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

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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 IgGs. 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.
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(1)), or both GFP and CD20 (CEM-GFP/CD20(2)), Raji, DOHH-2, DHL-4, Jeko1, and Karps 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% antibiotics, and 0.1% heat-inactivated FBS (Euroclone), 0.1% antibiotics, and 0.1% heat-inactivated FBS (Euroclone). culture. In the experiments with conditioned medium, human rIL-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.

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 on 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 × 10^7/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 PE Abs (BD Biosciences) and quantification of the percentage of cells with CD20 PE Abs and quantification of 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 calcein 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, cytoxicity 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 CSFSE 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. 1A, in
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. 1B). 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. 1C).

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. 2C). Phagocytosis was specific because trastuzumab or cetuximab was unable to mediate the effect (Fig. 2C). Interestingly, no phagocytosis could be observed in presence of rituximab using CD20+ cell lines such as BJAB or CEM-GFP-CD20 (Fig. 2D 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. 2F). 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. 2F). 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. 2G). In contrast, monocytes did not show detectable phagocytic activity at any dose tested (Fig. 2G). 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. 2D), 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.
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. 3A). Similarly, CD20 expression levels did not significantly modify the efficacy of phagocytosis (Fig. 3B). The two groups of B-CLL samples used in this set of experiments were confirmed to carry different amounts of CD20 by experiments were confirmed to carry different amounts of CD20 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 IgGs (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.

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. 4A). This suggests that different FcγRs may participate in the phagocytosis process. Indeed, human Ig prepreparations were able to strongly inhibit phagocytosis already at 20 µg/ml, confirming the role of FcγRs (Fig. 4B).

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 Val158, 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).
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. 5C). We next investigated their phagocytic capacity. As shown in Fig. 5A, 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. 5B).

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 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. 6A). 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. 6A). 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. 6B). IL-10 further up-regulated expression of all FcγRs. In contrast, IL-4 diminished CD64 expression and to some extent CD16 (Fig. 6B). 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. 7A). 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. 7B) 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. 7C), was added to separate wells. As shown in Fig. 7, B–D, BJAB supernatant significantly increased phagocytosis of rituximab-opsonized targets, compared to that observed with IL-10 at equivalent concentration. In contrast, the conditioned medium from the DOHH2,
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 the 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 FcγRIIB (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.
References


