR, as previously described, using 1 μg of DNA/sample (21).

Western blots

Western blots were performed, as described previously, using anti-CD32A/C, CD32B, and actin-specific Abs (Santa Cruz Biotechnology) (22).

Statistical analysis

Statistical significance was determined using Student's t test, and set at p values less than 0.05. In the figures: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Results

Rituximab mediates the binding and phagocytosis of rituximab-opsonized B-CLL cells by human macrophages

To study the effector functions of human macrophages, these cells were generated in vitro by culture of purified peripheral blood CD14$^+$ monocytes in presence of M-CSF. To assess E:T cell binding, B-CLL cells were first labeled with the CFSE fluorescent dye and then preincubated for 30 min with the rituximab or control Ab at 4°C. They were then put in contact with the macrophages for 5 min at 4°C at a 1:1 ratio. Binding was quantified by FACS analysis after detaching the cells and staining macrophages with CD11b-PE at 4°C. Macrophages that had bound the fluorescent B-CLL target could be measured as double-positive cells, whereas free macrophages were PE positive only. An example is shown in Fig. 1*A*, in

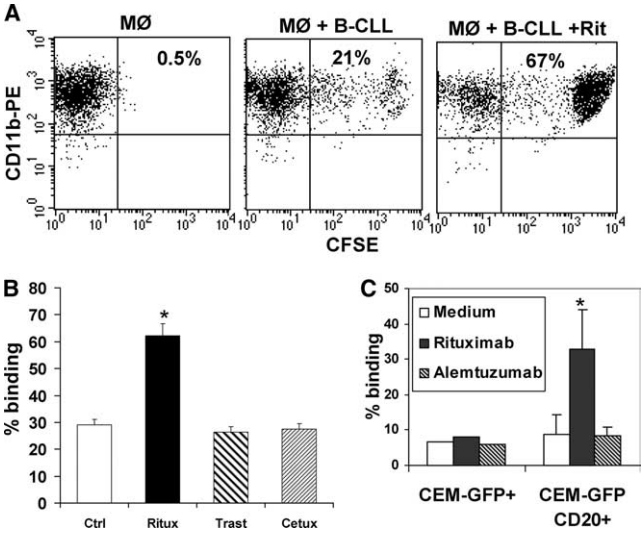

FIGURE 1. Macrophages bind specifically to rituximab-opsonized B-CLL targets. *A*, Example of binding assay. B-CLL cells were labeled with CFSE and stained with 1 μg/ml rituximab. M-CSF-differentiated macrophages were incubated for 5 min at 4°C in presence or absence of CFSE-labeled target cells (1:1 ratio), detached, and stained with anti-CD11b PE Ab. The percentage of double-positive macrophages with respect to total macrophages is shown in each panel. *B*, Results of binding assays using macrophages and CFSE-labeled B-CLL target cells incubated with either 1 μg/ml rituximab, trastuzumab, or cetixumab. *C*, Binding assay using macrophages and the CEM GFP$^+$ or CEM GFP$^+$CD20$^+$ target cell lines in presence or absence of rituximab or the control Ab alemtuzumab. The data of *B* and *C* are the mean and SD of three experiments.

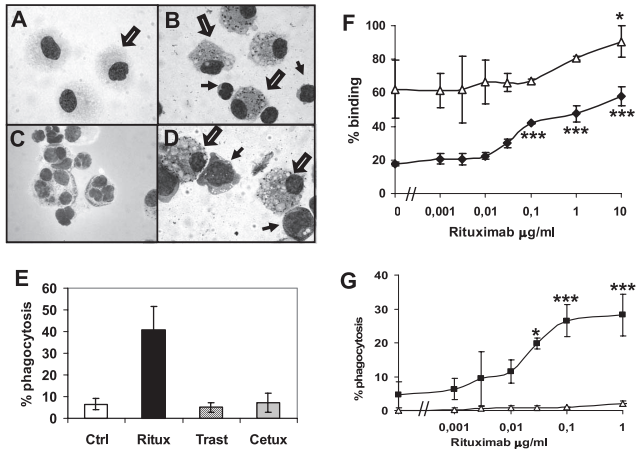

FIGURE 2. M-CSF-differentiated macrophages phagocytose rituximab-opsonized B-CLL, but not BJAB cells. Macrophages differentiated in vitro with M-CSF were used in binding (*F*) or phagocytosis assays (*A–E* and *G*) using B cell targets opsonized with rituximab or control Abs. *A*, Macrophages only; *B*, macrophages + B-CLL + 1 μg/ml trastuzumab; *C*, macrophages + B-CLL + 1 μg/ml rituximab; *D*, macrophages + BJAB + 1 μg/ml rituximab. *E*, Percentage of phagocytosis of B-CLL cells in presence of 1 μg/ml rituximab or control Abs trastuzumab and cetuximab. *F*, Dose-response curve of binding, using macrophages (■) or monocytes (△) and B-CLL targets incubated with increasing concentrations of rituximab. *G*, Dose-response curve of phagocytosis, using macrophages (■) or monocytes (△) and B-CLL targets incubated with increasing concentrations of rituximab. Data in *E–G* are the mean and SD of three experiments. Statistical analysis: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

which macrophages in absence of rituximab bound 21% of B-CLL target, which increased to 63% in presence of 1 μg/ml rituximab. The specificity of binding in presence of rituximab was demonstrated by the lack of effect of anti-HER2 Ab trastuzumab or anti-vascular endothelial growth factor Ab cetuximab (Fig. 1*B*). Furthermore, the CEM cell line stably transduced with human CD20 and the GFP (CEM-GFP-CD20$^+$) (20) showed increased binding to macrophages in presence of rituximab, but not the control Ab alemtuzumab, the target cell line being CD52 negative. Finally, rituximab did not mediate binding of the CEM-GFP control line that does not express human CD20 (Fig. 1*C*).

We then performed phagocytosis assays by standard cytospin techniques and microscopic evaluation of stained phagocytic cells. An example of phagocytosis assay using either B-CLL or BJAB cells as targets is shown in Fig. 2, *A–D*. Macrophages were easily distinguished from B-CLL or the BJAB cell line by their morphology (Fig. 2, *B* and *D*). Furthermore, in presence, but not absence of rituximab, macrophages could be clearly seen to have phagocytosed the B-CLL targets, with one to five ingested B-CLL/macrophage (mean of 2.3 B-CLL cells/phagocytosing macrophage) (Fig. 2*C*). Phagocytosis was specific because trastuzumab or cetuximab was unable to mediate the effect (Fig. 2*E*). Interestingly, no phagocytosis could be observed in presence of rituximab using CD20$^+$ cell lines such as BJAB or CEM-GFP-CD20 (Fig. 2*D* and data not shown), but only tight binding of E:T cells, presumably due to the large size of the target cells compared with the macrophage. The same results were obtained by plating monocytes in chamber slides and performing phagocytosis directly in the chambers after their in vitro differentiation to macrophages (data not shown and see below).

Having set up the techniques, we performed binding and phagocytosis assays with increasing concentrations of rituximab and either freshly isolated CD14$^+$ monocytes or macrophages as effectors, and B-CLL as targets. The data show that specific binding of B-CLL to macrophages was already detectable at a concentration as low as 0.03 μg/ml, with increased binding up to 35–40% above background with 0.1–10 μg/ml Ab (Fig. 2*F*). In contrast, purified monocytes showed a high nonspecific binding (~60% in absence of Ab), which increases by only 15–20% at 1–10 μg/ml rituximab (Fig. 2*F*). Similarly to binding, phagocytosis by macrophages started to be measurable at ~0.01 μg/ml and was maximal at 0.1 μg/ml, with a 20–40% increase in phagocytic cells over control at these doses (Fig. 2*G*). In contrast, monocytes did not show detectable phagocytic activity at any dose tested (Fig. 2*G*). These data clearly demonstrate that binding is not sufficient to trigger phagocytosis.

Macrophages do not mediate ADCC

To evaluate whether macrophages can also kill target cells by cell lysis, we performed ADCC assays in presence or absence of rituximab, using M-CSF-differentiated macrophages and the BJAB cell line as target. In three separate experiments, we were unable to detect significant lysis of rituximab-coated target cells by macrophages, after incubations for 4 or 24 h and at E:T ratio ranging from 10:1 to 90:1 (data not shown). Because BJAB cells are not phagocytosed by macrophages (Fig. 2*D*), any killing through cellular lysis should have been detected with this CD20$^+$ target, because it would not have been masked by the phagocytosis process. In contrast, BJAB cells were target of ADCC in presence of rituximab and NK cells (data not shown) (19). These results suggest that macrophages do not mediate ADCC, at least under the conditions used.

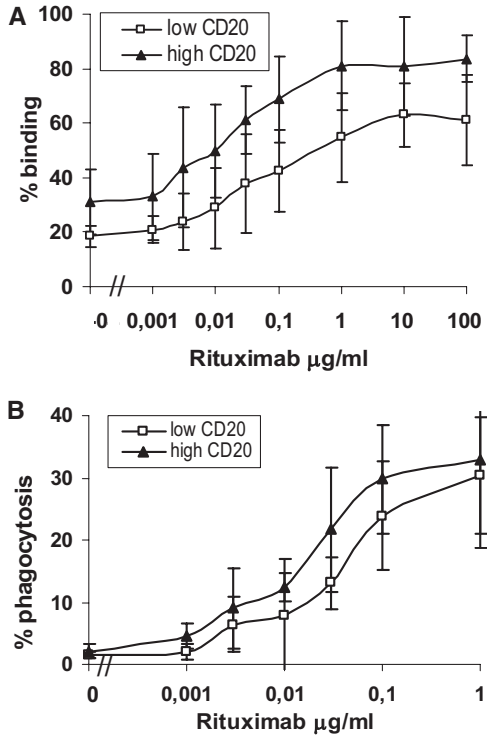

FIGURE 3. CD20 levels do not affect phagocytosis. Macrophages differentiated in vitro with M-CSF were used in binding (*A*) or phagocytosis assays (*B*) using B-CLL/MCL targets expressing either low levels (mean MFI 91) or high (mean MFI 457) levels of CD20. The results are the mean and SDs of three independent experiments.

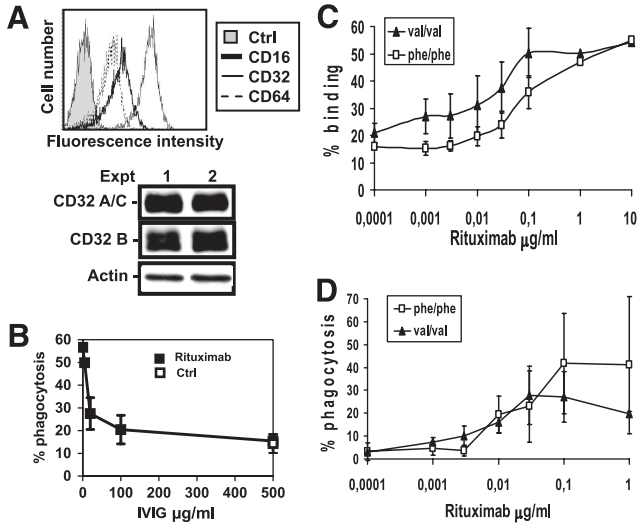

FIGURE 4. Role of FcγRs and CD16A polymorphism. *A*, Expression of CD16, CD32, and CD64 on M-CSF-differentiated macrophages was analyzed by FACS (*upper histogram*), and that of CD32A/C and CD32B in M-CSF-differentiated macrophages from two donors in Western blot (*lower panel*). *B*, Phagocytosis of rituximab-opsonized B-CLL (■) was performed in presence of increasing concentrations of human Igs (intravenous Ig IVIG). Control (□) was in absence of rituximab and presence of highest dose of IVIG. *C* and *D*, Monocytes were purified from donors homozygous for the Val/Val (▲) or Phe/Phe genotype (□) at position 158 of CD16A gene and differentiated in vitro with M-CSF. These were then used in binding (*C*) or phagocytosis assays (*D*) with B-CLL targets and increasing concentrations of rituximab. The experiments were done in parallel with pairs of Phe/Phe or Val/Val macrophages using the same B-CLL targets. The results are the mean and SDs of at least five independent experiments with at least three different donors for each genotype.

CD20 expression levels do not affect the efficacy of phagocytosis in vitro

CD20 expression levels have been shown previously to be one of the most important factors regulating the efficiency of complement-mediated lysis of B-CLL cells by rituximab. We therefore investigated whether this was the case also for phagocytosis. B-CLL and prolymphocytic leukemia/MCL samples expressing either low or high levels of CD20 (mean fluorescence intensity (MFI) 91 ± 11 vs 457 ± 192) were used in binding and phagocytosis dose-response curves. High CD20 samples bound macrophages slightly better than low CD20 cells, but this difference was not statistically significant (Fig. 3*A*). Similarly, CD20 expression levels did not significantly modify the efficacy of phagocytosis (Fig. 3*B*). The two groups of B-CLL samples used in this set of experiments were confirmed to carry different amounts of CD20 by quantitative analysis of CD20 using calibrated beads. Dull CD20 samples carried a mean of 4,404 (±254) CD20 molecules compared with 18,662 (±7,311) for bright CD20 samples. Furthermore, the two groups showed a different capacity to be lysed by rituximab in presence of human complement (11.9 vs 73.7%, respectively), in agreement with previous data (23). We can conclude from these data that the efficacy of phagocytosis, in contrast to complement-mediated lysis, is not significantly affected by CD20 expression levels.

Role of FcγRs and CD16A polymorphism

M-CSF-differentiated macrophages expressed all three activating FcγRs, CD16, CD32A/C, and CD64, as well as the inhibitory CD32B molecule, as determined by FACS analysis and Western blot analysis (Fig. 4*A*). This suggests that different FcγRs may participate in the phagocytosis process. Indeed, human Ig preparations were able to strongly inhibit phagocytosis already at 20 μg/ml, confirming the role of FcγRs (Fig. 4*B*).

The CD16A polymorphism at position 158 (Phe/Val) has been shown previously to affect IgG binding as well as ADCC of rituximab-opsonized targets by NK cells (24). We therefore investigated whether this polymorphism also affected binding or phagocytosis. A panel of normal donors was genotyped by PCR and sequencing analysis. Four Phe/Phe homozygotes and three Val/Val homozygotes were selected to generate macrophages for binding and phagocytosis experiments. Donors were used in pairs so as to compare Phe/Phe and Val/Val effectors against the same B-CLL target within each experiment. As shown in Fig. 4, *C* and *D*, the CD16A polymorphism analyzed did not significantly affect binding or phagocytosis: although slightly higher binding was observed in Val/Val donors compared with Phe/Phe, as may be expected from the higher affinity for IgG of CD16A Val^{158}, the difference was not statistically significant. Moreover, phagocytosis using Val/Val macrophages was similar to that observed with Phe/Phe donors. Altogether, these data show that CD16A polymorphism at position 158 does not affect significantly the efficiency of phagocytosis by human macrophages in vitro.

M2 macrophages, differentiated in presence of M-CSF and IL-10, show a high phagocytic capacity

Macrophages are known to be heterogeneous in vivo and in vitro (12, 16, 25). We have therefore investigated whether different macrophage populations generated in vitro varied in their capacity to mediate binding and phagocytosis with rituximab. The classical method of generating M1-type macrophages is culture in presence of GM-CSF, followed by activation with IFN-γ and LPS. In contrast, M-CSF, as used above, drives macrophage differentiation toward M2-type cells (26).

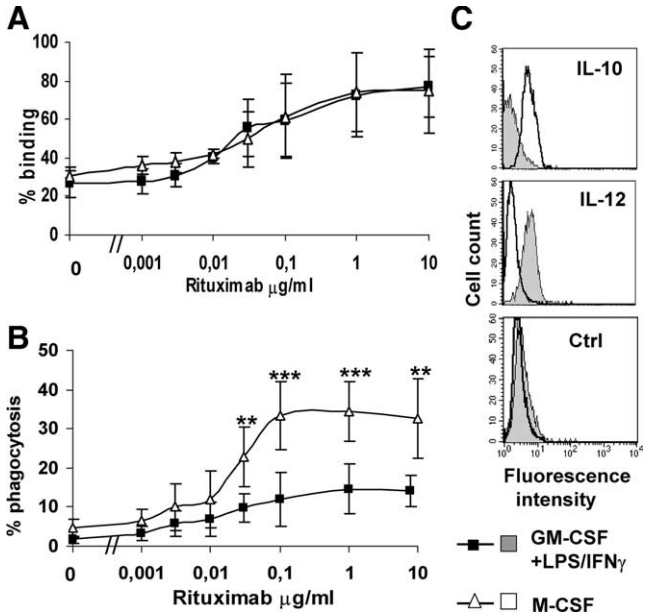

FIGURE 5. M2 macrophages phagocytose rituximab-opsonized B-CLL more efficiently than M1 cells. Monocytes were differentiated with GM-CSF, IFN-γ, and LPS (M1 cells; ■), or M-CSF (M2 cells; △), and used in binding (*A*, mean and SDs of three experiments) or phagocytosis assays (*B*, mean and SDs of five experiments) using B-CLL targets. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. *C*, Differentiation toward M1 (gray histograms)- or M2-type macrophages (white histograms) was checked by intracellular staining with IL-10 and IL-12 Abs.

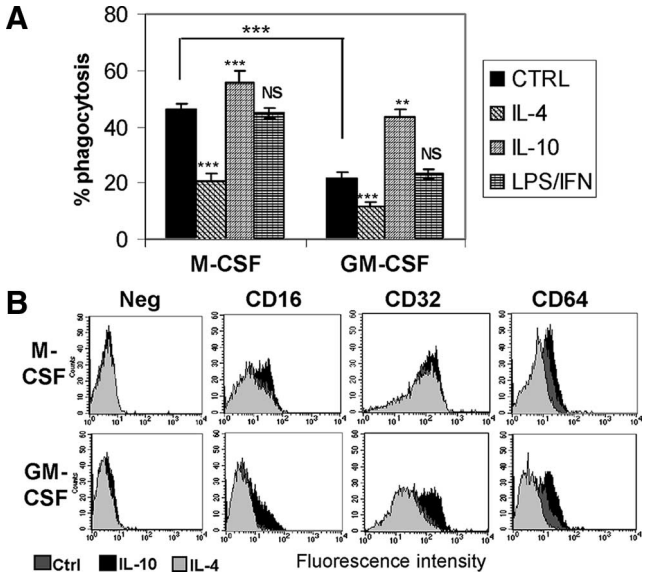

FIGURE 6. IL-10 increases and IL-4 decreases phagocytosis and FcγR expression by M-CSF- or GM-CSF-differentiated macrophages. Macrophages were differentiated in chamber slides with M-CSF or GM-CSF for 7 days. A total of 20 ng/ml IL-4 or 10 ng/ml IL-10 was added during the last 48 h. LPS/IFN-γ were added during the last 24 h. Phagocytosis was then assayed in presence of B-CLL and 0.1 μg/ml rituximab (*A*). Data are the mean and SEs of duplicate wells. Expression of CD16, CD32, and CD64 in the different macrophage populations was analyzed by FACS (*B*). All data shown are representative of three independent experiments.

We therefore generated from the same monocytes M1- and M2-type macrophages in these conditions and measured their secretion of IL-12 and IL-10 to verify their effective polarization. As expected, GM-CSF- and LPS/IFN-γ-differentiated macrophages secreted IL-12, whereas M-CSF-derived cells produced IL-10 (Fig. 5*C*). We next investigated their phagocytic capacity. As shown in Fig. 5*A*, both cell types bound rituximab-opsonized targets with similar efficiency, with ~40% increase in binding at optimal rituximab concentrations. M-CSF-differentiated macrophages (M2), however, were significantly more efficient at phagocytosis than GM-CSF plus LPS/IFN-γ-induced M1 cells, with a ~2- to 3-fold higher percentage of phagocytic cells at all effective rituximab concentrations (0.1–10 μg/ml) (Fig. 5*B*).

M-CSF-cultured macrophages can be further activated toward different M2 phenotypes (called M2a and M2c) by addition of IL-4 and IL-10, respectively (12). We therefore evaluated the phagocytic capacity of macrophages differentiated for 5–6 days with either M-CSF or GM-CSF, followed by incubation with IL-4, IL-10, or LPS/IFN-γ for 24–48 h. Activation of the cells along the different programs was verified by immunophenotyping with CD14 and HLA-DR. As expected (27–30), IFN-γ/LPS up-regulated HLA-DR, whereas IL-4 decreased and IL-10 increased CD14 (data not shown), confirming that the cytokines had induced different macrophage activation states. The cells were then used in phagocytosis assays with rituximab. Macrophages differentiated in M-CSF alone showed a 2-fold increased phagocytosis with respect to cells cultured in only GM-CSF ($p < 0.001$) (Fig. 6*A*). Interestingly, IL-4 significantly decreased and IL-10 increased phagocytosis in both M-CSF- and GM-CSF-differentiated macrophages. IFN-γ/LPS activation had no significant effect (Fig. 6*A*). Down-modulation by IL-4 and up-regulation by IL-10 were particularly evident in M-CSF- and GM-CSF-differentiated macrophages, respectively. Similar differences were observed also at higher (1 μg/ml) and lower doses of rituximab (0.01 μg/ml) (data not shown).

We also investigated the expression of FcγRs in the same culture conditions. CD16, CD32, and CD64 were more intensely expressed in M-CSF with respect to GM-CSF-differentiated macrophages (Fig. 6*B*). IL-10 further up-regulated expression of all FcγRs. In contrast, IL-4 diminished CD64 expression and to some extent CD16 (Fig. 6*B*). IFN-γ/LPS had no significant effect (data not shown).

We conclude that M-CSF- and IL-10-differentiated M2c-type macrophages are most efficient at phagocytosing rituximab-opsonized targets. Both GM-CSF and IL-4 diminish the capacity of macrophages to mediate phagocytosis. The efficacy of phagocytosis correlates with the levels of expression of FcγRs.

Some lymphoma cells secrete IL-10, and their conditioned medium increases the phagocytic capacity of macrophages

Given the role of cytokines in modulating macrophage differentiation, we analyzed the mediators secreted by several B lymphoma cell lines. Of seven non-Hodgkin B lymphoma cell lines analyzed, two (BJAB and Raji) were good producers of IL-10, with 400-1300 pg/ml IL-10 produced in the supernatant in 24–48 h (Fig. 7*A*). In contrast, none of the cell lines produced M-CSF, IL-4, or IL-12 (data not shown). To determine whether the IL-10 produced by lymphoma cells could favor differentiation of macrophages to a high phagocytic capacity, we cultured peripheral blood monocytes for 7 days in presence of GM-CSF and added BJAB conditioned medium for the whole culture period (days 1–7; Fig. 7*B*) or only during the last 2 days of culture (days 5–7; Fig. 7, *B–D*). As control, IL-10, at approximate equivalent concentration as that present in the diluted BJAB conditioned medium (100 pg/ml; Fig. 7, *B–D*) or at the standard concentration of 10 ng/ml (Fig. 7*C*), was added to separate wells. As shown in Fig. 7, *B–D*, BJAB supernatant significantly increased phagocytosis of rituximab-opsonized targets, comparably to that observed with IL-10 at equivalent concentration. In contrast, the conditioned medium from the DOHH2,

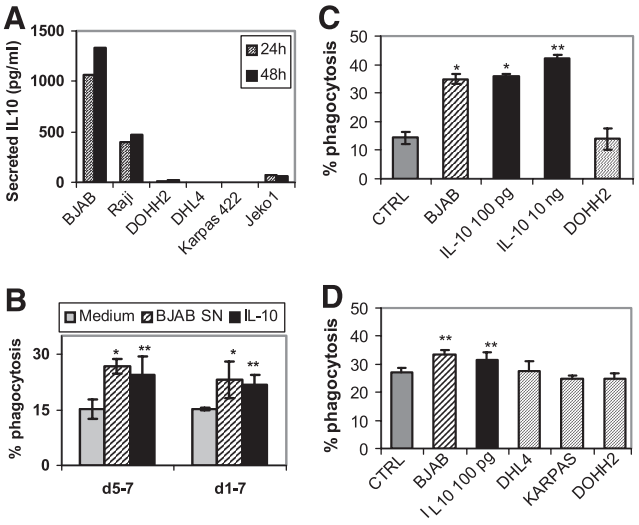

FIGURE 7. The BJAB lymphoma cell line secretes IL-10, and its conditioned medium increases the phagocytic activity of cultured macrophages. *A*, The indicated B lymphoma cell lines were cultured at 1×10^6/ml, and conditioned medium was collected after 24 or 48 h. IL-10 was measured using the flex set kit and FACS analysis. *B–D*, $CD14^+$ monocytes were cultured in chamber slides in GM-CSF either alone (▨), or supplemented with conditioned medium from either BJAB (thick striped bars) or DOHH2, DHL4, or KARPAS 422 (thin striped bars), or supplemented with 100 pg/ml or 10 ng/ml human rIL-10 (■) during the last 2 days of culture (*B*, days 5–7, and *C* and *D*) or during the whole culture (*B*, days 1–7). The generated macrophages were then used in phagocytosis assays using B-CLL targets opsonized with 0.1 μg/ml rituximab. Data are mean and SD of duplicate (*B* and *C*) or quadruplicate wells (*D*) and are representative of at least three independent experiments.

DHL-4, and Karpas 422 cell lines, which do not produce IL-10, had no effect on the efficacy of phagocytosis (Fig. 7, *C* and *D*). Similar results were obtained with lymphoma conditioned medium added to M-CSF-differentiated macrophages (data not shown).

We conclude that the conditioned medium of some lymphoma cell lines allows differentiation of macrophages toward cells with higher phagocytic capacity.

Discussion

In this study, we have investigated some of the factors that regulate binding and phagocytosis of rituximab-opsonized tumor cells by human macrophages. We show that macrophages differentiated in vitro with M-CSF, but not peripheral blood $CD14^+$ monocytes, can mediate specific binding and phagocytosis of rituximab-coated B-CLL cells, with 30–50% of macrophages having ingested one to five target cells after 2-h contact. In contrast, $CD20^+$ lymphoma cell lines could bind the macrophages, but were not ingested in the same conditions. Phagocytosis is known to take place through a complex series of signaling events, coordinated spatially and temporally (31). Target cell size and shape have been shown to affect the capacity and molecular mechanism of phagocytosis (31). Thus, our observations confirm that the macrophage can sense target cells that are presumably too large to be ingested, and suggest that the choice of target is important in the study of Ab-mediated phagocytosis in vitro. We could also measure phagocytosis of primary follicular and MCL, similarly to that of B-CLL, suggesting that phagocytosis may take place against these cellular targets also in vivo.

Of interest in this context is that, in standard ADCC assays using the same target cell lines, we could not detect significant lysis through ADCC of neoplastic B cells by M-CSF-differentiated macrophages. Also, GM-CSF-differentiated macrophages activated with IFN-γ and LPS (M1) were unable to mediate ADCC in our conditions (M. Leidi, unpublished observation). The target used in ADCC assays was BJAB, which we have shown previously to be lysed by rituximab and NK cells (19), suggesting that it is an adequate target for ADCC. Thus, altogether, our data suggest that the major killing mechanism mediated by human macrophages is phagocytosis and not ADCC. This is in agreement with the previous reports by Lefebvre et al. (32), who used GM-CSF- and IFN-γ-activated macrophages.

Both target cell binding and phagocytosis were specific and dose dependent, with maximal effect observed at ~0.1 μg/ml mAb, which is ~10 times below saturation level of CD20 on target cells. The requirement for mAb concentrations well below saturation level suggests that phagocytosis takes place efficiently with relatively few CD20 molecules occupied. These conclusions are in agreement with our data that CD20 expression levels in different target cells did not significantly influence the efficacy of phagocytosis. These conclusions suggest that phagocytosis, unlike CDC, may be effective in vivo also with cellular targets expressing low CD20, like B-CLL.

The phenotype and functional activities of macrophages can be modulated by a number of cytokines and microbial products, and form a continuum with, at the extremes, polarization toward either M1 (also called type I) or M2 (type II) macrophages (11, 12, 15, 17), differentiation modes that can also be observed in vivo (14, 15). Alternatively, activated M2-type macrophages are themselves a heterogeneous category (11, 12, 16, 17). M1- and M2-type cells express different levels of cell surface markers, adhesion molecules, scavenger receptors, chemokines, cytokines, and receptors, and show different effector functions: M1 macrophages are high producers of proinflammatory cytokines and IL-12 and mediate resistance toward intracellular parasites and tumors. In contrast, M2 macrophages produce less proinflammatory molecules and are involved in tissue remodeling, angiogenesis, and tumor promotion through a variety of mechanisms. We observed in this study that M-CSF combined with IL-10 were the best inducers of macrophages with high phagocytic activity. This is in agreement with previous report showing that either M-CSF or IL-10 increases the phagocytic capacity of macrophages toward apoptotic cells or pathogens (27–29, 33) and is likely to be related to the higher activity of M2 macrophages in scavenging debris and tissue remodeling (12, 34). The macrophages in this study clearly showed high plasticity in that either M-CSF or IL-10 could efficiently differentiate macrophages to high phagocytic capacity and combination of both cytokines showed highest activity. In contrast, IL-4 significantly decreased phagocytosis in both cases. Indeed, IL-10 and IL-4 have been described previously to drive alternative differentiation of macrophages with different phenotypic and genotypic characteristics, and these different populations have been called M2a (IL-4) and M2c (IL-10) (12, 35). This study supports the notion that IL-4 and IL-10 activate different programs in macrophages, and suggests that, indeed, IL-10-derived M2c cells are much more phagocytic than IL-4-differentiated M2a cells also for Ab-opsonized target cells (27, 33).

Expression of all three FcγRs, CD16, CD32, and CD64, paralleled phagocytosis, suggesting a role for all three receptors in this process. Indeed, human Ig preparations were also strongly inhibitory on phagocytosis. Previous studies have demonstrated that FcγRI (CD64), FcγRIIA (CD32A), and FcγRIIIA (CD16A) can mediate IgG-driven phagocytosis (31, 36, 37). With regard to the polymorphism of CD16A, previously reported to play a role in the clinical response of some patients to rituximab (38, 39), we did not detect a significant difference in binding or phagocytosis of target cells, using macrophages homozygous for valine or phenylalanine

at position 158 of the FcγRIIIA (CD16A). This would be in agreement with our conclusion that CD16A is not the only receptor involved in this process and a potential effect of CD16A polymorphism may be masked by the presence of CD64 and CD32A.

We report in this study that some B lymphoma cells lines, such as BJAB and Raji, secrete high levels of IL-10 in vitro. This is in agreement with previous reports suggesting that a significant proportion of Burkitt's lymphomas as well as follicular and diffuse large B cell lymphomas secrete IL-10 (33, 40). Interestingly, conditioned medium from the IL-10-producing BJAB cell line, but not that of other nonproducer lymphoma lines, increased significantly the phagocytic capacity of in vitro differentiated macrophages toward rituximab-opsonized target cells. These data suggest that the tumor microenvironment, and in particular lymphoma-derived IL-10, can drive alternate activation of macrophages to M2-like cells. Indeed, TAMs have indeed been previously reported to display a M2-like phenotype (13, 41–43). In agreement with this, preliminary experiments in our laboratory suggest that macrophages infiltrating the BJAB tumor in vivo in mice show typical M2 markers (M. Roncalli and E. Miranda, unpublished data). Present evidence suggests that TAMs usually play a tumor-promoting function by a variety of mechanisms, including immune suppression and promotion of angiogenesis (13, 18, 25, 42). Thus, in the case of lymphomas treated with rituximab, M2 macrophages infiltrating the tumor would be ideally placed to mediate a strong antitumor response through phagocytosis of the rituximab-coated tumor cells. Interestingly, recent data support the likely positive effect of TAMs in the clinical response to rituximab (44, 45). Whereas in FL patients treated with chemotherapy alone, higher levels of macrophage infiltration correlates with poor prognosis (46), this correlation is reverted in patients treated with rituximab in addition to standard chemotherapy (44, 45). Importantly, the types of patients and methods used in these studies were comparable, suggesting indeed that lymphoma-associated macrophages switch from a tumor-promoting to a tumor-inhibiting function with the addition of rituximab. These data support a therapeutic activity of TAMs during rituximab treatment. Our data suggest that this therapeutic activity includes phagocytosis of lymphoma cells that may be favored by the lymphoma microenvironment (32). These results offer further insight into the mechanism of action of rituximab and open the way to novel in vivo studies to try and modulate the macrophage-mediated therapeutic response to rituximab or other Abs.

Disclosures

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