Within Peripheral Blood Mononuclear Cells, Antibody-Dependent Cellular Cytotoxicity of Rituximab-Opsonized Daudi cells Is Promoted by NK Cells and Inhibited by Monocytes due to Shaving

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Treatment of chronic lymphocytic leukemia patients with anti-CD20 mAb rituximab (RTX) leads to substantial CD20 loss on circulating malignant B cells soon after completion of the RTX infusion. This CD20 loss, which we term shaving, can compromise the therapeutic efficacy of RTX, and in vitro models reveal that shaving is mediated by effector cells which express FcγRI. THP-1 monocytes and PBMC promote shaving, but PBMC also kill antibody-opsonized cells by antibody-dependent cellular cytotoxicity (ADCC), a reaction generally considered to be due to NK cells. We hypothesized that within PBMC, monocytes and NK cells would have substantially different and competing activities with respect ADCC or shaving, thereby either enhancing or inhibiting the therapeutic action of RTX. We measured ADCC and RTX removal from RTX-opsonized Daudi cells promoted by PBMC, or mediated by NK cells and monocytes. NK cells take up RTX and CD20 from RTX-opsonized B cells, and mediate ADCC. Pre-treatment of RTX-opsonized B cells with THP-1 cells or monocytes suppresses NK cell-mediated ADCC, and blockade of FcγRI on monocytes or THP-1 cells abrogates their ability to suppress ADCC. Our results indicate NK cells are the principal cells in PBMC that kill RTX-opsonized B cells, and that monocytes can suppress ADCC by promoting shaving. These results suggest that RTX-based immunotherapy of cancer may be enhanced based on paradigms which include infusion of compatible NK cells and inhibition of monocyte shaving activity. The Journal of Immunology, 2008, 181: 2916–2924.

The application of mAbs in cancer immunotherapy has evolved as a key targeting strategy over the past 25 years (1–9). The chimeric humanized anti-CD20 mAb rituximab (RTX), specific for B cells, was the first such mAb approved by the FDA; RTX has been used successfully in the treatment of several B cell lymphomas and of rheumatoid arthritis (10–17). A considerable body of evidence, based on in vitro studies, mouse models, and clinical reports, reveals that RTX can mediate cytotoxicity of both normal and malignant B cells by harnessing effector functions of the immune system (18–36). These include complement-dependent cytotoxicity, Ab-dependent cellular cytotoxicity (ADCC) and/or phagocytosis; these latter two processes are promoted by activating FcγR on effector cells such as monocyte/macrophages and NK cells (21, 22, 27, 30–42). However, more than thirty years ago Griffin et al. demonstrated that under certain conditions, instead of phagocytosing Ab-opsonized lymphocytes, macrophages simply removed clusters of cell-bound IgG and spared the lymphocyte (43).

In principle multiple processes mediated by effector cells, i.e., ADCC, phagocytosis or removal of bound IgG, may occur simultaneously under physiologic conditions. In fact, we recently reported that although treatment of chronic lymphocytic leukemia (CLL) patients with low doses (20 mg/m2) of RTX promotes rapid clearance of most malignant B cells from the bloodstream, the small fraction of circulating cells that is not immediately cleared undergoes a substantial reduction in levels of CD20 (44). However, when CLL patients are treated with the usual, higher 375 mg/m2 dose of RTX, saturation of effector cell-based killing mechanisms can occur because high concentrations of RTX are present in the circulation after completion of the infusion. The reaction which then predominates, as malignant B cells re-equilibrate into the bloodstream, is one in which both bound RTX and CD20 are stripped (“shaved”) off of the targeted circulating cells (44, 45). These observations have important clinical implications because loss of the target CD20 Ag is likely to interfere with, and reduce the effectiveness of, RTX-mediated killing of malignant B cells.

Identification of the cell types that execute shaving and thus potentially inhibit killing of mAb-opsonized cancer cells should aid in the design and optimization of future mAb-based immunotherapeutic modalities. We have previously shown, in an in vitro model system using THP-1 monocytes and PBMC, that binding of RTX to B cells will also promote shaving of the CD20 Ag and bound RTX from the cell surface. This reaction is mediated by FcγRI on the effector cells (46); although the THP-1 monocytes mediated substantial shaving, they did not kill the B cells. We now examine the ability of subpopulations of PBMC to mediate the processes of ADCC and/or CD20 shaving of RTX-opsonized B cells.
We find that PBMC and purified NK cells both kill RTX-opsonized B cells, whereas purified monocytes only promote shaving and do not kill opsonized B cells. We also found that NK cells are able to remove RTX and CD20 from RTX-opsonized cells, but usually to a lesser degree than observed for monocytes. Kinetic experiments reveal that purified NK cells kill RTX-opsonized cells at a much greater rate than do PBMC, suggesting the presence of an inhibitory cell type in the PBMC, most likely monocytes. Indeed, monocyte-depleted PBMC have greater ADCC activity than the original PBMC. Moreover, PBMC depleted of NK cells display little ADCC, and pre-treatment of RTX-opsonized B cells with THP-1 cells or monocytes suppresses NK cell-mediated killing due to shaving. However, blockade of FcγRI on monocytes or THP-1 cells abrogates their ability to suppress ADCC. Our findings may provide general support for enhancing mAb-based targeting immunotherapies for cancer by modulating the action of specific FcγRI on effector cells as well as by increasing the number of NK cells by infusion.

Materials and Methods

Cells

The Daudi and Raji B cell lines and the THP-1 monocytic cell line were obtained from American Type Culture Collection. "B" cells were cultured as previously described (28). THP-1 cells were cultured in RPMI 1640 containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μM 2-ME, and were activated by incubation with PMA or all-trans retinoic acid as previously described (46). Heparinized blood was obtained from normal healthy donors, and PBMC were isolated as described (28, 45). University of Virginia Institutional Review Board approved all protocols. Monocytes were isolated from PBMC by allowing them to adhere to tissue culture plastic (47, 48) and the purity of different preparations varied between 60 and 90%, based on staining for CD11b and CD14 (46). NK cells were isolated from PBMC using CD56 microbeads or a negative selection protocol followed by purification using an AutoMACS magnetic cell separator, according to the manufacturer’s directions (Miltenyi Biotech). The NK cells were greater than 90% pure, based on either protocol, and NK cells isolated by the two methods gave the same results in the measurements we report. Seven normal donors provided blood for PBMC, monocyte and NK cell isolation on multiple occasions. Neutrophils were isolated from whole blood based on a procedure developed by Mandell and Sullivan (49).

Reagents and Abs

PMA, retinoic acid, cytochalasin D, and red fluorescent dye PKH 26 were purchased from Sigma-Aldrich. RTX was obtained from the University of Virginia hospital pharmacy and was labeled with Alexa (Al) 488 (Invitrogen), according to the manufacturer’s instructions. mAb 10.1, FITC anti-CD19, and FITC anti-CD45 were purchased from Caltag; APC anti-CD56 was obtained from Miltenyi Biotech; PE anti-CD16 was purchased from Caltag. Human IgG was obtained from Lampire Biological Laboratories. 51Cr-labeled sodium chromate was obtained from Amersham Biosciences. TO-PRO-3 was purchased from Invitrogen.

51 Cr Labeling, opsonization of cells with RTX, and measurement of ADCC by 51 Cr release

Daudi B cells (3 × 10⁶ cells in 1 ml RPMI 1640) were incubated for 2.5 h at 37°C with 0.5 mCi 51Cr sodium chromate, then washed three times with RPMI 1640 and the degree of 51Cr labeling was determined. The Daudi cells were then opsonized ± 10 μg/ml RTX in RPMI 1640 media for 20 min at 37°C with gentle shaking, and washed three times with BSA/PBS. Then 100 λ of 51Cr-labeled Daudi cells (1 × 10⁶ cells) were combined with 100 λ of effector cells in 96-well plates with V-shaped wells. Typically, a 1:40 ratio of Daudi cells to PBMC or a 1:2 ratio of Daudi cells to purified NK cells or purified monocytes was used for the ADCC reactions. The plate was centrifuged for 5 min at 200 × g and incubated for up to 5 h at 37°C, centrifuged again for 5 min at 200 × g, and then 100 λ supernatant was removed and counted in a γ counter. Negative controls included samples that were incubated with RPMI 1640 media in the absence of effector cells. Total lysis controls consisted of 100 λ Daudi cells incubated with 10 λ 5% Triton X-100. ADCC reactions were done in triplicate. The percent ADCC was calculated using the following formula: % ADCC = ((exp sample cpm − neg control cpm)/(total lysis control cpm − neg control cpm)) × 100%.

Flow cytometry assays for ADCC and loss of RTX

Alternatively, samples of Daudi or Raji cells were analyzed in a non-radioactive, flow cytometry assay to measure ADCC and loss of RTX after incubation with the effector cells. These B cells were labeled with PKH 26 fluorescent membrane dye (46) and then opsonized with 10 μg/ml Al488 RTX. Then 100 λ of PKH 26-labeled and Al488 RTX-opsonized B cells were combined with 100 λ of effector cells at the same ratios as used in the ADCC 51Cr release assay. Samples were centrifuged for 5 min at 200 × g and then incubated for up to 5 h at 37°C, and then quenched with 4 ml cold BSA/PBS. Negative controls consisted of PKH 26-labeled Al488 RTX-opsonized B cells incubated with RPMI 1640 in the absence of effector cells; after the incubation effector cells were added and the sample was immediately quenched. After the quenching step, cells were stained with 2 μM TO-PRO-3 iodide (46) and then analyzed by flow cytometry to measure the degree of cell killing, based on gating on the FL2-positive PKH 26-labeled B cells. All flow cytometry assays that measured ADCC based on TO-PRO-3 uptake were done in duplicate. In some experiments cells were incubated together for just 1–2 h, and uptake of Al488 RTX by monocytes or NK cells was then measured. FL2-low monocytes or NK cells were distinguished from FL2-high PKH26-labeled B cells. Mean fluorescence intensities for Al488 RTX binding were converted to molecules of equivalent soluble fluorochrome (MESF) using standardized fluorescent beads (45) (Spherotech, Libertyville, IL). The degree of loss of Al488 RTX was determined as follows: % RTX loss = ((neg control RTX MESF − exp sample RTX MESF)/(neg control RTX MESF)) × 100%.

To measure loss of CD20, CD19, or CD45 caused by killing of Al488 RTX- (or unlabeled RTX)-opsonized Daudi cells by NK cells, after the reactions we secondarily probed the Daudi cells with Al488 RTX, or with FITC anti-CD19, or with FITC anti-CD45. The reduced capacity of the cells to bind these mAbs relative to zero time controls was taken as a measure of epitope loss. To determine loss of epitope on both live and dead cells, the cells were also probed with TO-PRO-3, and gated separately on live and dead populations.

In some experiments we evaluated monocyte or THP-1 cell-mediated inhibition of ADCC by pre-incubating RTX-opsonized Daudi cells for 45 min at 37°C with either of these cell types. Purified NK cells were then added to the cell mixtures and the incubation continued for 90 min, and then cell killing was determined by flow cytometry based on TO-PRO-3 staining. To determine the role of FcγRI in these reactions, the THP-1 cells or monocytes were first incubated with either human IgG (2 mg/ml) or with anti-FcγRI mAb 10.1 (30 μg/ml). In experiments with cytochalasin D, NK cells were incubated with 5 μg/ml cytochalasin D for 60 min before addition of RTX-opsonized Daudi cells.

Analyses of NK cells for uptake of CD20

After incubation with RTX-opsonized or naive donor cells, NK cells were separated by magnetic separation, washed, and then fixed and permeabilized according to the manufacturers directions (Beckman Coulter). The NK cells were then probed with mouse anti-CD20, specific for the cytoplasmic domain (Lab Vision) followed by secondary development with Al488 anti-MsIgG, and analyzed by flow cytometry. Western blotting on lysates of purified NK cells was performed as previously described (46) with rabbit polyclonal anti-CD20, C terminus specific (Lab Vision). Band intensities on exposed x-ray films were determined with a laser densitometer (Molecular Dynamics) using ImageQuant software. NK cells were also examined for uptake of Al488 RTX after reaction with Al488 RTX-opsonized Daudi cells based on probing with APC anti-CD56 followed by fluorescence microscopy at 100x under oil (Olympus BX40 microscope and digital camera with Magnafire software).

Statistical analysis

Statistical significance was determined using t tests performed with Sigma Stat software (Jandel).

Results

Comparison of NK cells and monocytes with respect to ADCC

We first measured ADCC and loss of RTX from opsonized Daudi cells, mediated by either unfractonated PBMC, or by NK cells or monocytes isolated from the PBMC. Fig. 1A reveals that both PBMC and NK cells can execute ADCC as measured by 51Cr release, but cells are not killed by isolated monocytes. Similar
results were obtained when ADCC was measured by flow cytometry (see below). Moreover, as is evident in Fig. 1A, all three cell populations, including the isolated NK cells, promoted loss of bound RTX.

We next analyzed the kinetics of ADCC of RTX-opsonized Daudi cells mediated by PBMC, or by purified NK cells or purified monocytes. In agreement with Fig. 1A, Fig. 1B shows that killing by monocytes is very low at all time points, and that killing by NK cells is greater than killing by PBMC. In fact, within 1 h, the degree of ADCC mediated by isolated NK cells is already equal to the level of ADCC mediated by PBMC for the full 5 h reaction period. ADCC mediated by the PBMC and NK cells does not appear to level off after 5 h, and this finding could indicate that individual NK cells may kill more than one substrate cell during this time period (50). Finally, Fig. 1C reveals that incubation of Al488 RTX-opsonized Daudi cells with each cell population leads to loss of bound RTX, and the unfractionated PBMC are most effective in promoting this loss.

We examined the dependence of ADCC mediated by NK cells on the concentration of RTX used in the initial opsonization step. Fig. 2A shows that ADCC of Daudi cells by NK cells is quite low in the absence of opsonizing RTX, but that small amounts of RTX increase cell killing substantially; ADCC levels off at RTX concentrations of 0.5–1 µg/ml. Strikingly, we observe a similar isotherm with respect to loss of Al488 RTX (Fig. 2B). It would thus appear that the amount of bound RTX necessary to trigger ADCC is quite comparable to the amount that triggers RTX removal. In comparable experiments with NK cells from two other donors for RTX inputs of 10 µg/ml, ADCC was 23 ± 1% and 40 ± 5%. Finally, similar dose-response results for ADCC were obtained when we used a second RTX-opsonized substrate, Raji B cells (Fig. 2C).

In most of our ADCC reactions we used PBMC in approximately 40-fold excess and NK cells in 2-fold excess over the target RTX-opsonized Daudi cells, based upon the ratios used in previous ADCC studies and on our observations that NK cells make up
approximately 3–5% of PBMC. We next investigated whether altering these ratios would significantly affect the degree of killing of the RTX-opsonized Daudi cells. Fig. 3A shows that PBMC can kill as effectively at a 40-fold excess over the RTX-opsonized Daudi cells as they can at a 10-fold excess, while NK cells are able to kill more effectively at a 10-fold excess than a 2-fold excess (Fig. 3B).

Of more importance, it is clear that even at very high PBMC input ratios, ADCC is far less than ADCC mediated by purified NK cells at lower inputs. This finding suggests that an inhibiting cell type within the PBMC, most likely monocytes, may reduce the ability of NK cells within the PBMC to kill Daudi cells.

**Monocytes inhibit NK cell-mediated ADCC**

We obtained evidence indicating monocytes can inhibit NK cell-mediated ADCC based on a series of cell depletion and mixing experiments. Addition of either monocytes or THP-1 cells to purified NK cells reduces NK cell-mediated ADCC (Fig. 4A). We assayed the ability of PBMC to kill RTX-opsonized Daudi cells after they had been depleted of NK cells; as illustrated in Fig. 4B, after NK cells are removed from PBMC, the ADCC activity of the remaining cells is almost totally abrogated. Moreover, the NK cells isolated from the original PBMC have much greater ADCC activity than the starting PBMC population. Perhaps of most relevance for future RTX-based therapies (see discussion), we find that addition of NK cells to the original PBMC leads to an increase in ADCC activity (PBMC/NK).

Because monocytes isolated from PBMC shave but do not kill RTX-opsonized cells, we next investigated the effect of monocyte depletion on the ADCC activity of PBMC. PBMC depleted of NK cells have virtually no ADCC activity, while depletion of monocytes from PBMC actually increases the ADCC activity of the remaining cells (Fig. 4C, second and third filled bars, respectively). If both NK cells and monocytes are removed, the remaining cells exhibit little ADCC (first striped bar on the left). Moreover, addition of NK cells to this twice-depleted mixture substantially restores ADCC (second stripped bar), but addition of only monocytes has no discernible effect (third striped bar). If both NK cells and monocytes are added, the net amount of ADCC is somewhat less than ADCC observed when only NK cells are added. We also performed parallel flow cytometry experiments, based on staining cells with TO-PRO-3, that evaluated Daudi cell killing for all of the conditions illustrated in Fig. 4C. The results were in excellent agreement (Fig. 4D), thus ruling out any potential artifacts due to internalization of the $^{51}$Cr label by the mono-

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**FIGURE 3.** Dose-response experiments demonstrate that NK cells, isolated from PBMC, mediate ADCC of RTX-opsonized Daudi cells much more effectively than the PBMC. A, PBMC; B, NK cells. $^{51}$Cr release assay.

**FIGURE 4.** Both monocytes and THP-1 cells inhibit ADCC of RTX-opsonized cells mediated by NK cells as revealed by reconstitution and mixing experiments. A, NK cell-mediated ADCC (E/T = 4:1) is suppressed by addition of monocytes (E/T = 4:1) or THP-1 cells (E/T = 4:1). Statistical differences compared with NK cells are noted. B, NK cells (E/T = 2:1) increase PBMC (E/T = 40:1)-mediated ADCC. Removal of NK cells from PBMC reduces ADCC, and addition of NK cells restores ADCC. Statistical differences compared with PBMC are noted. C and D, Mixing experiments demonstrate that ADCC is principally mediated by NK cells, and inhibited by monocytes. Filled bars show ADCC for intact PBMC or singly depleted PBMC; striped bars denote doubly depleted PBMC, and show the effect on ADCC of additions of different populations of cells. Depletion of monocytes from PBMC enhances killing, and adding NK cells back to the doubly depleted PBMC leads to more ADCC than seen in the original PBMC. The E/T ratios for PBMC, NK cells and monocytes were 30:1, 2:1, and 5:1, respectively. D, shows the results of parallel flow cytometric TO-PRO-3 assay measurements of ADCC for the seven conditions illustrated in C. Compared with PBMC, all other treated samples were statistically different at $p < 0.01$. Each figure is representative of at least two independent experiments with similar findings.
The results in Fig. 4 provide substantial evidence that THP-1 cells and monocytes block NK cell-mediated ADCC, presumably by removing bound RTX and CD20 from the B cells and therefore decreasing their interaction with Fc/H9253 RIII on the NK cells. To directly examine the effect of RTX loss promoted by monocytes or THP-1 cells on NK cell-mediated ADCC, we pre-incubated substrate RTX-opsonized B cells with THP-1 cells or monocytes, and then added NK cells and performed the ADCC assays. The results in Fig. 5 indicate that pre-treatment of RTX-opsonized B cells with monocytes or THP-1 cells leads to effective inhibition of ADCC, for both RTX-opsonized Raji cells (Fig. 5A) and RTX-opsonized Daudi cells (Fig. 5B–D). This inhibition is likely due to loss of Al488 RTX from the pre-treated Daudi B cells, which was verified by flow cytometry (Fig. 5B, open bars). In a separate control experiment we show that cells that are not opsonized with RTX are not killed in any of these paradigms (Fig. 5C, open bars). Previously we reported that Fc/H9253 R plays a major role in promoting shaving that is mediated by THP-1 cells. Therefore, we examined the effect of blocking this receptor on both THP-1 cells and on monocytes, either with mAb 10.1, specific for Fc/H9253 R, or with human IgG. THP-1 cells or monocytes, either untreated or first reacted with these inhibitors, were then pre-incubated with RTX-opsonized Daudi cells followed by addition of NK cells as in Fig. 5B. We have reported that reaction of monocytes with either blocking agent has the net effect of reducing shaving (46), and here we indeed find that blockade of Fc/H9253 R restores NK cell-mediated ADCC (Fig. 5D). Furthermore, neither human IgG nor mAb 10.1 increased ADCC in the absence of THP-1 cells or monocytes (not shown).

To gain additional insight as to the mechanism by which NK cells mediate ADCC and remove RTX from RTX-opsonized Daudi cells, we examined the effect of treating the cells with cytochalasin D, an inhibitor of actin polymerization. The results in Fig. 6 clearly indicate that both ADCC and loss of RTX are substantially inhibited, suggesting a role for actin polymerization in both NK cell-mediated ADCC and in removal of cell-bound RTX. These findings are consistent with the results we reported for THP-1 cell-mediated shaving (46).

**FIGURE 5.** Pre-treatment of RTX-opsonized B cells with monocytes or THP-1 cells inhibits NK cell-mediated ADCC, and blockade of FcyRII abrogates this inhibition. Four different donors were used for the experiments illustrated in A–D, respectively. Raji cells (A) or Daudi cells (B) were opsonized with RTX and then incubated for 45 min with either THP-1 cells or monocytes at a E/T ratio of 5/1, or no effector cells were added (“none”). NK cells were then added to the three mixtures (E/T = 3:1) and, after a 90 min incubation, %ADCC was determined by flow cytometry with TO-PRO-3 (filled bars). Aliquots of the pre-treated Daudi cells in panel B were assayed separately to verify % RTX loss (open bars). C. In a similar experiment, RTX-opsonized (filled bars) or naive (open bars) Daudi cells were pre-treated with THP-1 cells or monocytes (E/T = 5:1), then incubated with NK cells (E/T = 3:1) and assayed as in A and B above. D. In a similar experiment, aliquots of the THP-1 cells and monocytes (E/T = 5:1) were first reacted with either anti-FcγRI (mAb 10.1, 30 μg/ml), or with human IgG (2 mg/ml) and then RTX-opsonized Daudi cells were added and the samples were incubated for 45 min. Finally, NK cells (E/T = 3:1) were added, and the mixture incubated and assayed as in A and B above. Each figure is representative of at least three independent experiments with similar findings.

**FIGURE 6.** Pretreatment of NK cells with cytochalasin D abrogates ADCC and loss of RTX from RTX-opsonized Daudi cells. E/T = 3:1. TO-PRO-3 assay. Each figure is representative of two independent experiments with similar findings.
two different donors, cell-surface CD16 on NK cells was reduced by 97 and 96%. On this basis, as well as on our observations that RTX is lost from Daudi cells after incubation with NK cells (Figs. 1, 2, 5 and 6), we suspected that the NK cells may simultaneously remove (i.e., shave) and internalize bound RTX and CD20 from the RTX-opsonized cells, in addition to promoting ADCC. We tested this hypothesis by examining NK cells by flow cytometry after the usual ADCC assay. The results in Fig. 7A reveal that after 1 h both monocytes (positive control) and NK cells take up Al488 RTX from opsonized Daudi cells, but only the NK cells promote killing (see Fig. 7 legend). Indeed, kinetic experiments (Fig. 7B) indicate that the rate at which NK cells take up Al488 RTX from the Daudi cells tracks closely with the rate at which they execute ADCC.

In additional experiments to test for CD20 shaving mediated by NK cells, Al488 RTX-opsonized Daudi cells were reacted with NK cells for 1 h at 37°C. The cell mixture was then stained with APC CD56 to identify the NK cells and analyzed by fluorescence microscopy (Fig. 7C). The merged image in the right-most panel shows that the green Al488 RTX is indeed associated with the red NK cell. In a separate experiment, purified NK cells obtained from two different donors were incubated with RTX-opsonized or naive Daudi cells. After 2 h at 37°C, the NK cells were purified by magnetic separation. The recovered NK cells were then lysed and subjected to SDS-PAGE and immunoblotting for CD20. Fig. 7D shows the blot image, and Fig. 7E gives the ratios of the integrated intensities of CD20 relative to the loading control tubulin, thus demonstrating uptake of CD20 by NK cells from RTX-opsonized Daudi cells. The purified NK cells from this experiment were also probed, or fixed, permeabilized and probed, for CD20 with a mouse mAb specific for the cytoplasmic domain of CD20, followed by a secondary Al488 polyclonal goat anti-mouse Ab. As illustrated in Fig. 7F, flow cytometric analysis of the NK cells demonstrated internalized CD20 in the permeabilized NK cells reacted with RTX-opsonized Daudi cells, relative to NK cells reacted with either naive Daudi cells or with no donor cells, and relative to controls which were not permeabilized.

Experiments which focused on obtaining quantitative measures of shaving of CD20 from RTX-opsonized Daudi cells after reaction with NK cells were confounded by substantial difficulties in interpreting the results of flow cytometry experiments on killed cells. Al488 RTX- (or unlabeled RTX-) opsonized Daudi cells were reacted with NK cells and then probed with either Al488 RTX (for CD20), or with FITC- mAbs specific for CD19 or CD45 as controls. However, levels of all three epitopes on dead cells were reduced to less than 30% of the levels on naive, untreated cells. To independently evaluate the effect of cell killing on measurement of epitopes, we reacted Daudi cells with normal human serum and RTX, to promote complement-dependent killing. We then probed the cells in a similar fashion, and we found that levels

![FIGURE 7.](The Journal of Immunology)
of CD20, CD19, and CD45 on dead cells were substantially reduced to ~ 20% of the levels on naive, untreated cells (not shown). Therefore, based on analyses of the Daudi cells, after ADCC we cannot quantitatively determine the amount of NK cell-mediated CD20 removal from RTX-opsonized Daudi cells, although the results of the western blotting and permeabilization experiments (Fig. 7) clearly demonstrate that the NK cells do take up CD20 from the opsonized Daudi cells.

**Neutrophils take up RTX from opsonized Daudi cells**

In common with NK cells, neutrophils also express FcγRIII, and we therefore evaluated the action of neutrophils in the RTX/Daudi cell system. We isolated neutrophils from whole blood (49), and found that these cells do not promote killing of RTX-opsonized cells, in agreement with the reports of Stockmeyer et al. (52, 53), but they do remove Al488 RTX from RTX-opsonized Daudi cells. In separate experiments with neutrophils isolated from the blood of two different donors, we found that between 50 and 75% of the bound RTX was removed from Al488 RTX-opsonized Daudi cells. This result is in agreement with the findings of Whala et al. which revealed that neutrophils can indeed remove ligands from other cells (54).

**Discussion**

**Monocytes inhibit ADCC by shaving**

The ADCC activity in PBMC is generally considered to be principally mediated by NK cells (22, 27, 30, 32), and our results (Figs. 1–6) are consistent with this concept. The most important finding in this report is that within the PBMC population, monocytes are not inert, but rather have an inhibitory effect on ADCC (Figs. 4 and 5), and this effect is most directly related to the ability of monocytes to remove RTX from opsonized cells via the shaving reaction. Thus, if sufficient RTX is removed from the B cells by monocytes, the B cells will no longer be engaged by FcγRIII on NK cells, and ADCC will be reduced and the potential therapeutic activity of RTX will be compromised. Moreover, removal of monocytes from PBMC populations did enhance ADCC (Fig. 4, C and D), presumably because substrate cells were no longer subject to monocyte-mediated loss of bound RTX.

Our previous work demonstrated that the high affinity monocyte receptor for IgG, FcγRII, plays a key role in shaving (46). It is therefore likely that to the higher affinity of this receptor, monocytes will have a competitive advantage in binding to RTX-opsonized cells: they may therefore hinder access of NK cells to target cells and/or induce shaving and thus down-modulate ADCC. Indeed, blockade of FcγRI on monocytes or THP-1 cells by human IgG or by mAb 10.1, specific for this receptor, abrogated the ability of these cells to inhibit ADCC mediated by NK cells (Fig. 5D). The observation that human IgG actually increased ADCC is in apparent contrast with the findings of Preithner et al. who investigated ADCC in several other mAb-target cell systems (40). They reported that ADCC mediated by PBMC is inhibited in 50% serum by endogenous human IgG (4–9 mg/ml). Several differences in experimental conditions may explain this discrepancy. In our experiments the target cells were reacted with saturating concentrations of RTX (10 µg/ml) to insure a maximum level of opsonization and we used lower concentrations of human IgG (2 mg/ml). Indeed, Preithner et al. reported that the ability of human IgG in serum to inhibit ADCC was decreased when the opsonizing mAbs were used at concentrations of 10 µg/ml. Finally, in the experimental system we have studied, NK cell-mediated ADCC is inhibited upon addition of either THP-1 cells or monocytes. The added mAb 10.1 or human IgG should bind with high avidity to FcγRI on these cells, thus reducing shaving and restoring ADCC; this effect may outweigh the potential of human IgG, especially if it is monomeric, to block interaction of FcγRIII on NK cells with Daudi cells which are highly-opsonized with RTX.

Although under a variety of conditions macrophages can phagocytose intact IgG-opsonized cells (43, 55), our results, taken in context with Griffin’s original report (43), demonstrate that monocytes can also execute the shaving reaction, and remove small amounts of material from the surface of an IgG-opsonized cell. A similar process has been described by Wallace et al. who reported that macrophages appeared to be capable of “chipping away” small pieces of opsonized target cells (51). This process is not unique to monocytes/macrophages: “nibbling” has also been reported to be performed by dendritic cells (56), and “trogocytosis” is mediated by other cells including B cells, T cells and NK cells (57–60). In each case these reactions require recognition of ligands on the substrate cell by cognate receptors on the effector cell. An immunologic synapse is formed, followed by transfer of membrane-associated ligands on the substrate cell to the acceptor cell, and then the transferred complexes, including the effector cell receptors, are internalized.

Our studies of the interaction of NK cells with RTX-opsonized Daudi cells demonstrate that both ADCC of RTX-opsonized Daudi cells, as well as transfer of RTX and CD20 from the Daudi cells to the NK cells can occur (Fig. 7). Presumably an immunologic synapse is formed in which several low affinity FcγRIII on the NK cells chelate clusters of RTX-CD20 complexes on the Daudi cells, thereby activating the NK cells to promote killing of the Daudi cells. However, coincident with this reaction, it is quite likely that a fraction of the Daudi cell-associated RTX-CD20 complexes, as well as NK cell-associated FcγRIII, are internalized by the NK cells, thus giving rise to shaving as well. Independent support for this hypothesis is provided in the reports from Weiner’s laboratory that NK cell-mediated ADCC of RTX-opsonized B cells occurs concomitantly with substantial down-regulation of FcγRII (CD16), due to internalization of this receptor (32, 61, 62). Our finding that CD16 is down-regulated on NK cells in the ADCC assays is in agreement with these reports; the CD20 western blots, fluorescence microscopy pictures, and permeabilization studies demonstrate that NK cells can engage in shaving/trogocytosis, as defined by uptake of Al488 RTX and CD20 by these cells (Fig. 7). However, quantitative measurements of CD20 shaving of killed substrate B cells is uncertain, due to parallel decreases in CD19 and CD45. The mechanism of killing mediated by NK cells includes secretion of granules containing granzymes and perforin (50), and the action of these cytotoxic agents appears to compromise measurements of epitopes on the killed cells. Indeed, we also encountered a similar problem in independent experiments which documented decreased levels of these proteins on RTX-opsonized Daudi cells killed via complement-dependent cytotoxicity.

**Parallel findings in the clinic**

We have reported that when CLL patients are treated with low doses of RTX (20 mg/m²), clearance of opsonized B cells in the circulation appears to occur coincident with some loss of CD20 from surviving cells (44). Fixed tissue macrophages in the spleen and especially the liver are most likely to mediate clearance of the opsonized cells (34, 35, 63–65), and it is reasonable to speculate that RTX-opsonized B cells are not necessarily cleared in a single pass through either organ. Rather, depending on how the RTX-opsonized cells make contact with fixed tissue macrophages, it is possible that some cells are partially shaved but escape from the liver or spleen and are cleared after a second or third pass, as long
as sufficient numbers of RTX IgG molecules are still bound to the cells (63).

However, when patients receive the usual higher doses of RTX (375 mg/m²), large numbers of circulating B cells which have greatly reduced levels of CD20 are easily demonstrable soon after completion of the RTX infusion (44, 45). Under these conditions temporary exhaustion of the ADCC capacity of NK cells and of the clearance capacity of splenic and hepatic macrophages is likely to have occurred (36), due to saturation and/or down-modulation of the low affinity receptors for the Fc region of IgG, FcγRII and FcγRIII (65). Indeed, Kavai et al. (66) reported that high levels of circulating immune complexes chronically found in patients with systemic lupus erythematosus can reduce the levels of these two low affinity receptors on monocytes. These results, taken together with the down-regulation of FcγRIII reported by Weiner’s laboratory (32, 62), suggest that exhaustion of the body’s clearance capacity in CLL after high doses of RTX are given will also be manifested by down-regulation of the low affinity FcγR on effector cells. In contrast, the high affinity receptor FcγRI can rapidly recycle and promote very efficient endocytosis of small IgG-containing immune complexes (67–70). Our in vitro experiments and findings in mouse models (46, 71) suggest that it is this high affinity receptor that is primarily responsible for CD20 shaving when patients receive high doses of RTX, which temporarily saturates the clearance capacity of the body (46).

In principle reagents which can specifically block FcγRII (46) could be used clinically to prevent shaving and allow time for FcγRII and FcγRIII to be re-expressed on effector cells, thus allowing for more ADCC and cell clearance without shaving. Another approach, which currently is being explored in the clinic, is to enhance NK cell-mediated ADCC by treating patients with IL-2 along with RTX (27, 38, 72). Finally, several methodologies are being developed to treat cancer patients by infusion of compatible NK cells (73–76), and it would seem reasonable that patients could be given these NK cells to increase ADCC of RTX-opsonized NK cells (73–76), and it would seem reasonable that patients could be given these NK cells to increase ADCC of RTX-opsonized malignant B cells.

In summary, we find that within a PBMC population, monocytes can inhibit NK cell-mediated ADCC of RTX-opsonized cells by removing RTX from the opsonized cells. NK cells are able to both kill and shave RTX-opsonized cells, and it is likely that the killing capacity of these cells may be limited at high cell burdens because of internalization/down-regulation of FcγRIII when the cells come into contact with large numbers of RTX-opsonized B cells. Saturation of the body’s effector mechanisms for removing IgG antibody-targeted malignant cells may pose a general obstacle to mAb-based therapies for cancer, but there are possible strategies to address this problem.

**Disclosures**

The authors have no financial conflict of interest.

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