The Direction of Plasma Membrane Exchange between Lymphocytes and Accessory Cells by Trogocytosis Is Influenced by the Nature of the Accessory Cell

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The Direction of Plasma Membrane Exchange between Lymphocytes and Accessory Cells by Trogocytosis Is Influenced by the Nature of the Accessory Cell

Sandrine Daubeuf,*† Margaret A. Lindorfer,‡ Ronald P. Taylor,‡ Etienne Joly,*† and Denis Hudrisier*†

Exchange of plasma membrane fragments, including cell-surface proteins and lipids, in conjugates formed between lymphocytes and their cellular partners is a field of intense investigation. Apart from its natural occurrence during Ag recognition, the process of membrane transfer can be triggered in experimental or therapeutic settings when lymphocytes targeted by Abs are conjugated to FcγR-expressing accessory cells. The direction of membrane capture (i.e., which of the two cells is going to donate or accept plasma membrane fragments) can have important functional consequences, such as insensitivity of tumor cells to treatment by therapeutic mAbs. This effect, called antigenic modulation or shaving, occurs as a result of a process in which the FcγR-expressing cells remove the mAb and its target protein from the tumor cells. We therefore analyzed this process in conjugates formed between various FcγR-expressing cells and a series of normal or tumor T and B cells opsonized with different Abs capable of triggering membrane exchange (including the therapeutic Ab rituximab). Our results show that the direction of membrane capture is dictated by the identity of the FcγR-expressing cell, much more so than the type of lymphocyte or the Ab used. We found that monocytes and macrophages are prone to be involved in bidirectional trogocytosis with opsonized target cells, a process they can perform in parallel to phagocytosis. Our observations open new perspectives to understand the mechanisms involved in trogocytosis and may contribute to optimization of Ab-based immunotherapeutic approaches.  


Upon recognition of cognate Ags on APC surfaces, T and B cells can capture that Ag, and it can then be detected on the surface of the acceptor T or B cell (1–3). We and others have shown that fragments of plasma membrane of the APC, together with a variety of other molecules (4), are also transferred to the acceptor B or T cell in a process we termed trogocytosis (5–8). Trogocytosis was first described on CD8+ T cells as a unidirectional, TCR-mediated capture process (9, 10) and was rapidly confirmed to be also performed by CD4+ cells, B cells (11), and γδ T cells (12). In all these cases, recognition of cognate Ag by a specific receptor on the acceptor cell was clearly the triggering event, because trogocytosis occurred only when the Ag was present, and the process was inhibited by mAbs that blocked the Ag receptor (13, 14). Other hematopoietic cells also capture plasma membrane fragments from cells they encounter, in a process similar to trogocytosis. This is indeed the case for NK cells (15) or for dendritic cells (DC) in contact with tumor cells (16), virus infected cells (17), T cells (16, 18), B cells (18), macrophages (18) or other DCs (18–20). Macrophages have been reported to acquire Ags from B cells in a membrane-associated form (21), and monocytes were also shown to capture plasma membrane fragments from endothelial cells and to gain T cell activation properties (14). In addition, interaction of neutrophils with Raji cells opsonized with bispecific Abs leads to both neutrophil-mediated Ab-dependent cellular cytotoxicity and capture of membrane fragments from the Raji cells by the neutrophils (22).

Observations in the clinic, based on the loss of CD20 from circulating malignant B cells after infusion of the usual 375-mg/m2 dose of rituximab (RTX), a humanized anti-CD20 mAb used in the treatment of chronic lymphocytic leukemia (CLL), suggest that trogocytosis can occur in vivo (23, 24). Confirming this latter conclusion, trogocytosis by CD8+ T cells could also be detected in vivo in mice immunized with DC loaded with Ags (25). Moreover, subsequent in vitro studies have revealed that Raji cells, as well as other lymphoma cell lines or CLL B cells, opsonized with RTX exhibit a marked loss of CD20 when incubated with the monocytic cell line THP-1 (26). Transfer of RTX/CD20 complexes from Raji cells to THP-1 cells (an example of the shaving reaction) is accompanied by the concomitant transfer, to the FcγR-expressing acceptor THP-1 cells, of other Raji cell determinants, and of fluorescent lipophilic probes incorporated in the Raji cell membrane, thus suggesting that shaving and trogocytosis are terms describing the same process (26). This reaction may have important clinical implications, because CLL cells, having lost CD20, would not be eliminated because they would become insensitive to RTX treatment (27). However, repeated administration of low doses of RTX decreases the loss of CD20 and therefore promotes enhanced targeting in CLL (27).
In conjugates involving RTX-opsonized CD20+ cells and acceptor cells, shaving was inhibited at least in part by anti-FcγR Abs and/or by human IgG, both in vitro (26) and in a mouse model (24). These results strongly suggest that the cognate Ag recognized by the receptors (FcγR) on the acceptor monocyte is indeed RTX IgG. In other cellular systems, in which no identified stimulus is present in the coculture, class A scavenger receptors were implicated in the capture of plasma membrane fragments by DCs (28), monocytes (14), and macrophages (21), as shown by the inhibitory effects of blocking mAb and/or pharmacologic agents.

Interestingly, we recently found that after opsonization with mAbs directed against certain surface molecules, murine T and B cells could function as acceptor cells and promote trogocytosis when these cells were allowed to react with FcγR-expressing cells, such as P815 cells (29). Combined with the results described above, these studies suggest that in conjugates formed between (on the one hand) mAb-coated cells such as T cells, B cells, or Raji and other lymphoma or leukemia cells, and on the other hand FcγR-expressing cells [P815 or THP-1 cells]), the transfer of plasma membrane components may proceed either from the FcγR-expressing cell toward mAb-coated lymphocyte (29) or in the reverse direction (26). There are no obvious explanations to reconcile this apparent contradiction because the parameters (target cells, effector cells, mAb) were different in the experiments. However, given the potential importance of the functional consequences of trogocytosis, we investigated the molecular and cellular mechanisms determining which cell in a conjugate will take up portions of the plasma membrane of its partner.

Materials and Methods

Cells

The mouse mastocytoma cell line P815, which expresses FcγRII/III naturally, the human monocytic cell line THP-1, and Burkitt’s lymphoma Raji (CD20+) cells were all cultured in RPMI 1640 with 10% heat-inactivated FBS, penicillin-streptomycin (100 U/ml) and 2 mM glutamine. CD20-transfected 38C13 mouse B cells (30) were a kind gift from Dr. Josee Golay (Ospedali Riuniti di Bergamo, Bergamo, Italy). Elutriated mouse peritoneal macrophages were harvested from female C3H/HeN mice previously injected i.p. with thioglycolate (30) were a kind gift from Dr. Josee Golay (Ospedali Riuniti di Bergamo, Bergamo, Italy). RTX was obtained from the University of Virginia hospital pharmacy. PKH-26 were acquired from Sigma-Aldrich (Lyon, France). The mouse mastocytoma cell line P815, which expresses FcγRII/III naturally, the human monocytic cell line THP-1, and Burkitt’s lymphoma Raji (CD20+) cells were all cultured in RPMI 1640 with 10% heat-inactivated FBS, penicillin-streptomycin (100 U/ml) and 2 mM glutamine. CD20-transfected 38C13 mouse B cells (30) were a kind gift from Dr. Josee Golay (Ospedali Riuniti di Bergamo, Bergamo, Italy). Elutriated mouse peritoneal macrophages were harvested from female C3H/HeN mice previously injected i.p. with thioglycolate (31). Leukocytes (PBL) from healthy donors were isolated by dextran sedimentation and centrifugation through Ficoll-Hypaque. The interface band containing mononuclear cells was removed and washed twice with saline. The leukocytes were magnetically separated from normal human leukocytes using a monocyte isolation kit (Miltenyi, Paris, France).

Abs and other reagents

Purified mAbs against human Ags used in trogocytosis experiments were from Immunotools (Friesoythe, Germany); CD2, CD3, CD4, CD5, CD8, CD9, CD11a, CD11b, CD11c, CD16, CD19, CD25, CD27, CD28, CD30, CD40, CD43, CD44, CD45, CD56, CD62L, CD63, CD69, CD71, CD80, CD11b, CD14, CD16, CD18, CD19, CD20, and CD80 of BD Pharmingen (San Diego, CA; FLADRA"). Fluorescent mAbs against mouse FcγRII/III (2.4G2), human CD19 (SI25C1) and CD4 (RPA-T4) were acquired from BD Pharmingen. Alexa 647 (A647) goat anti-mouse IgM was acquired from Molecular Probes (Eugene, OR). Mabs 5C6, specific for mouse CD11b was acquired from the American Type Culture Collection (Manassas, VA). Mabs 11G6 and 5C6 were labeled with Alexa dyes according to the manufacturer’s directions. CD80 against the 31313 BCR (11G6) (32) was a gift from Prof. George Weiner (University of Iowa, Iowa City, IA). mAbs against human β2m (B2.62.23) (33) and MHC class I (W6/32) were produced from hybridoma supernatants in our laboratory. A summary of all mAbs used is presented in Table I. Fluorescently labeled Abs (anti–CD4-APC, anti–CD8-AL647, anti–CD19-APC) used for the detection of human lymphocytes and fluorescent streptavidin were acquired from BD Pharmingen (Le-Pont-de-Claux, France) and anti–CD14 used to detect human monocytes was from Dako (Trappes, France). RTX was obtained from the University of Virginia hospital pharmacy. RTX was labeled with biotin, Alexa 488 (A488), or AL647. DIO and PKH-26 were acquired from Sigma-Aldrich (Lyon, France).

Determination of surface receptor expression

Mouse IgGl or IgG2a mAbs against human Ags were labeled with Fab-Al488 using the Zenon kit Z-25000 or Z-25180 (Molecular Probes, Invitrogen, Cergy Pontoise, France) using the instructions provided by the manufacturer. Briefly, 1 μg mAb was incubated with 5 μl of the chosen Zenon labeling reagent (200 μg/ml). The labeled Fab fragments were then mixed with the intact primary mAb at a molar ratio of 3:1 and incubated at room temperature for 5 min in the dark. At the end of that incubation, 5 μl Zenon blocking reagent was added and incubated at room temperature for 5 min. Unbound Fab-Al488 fragments were absorbed by the nonspecific IgG in the blocking reagent included in the kit. The Zenon-labeled complexes were then used to label cells together with the mAb directed against CD4, CD8, or CD19.

Incorporation of DIO in cells

Cells were labeled with DIO as previously described (8). Briefly, 5 × 10⁶ cells were washed in PBS and resuspended in Diluent C (Sigma-Aldrich). A stock solution of DIO was diluted in diluent C and mixed with the cells at 2 μM final concentration for 5 min. Labeled cells were then washed three times in culture medium.

Cell surface biotinylation

Cell surface biotinylation was carried out as previously described (8). Briefly, 5 × 10⁶ cells were washed in PBS and resuspended in 0.5 ml PBS containing 1 mg/ml Sulfo-NHS-LC-biotin (Pierce, Rockford, IL) and then incubated for 10 min at room temperature. An equal volume of FCS was then added, and the cells were incubated for an additional 10 min at 4°C. Labeled cells were then washed three times in culture medium.

Trogocytosis experiments

In experiments in which trogocytosis mediated by FcγR+ cells was analyzed, donor cells (Raji cells or PBL) labeled with DIO (or cell surface biotinylation in some experiments) were opsonized for 30 min at 4°C with 5 μg/ml of the indicated mAbs, washed, and incubated with FcγR-expressing acceptor cells (primary monocytes, J774, THP-1, or P815 cells). For these experiments, mAbs labeled cells were resuspended in RPMI 1640 medium and 100 μl (corresponding to 0.5 × 10⁶ cells per well) were placed in wells of V-bottom, 96-well plates. FcγR-expressing cells were then added to the wells (0.1 × 10⁶ cells per well), giving a ratio of 5:1 between DIO-labeled donor and unlabeled acceptor cells. The plates were centrifuged for 30 s at 150 × g to promote conjugate formation and left at 37°C for 1 h. Conjugates were then dissociated by washing the cells in PBS containing 2 mM EDTA before analysis on an LSRII cytometer. This treatment results in the gentle but efficient disruption of virtually all cell conjugates. Trogocytosis is, moreover, easily distinguished from cell doublets (or from phagocytosis) because trogocytosis results in the acquisition of only a fraction of the fluorescent present on donor cells, whereas a doublet between a donor and an acceptor cell results in a signal that corresponds to the total fluorescence present on a donor cell (22, 34, 35). In addition, when cocultures were performed between small recipient cells and large donor cells, we restricted our analysis to the small cell population based on FSC/SSC gating (8). In some cases, we also formally excluded doublets using SSC-A and SSC-W parameters; dyeing, permeable cells were excluded using DAPI staining.

In some assays, capture of the CD20/RTX complex was determined by using biotinylated RTX to opsonize Raji cells and monitoring its transfer to FcγR-expressing acceptor cells by development with fluorescent streptavidin. Similarly, the capture of other proteins endogenously expressed by the cells used in cocultures was analyzed using specific mAbs directed against these proteins.

In experiments in which trogocytosis mediated by mAb-coated acceptor cells was analyzed, acceptor cells (Raji cells or PBL) were opsonized for 30 min at 4°C with 5 μg/ml of the indicated mAbs, washed, and incubated with FcγR-expressing acceptor cells (primary monocytes, J774, THP-1, or P815 cells). For this procedure, mAb-coated acceptor cells were resuspended in RPMI 1640 medium and 100 μl (corresponding to 0.1 × 10⁶ cells per well) were placed in wells of V-bottom, 96-well plates. FcγR-expressing donor cells were then added to the wells (0.5 × 10⁶ cells per well), again resulting in a ratio of 5:1 between DIO-labeled donor and unlabeled acceptor cells. The other steps of the experiment were then performed as described above for trogocytosis mediated by FcγR+ cells.

Quantitative analyses of the capture of plasma membrane fragment

The efficiency of membrane capture was calculated and expressed in two different ways. One method calculates the fold induction, as the median fluorescence intensity (MFI) of DIO fluorescence on acceptor cells that were...
coclutred with mAb-opsonized donor cells, divided by the MFI of acceptor cells cultured with nonopsonized donor cells. Based on the variations obtained in different experimental systems with various negative control conditions (e.g., no mAb or various isotype controls) (29), we set an empirical threshold for trogocytosis of 1.5-fold that of the negative control. The second method expresses the percentage of DiO acquired from the donor cells and is calculated as follows: 100 × (MFI of DiO on acceptor cells cocultured in the presence of opsonizing mAb – MFI of DiO on acceptor cells cocultured without mAb)/MFI of DiO on donor cells cocultured without mAb. The incorporation of appropriate lipophilic dyes into the membrane of donor cells is the simplest method with which to follow the transfer of plasma membrane molecules, and this approach has been documented in numerous experimental systems both in vitro (6–8 and references therein) and in vivo (25). However, in this study, we also documented transfer of RTX and other membrane proteins, including biotinylated cell-surface proteins, from donor to acceptor cells to confirm the validity of this technique.

**Fluorescence microscopy and flow cytometric analysis of trogocytosis and phagocytosis by macrophages**

Elicited mouse peritoneal macrophages as well as J774 macrophages were adhered to and cultured on chamber slides. The 38C13–CD200R B cells were stained with PKH26 (36), and after multiple washes were opsonized with Al488- or Al647-RTX (26). The cells were then incubated with either adhered J774 macrophages or mouse peritoneal macrophages at 37°C for 10–15 h. At the end of the incubation, the 38C13–CD200R B cells were gently aspirated and examined by flow cytometry for loss of CD20/RTX complex. To provide a more rigorous index of loss, the recovered B cells were reopsonized with additional Al488 or Al647 RTX. The adhered macrophages were washed for 1 min with 0.05% trypsin/EDTA (Life Technologies 25300, Carlsbad, CA) to remove loosely bound B cells, rinsed once with media, and finally removed by scraping. After centrifugation and washing once more with media, the recovered macrophages were blocked with 2 ng/ml mouse IgG, stained with Al488 mAb 5C6 specific for mouse CD11b, and analyzed by flow cytometry (FacsCalibur, BD Biosciences) for capture of Al487 RTX and PKH26.

After the B cells were removed, the residual adherent macrophages were directly examined by fluorescence microscopy for captured Al488-RTX and PKH26. In these experiments, the slides were stained with Al647 anti-mouse IgM or Al647 mAb 11G6 (specific for the BCR on the 38C13–CD200R cells) to distinguish adhered B cells, which take up the stain, from phagocytosed cells or internalized trogocytosed fragments, which do not. Fluorescence microscopy was performed using an Olympus BX40 (Melville, NY) fluorescent microscope (40× objective). Images were captured with a digital camera and visualized with Magnafire analysis software (Optronics, Goleta, CA).

**Results**

**Trogocytosis in conjugates formed between THP-1 cells and Raji cells opsonized with RTX**

The coculture between a hematopoietic cell opsonized with certain mAbs and FcγR-expressing cells can lead to membrane exchange between these two cell types (26, 29). The direction of membrane exchange can proceed from the mAb-opsonized cell to the FcγR-expressing cell, or in the reverse direction (26, 29) (Fig. 1). The best example of trogocytosis mediated by FcγR+ cells is the interaction between RTX-opsonized B cells and monocyteic THP-1 cells (26). Therefore, we first analyzed the directions of trogocytosis for FcγR+ cells reacted with mAb-opsonized cells. For this determination, cocultures were performed in which either the RTX-opsonized Raji cells or the THP-1 cells were labeled with a fluorescent lipophilic dye to follow the transfer of plasma membrane component induced by RTX in either direction.

As shown in Fig. 2A, we confirmed that THP-1 cells capture plasma membrane components from Raji cells when the coculture was performed in the presence of RTX-opsonized Raji cells, but not in the absence of RTX [see also Neum et al. (26) for fluorescence microscopy and high-resolution fluorescence flow cytometric digital imaging analyses of this process]. After trogocytosis, THP-1 cells exhibit levels of DiO fluorescence that are markedly lower than those present on Raji cells, and this result indicates that this increased fluorescence is not due to cellular doublets or phagocytosis of whole Raji cells, which would both be predicted to result in levels of DiO fluorescence on THP-1 cells equivalent to those of Raji cells. Interestingly, the presence of RTX also induced THP-1 membrane capture by Raji cells (Fig. 2B). That is, RTX opsonization induced a reciprocal exchange of plasma membrane components between the FcγR+ THP-1 cells and the RTX-opsonized Raji cells. Analysis of both the ratios and percentages of trogocytosis by the two cell types (as described in Materials and Methods) revealed that, for the THP-1/RTX-Raji system, trogocytosis mediated by the THP-1 acceptor cells clearly predominates over the uptake of membrane when RTX-opsonized cells are used as acceptor cells. That is, under comparable conditions for DiO labeling, although there is some bidirectional transfer, more membrane dye moves from the Raji cells to the THP-1 cells than from THP-1 cells to the Raji cells.

**Identification of mAbs triggering trogocytosis by human T and B cells**

With the aim of identifying other mAbs directed against receptors present at the surface of human T or B cells able to trigger trogocytosis, we used the same approach that we have previously undertaken with murine T and B cells (29). The FcγR+ mastocytoma cell line P815 was labeled with DiO and cocultured with human PBL opsonized or not, with various mAbs directed against surface determinants expressed by T or B cells. The ability of various mAbs to trigger trogocytosis mediated by the opsonized B or T cells as acceptor cells was evaluated based on the capture, by these cells, of membrane fragments (increase in DiO signal) from P815 cells labeled with DiO. Based on previous knowledge that Ag recognition by the TCR triggers trogocytosis, we tested, as a positive control, the stimulatory OKT-3 anti-CD3e mAb and its isotype-matched control in our initial experimental system. As shown in Fig. 3, OKT-3 but not its isotype control triggered capture of membrane fragments from DiO-labeled P815 cells by both human CD4+ and CD8+ T cells, but not by the human CD19+ B cells. Having obtained this evidence that, like in murine systems (29), trogocytosis mediated by TCR recognition of its Ag can be promoted by anti-CD3e and with the objective to understand whether other surface molecules on effectors cells could trigger trogocytosis, we tested a series of surface molecules for which...
IgG mAbs were available. Results for three other representative examples are presented in Fig. 3A and a summary of our results is presented in Table I and Fig. 3B,3D. The expression of each of these receptors on T and B cells as well as their ability to induce cell activation (as assessed by CD69 upregulation) was determined by flow cytometry. As shown in Table I and in Fig. 3B,3D, we thus identified a selected set of surface receptors present on human CD4+ T cells, CD8+ T cells, or B cells capable of triggering trogocytosis upon interaction with donor P815 cells. Alternatively, CD69 upregulation, which is one of the earliest markers of lymphocyte activation, was only triggered by Ab binding to CD3 on T cells, and to β2 microglobulin on B and T cells. Finally, in results not depicted in Table I, when we used the humanized anti-CD20 RTX mAb in similar experiments, it induced a modest trogocytosis by B cells (ratio = 1.63 ± 0.21).

Target cell, not effector cell or triggering mAb, determines the direction of plasma membrane capture

With the aim of understanding what parameters influence the direction of plasma membrane capture, we performed cocultures between either P815 or THP-1 cells and either Raji cells or PBL. These incubations were performed in the presence or absence of either RTX, W6/32 anti-MHC class I or B2.62.2 anti-β2m Abs, the last two Abs having the advantage of targeting all the lymphocytes studied here (i.e., Raji cells, primary T and B cells). As shown in Fig. 4A and 4B, when THP-1 cells were cocultured with Raji cells in the presence of W6/32 or B2.62.2, the results we obtained were comparable to those with RTX (i.e., trogocytosis by both mAb-opsonized and FcγR+-cells was triggered), and transfer of membrane dye to the THP-1 cells, acting as acceptor cells, predominated. In contrast, when P815 cells were used in place of the donor cells, trogocytosis was only triggered by RTX-opsonized Raji cells, and membrane dye transfer was equal from both cell types. The results obtained in these studies are consistent with our earlier findings that the ability of the cell to capture and transport plasma membrane is determined by the membrane composition of the target cell, and not by the activating mAb or FcγR present on the THP-1 cells acting as acceptor cells. These data also indicate that the RTX mAb acts as a monocyte triggering Ab in this system, thereby triggering trogocytosis by both mAb-opsonized and FcγR+-cells, and not by RTX-opsonized donor cells alone.

FIGURE 2. Bidirectional trogocytosis occurs during THP-1 cell interaction with RTX-coated Raji cells. A, THP-1 cells were conjugated with DiO-labeled Raji cells that were coated (bottom panel) or not (top panel) with RTX to measure trogocytosis mediated by THP-1 cells. Numbers in the panels represent the MFI of DiO on Raji cells (brightly labeled cells) or on THP-1 cells (negative or dim cells). Numbers below the panels represent a quantitative analysis of trogocytosis measured as a fold induction (i.e., MFI on THP-1 cells incubated with RTX-coated Raji cells divided by MFI on THP-1 cells incubated with nonopsonized Raji cells) or as percentage (i.e., 100 × [MFI on THP-1 cells cocultured with RTX-opsonized cells – MFI of DiO on Raji donor cells]). B, As in A except that trogocytosis by Raji cells was assessed and DiO-labeled THP-1 cells (bright cells) were incubated with Raji cells coated (bottom panel) or not coated (top panel) with RTX. Similar results were obtained in three independent experiments.

FIGURE 3. A selective set of cell surface molecules on human CD4+, CD8+ T, and B cells trigger trogocytosis upon engagement with mAbs. A, Trogocytosis mediated by human CD19+, B, CD4+, T, and CD8+ T cells coated with the indicated mAb (white histograms) or its isotype control (gray histograms) was determined in the presence of DiO-labeled P815 cells. Histograms were obtained with cells gated as positive for CD19 (left panels), CD4 (middle panels) or CD8 (right panels). Only CD8bright cells were used to exclude CD8dim cells, which in humans are mostly NK cells. Data are representative of at least three independent experiments. Additional results are presented in Table I. B, Summary of the results obtained in trogocytosis and activation experiments for CD8+ T cells. Only the mAbs found to bind detectably to CD8+ T cells. The rectangles are used to indicate what mAbs trigger the indicated function. C, As in B but for human CD4+ T cells. D, As in B but for human B cells.
cells were used as donor cells in the coculture (Fig. 4C, not shown). Trogocytosis mediated by mAb-opsonized B, CD4, CD8, similar experiments (i.e., uptake of dye from P815 cells), and it was analyzed the transfer of proteins selectively expressed at the surface occurrence of trogocytosis, to confirm that our observations did not show the occurrence of trogocytosis in all cell lines, even in the absence of mAbs targeting surface proteins. This indicates that the trogocytosis mechanism may be more widespread than previously thought. Additionally, the transfer of proteins was assessed using flow cytometry, and the results were consistent with the fluorescence microscopy observations.

The pattern of transfer of lipophilic dye between cells correlates with the exchange of endogenously expressed proteins

Although lipophilic dyes have been widely used to demonstrate the occurrence of trogocytosis, to confirm that our observations did indeed reflect the exchange of plasma membrane fragments, we analyzed the transfer of proteins selectively expressed at the surface of the three cell lines, and for which intercellular transfer had already been reported or suggested (26, 37, 38). Those proteins were for Raji, CD99, and for which intercellular transfer had already been reported or suggested (26, 37, 38). Those proteins were for Raji, CD99, RTX-opsonized Raji cells, whereas capture of these molecules by B, CD4, and CD8 T cells was comparable when THP-1 or P815 cells were used as donor cells in the coculture (Fig. 4F). Therefore, occurrence of bidirectional trogocytosis does not seem to depend either on the mAb used or on the type of mAb-opsonized cell used. In our experimental setup, it is thus the nature of the FcyR-expressing cell, which appears to be the critical parameter in determining the direction of plasma membrane exchange.

**Table I. Summary of the effect of mAbs against the indicated determinants on trogocytosis mediated by CD4⁺, CD8⁺, and B cells**

<table>
<thead>
<tr>
<th>Determinant</th>
<th>Alternative Name</th>
<th>Clone</th>
<th>Isotype</th>
<th>Staining Detected</th>
<th>Trogocytosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD2</td>
<td></td>
<td>MEM-65</td>
<td>Mouse IgG1</td>
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<td>MEM-57</td>
<td>Mouse IgG2a</td>
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<td></td>
<td>MEM-115</td>
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<td>MEM-32</td>
<td>Mouse IgG1</td>
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<tr>
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<td>CD11a</td>
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<td>1.1 ± 0.1</td>
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<td>CD11b</td>
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<tr>
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<td>BU15</td>
<td>Mouse IgG1</td>
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<tr>
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<td>Mouse IgG2b</td>
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*Expression was assessed by flow cytometry using Zenon Technology to reveal lymphocytes incubated ± the indicated mAb. Subset indicates only a fraction of the cells reacted with the mAb, compared with isotype controls.

**The increased fluorescence recorded on acceptor cells reflects trogocytosis and cannot be attributed to cell doublets or to degraded products of a phagocytic process**

The observation that, after a coculture of two cell types, the fluorescent signals that were initially found on one cell type are found associated with the other cell type can, in principle, be...
Trogocytosis mediated by FcγR-expressing cells, and not by the mAb or mAb-coated cells. A, Trogocytosis mediated by FcγR⁺ cells (left panel) and by mAb-coated cells (right panel) between THP-1 cells and Raji cells that were coated (open histograms) or not (gray histograms) with RTX was measured as in Fig. 2. The arrows placed between THP-1 and RAJI indicate the direction of trogocytosis analyzed. Numbers represent a quantitative analysis of trogocytosis measured as fold induction or as a percentage (as in Fig. 2). B, As in A except that trogocytosis by FcγR⁺ cells (filled bars) or by mAb-coated cells (open bars) were assessed using Raji cells that were coated with three different mAbs. The experiment was performed three times, and the means of fold induction plus SD are presented. C, As in A except that P815 cells were used instead of THP-1 cells. D, As in B except that P815 cells were used instead of THP-1 cells. E, Trogocytosis mediated by THP-1 (filled bars) or P815 (open bars) cells conjugated to human PBL labeled with DiO and coated with the indicated mAb. The experiment was performed three times, and the means of fold induction plus SD are presented. F, Trogocytosis mediated by human PBL coated with the indicated mAb conjugated with DiO-labeled THP-1 (filled bars) or P815 (open bars) cells, shown as the means of fold induction plus standard deviations. To avoid overloading the figure, only the highest p value obtained is indicated for the various conditions tested. Trogocytosis was measured separately on B, CD4, or CD8 cells. Cells were stained at 4°C with the appropriate Abs at the end of the 37°C incubation with THP1 cells, and gating identified the positive cells. Student t test was used to assess the significance of the differences in capture in the presence or the absence of the indicated mAb. *p < 0.05; **p < 0.01; ***p < 0.001.

Both primary human monocytes and murine macrophages perform trogocytosis

To ascertain that our observations were not caused by a particular property of the THP-1 cell line of monocytic origin, we next determined whether primary monocytes were also able to perform trogocytosis and function as acceptor cells. To this end, we purified monocytes from human PBMCs, placed them in cocultures with various mAb-opsonized cells, and determined the direction of trogocytosis. As shown in Fig. 7A and 7B, in cocultures with Raji cells opsonized with RTX, B2.62.2 or W6/32, primary human monocytes behaved similarly to THP-1 cells, and although some bidirectional trogocytosis was observed, trogocytosis mediated by the FcγR⁺ acceptor monocytes predominated over that performed by mAb-coated cells. When monocytes were cocultured with PBL (Fig. 7C, 7D), we found that they performed trogocytosis even more efficiently than THP-1 cells, and that they could capture up to 25% of the total DiO originally present on the PBL. In addition, we found that the murine J774 macrophage cell line also performed trogocytosis and served as acceptor cells when cocultured with RTX-opsonized Raji cells and, like THP-1 cells (26), captured the CD20/RTX complex during this reaction (Fig. 7E).

Control experiments, similar to those depicted in Fig. 6 for THP-1 cells, were formally excluded using an SSC-A versus SSC-W gating, and phagocytosed materials. Furthermore, for this experiment, doublets were formally excluded using an SSC-A versus SSC-W gating, and an additional gating on DAPI⁻ cells was also used to ensure that fluorescent streptavidin detected biotinylated proteins at the surface of live cells, and not end products of phagocytosis in dying, permeable cells (Fig. 6A). We thus confirmed that the experimental procedures we have used in this study result in negligible percentages of doublets and permeabilized cells in our samples. As can be seen in Fig. 6B and 6C, the results obtained with this experimental setup were similar to those described in earlier figures with fluorescent membrane probes or by following membrane-bound endogenously expressed proteins: THP-1 cells efficiently captured biotinylated RTX and biotinylated membranes from opsonized Raji cells. In contrast, P815 cells only barely captured these molecules. Thus, based on these experiments, we can formally exclude the interpretation that the relatively high signals we record in THP1 cells correspond to products of phagocytosis, and we confirm that THP-1 cells perform trogocytosis much more efficiently than P815 cells when cocultured with opsonized Raji cells.
cells, were also performed with the J774 cell line and gave similar results (not shown). From these results, we conclude that it is an inherent property of monocytes/macrophages to predominate in trogocytosis (i.e., function primarily as acceptor cells) when they are cocultured with mAb-opsonized cells.

Monocyte/macrophages can perform both trogocytosis and phagocytosis

The results shown in Fig. 6 demonstrated that the rapid and efficient capture of membrane bound materials from opsonized Raji cells by FcR-expressing cells of the monocyte/macrophage lineage is due to trogocytosis rather than phagocytosis. Next, we wanted to explore whether trogocytosis and phagocytosis could occur simultaneously, and what type of signals resulted from each process. For this, we used the murine B cell lymphoma 38C13, engineered to express human CD20, and cocultured these cells with either J774 or primary murine macrophages. The 38C13-CD20+ B cells were stained with PKH26, and we first used fluorescence microscopy to examine the adhered J774 cells for incorporation of both Al488-labeled RTX and PKH26 (Fig. 8A).

The slides were also stained with Al647 anti-mouse IgM to differentiate between adhered and phagocytosed 38C13-CD20+ B cells, were also performed with the J774 cell line and gave similar results (not shown). From these results, we conclude that it is an inherent property of monocytes/macrophages to predominate in trogocytosis (i.e., function primarily as acceptor cells) when they are cocultured with mAb-opsonized cells.
Bidirectional trogocytosis occurs during interaction of RTX-coated Raji cells with primary monocytes or with the J774 macrophage cell line. A, Human monocytes, purified directly from blood obtained from healthy donors as described in Materials and Methods, were incubated conjugated with Raji cells that were opsonized (bottom panels) or not (top panels) with RTX to measure trogocytosis mediated either by FcγR+ cells (left panels) or by mAb-opsonized cells (right panels). Numbers in the panels represent the median fluorescence intensity of DiO on donor cells (brightly labeled cells) or on the recipients (dimmer cells), and those below the panels correspond to fold induction. The arrows placed between Monocytes and RAJI indicate the direction of trogocytosis analyzed. B, As in A except that trogocytosis mediated by FcγR+ cells (filled bars) or by mAb-coated cells (empty bars) was assessed using Raji cells that were opsonized with the indicated mAb and washed before the coculture. The experiment was performed three times, and the means of fold induction plus SD are presented. Student t test was used to assess the significance of differences in capture in the presence or the absence of the indicated mAb. **p < 0.01. C, Trogocytosis mediated by monocytes on DiO-labeled PBL coated or not coated with the anti-class I W6/32 mAb. D, As in B except that PBLs were used instead of Raji cells. Note that only trogocytosis by CD4+ T cells is presented; similar results were obtained in another independent experiment. E, As in A except that J774, a murine macrophage cell line was used instead of monocytes and conjugated with DiO-labeled Raji cells opsonized (bottom panels) or not (top panels) with biotinylated RTX. The capture of DiO (left panels) or biotinylated RTX/CD20 (right panels) was analyzed after staining with anti-C4 mAb (to identify J774 cells), and fluorescent streptavidin to follow RTX/CD20. Numbers indicate the fold induction of trogocytosis. Raji and J774 cells are indicated by the letters R and J in the appropriate quadrants. Data are from one representative experiment of three.

We combined these fluorescence microscopy results with flow cytometric analysis. Macrophages were positively identified by staining with Al488 mAb 5C6, specific for mouse CD11b, and analyzed for uptake of Alexa-labeled RTX and PKH26. The dot plot obtained for naive peritoneal macrophages alone stained with Al488 5C6 is shown in Fig. 9A. The signal in the channel for PKH26 is weak, and virtually all the cells in the R2 gate have an MFI <400 in both the PKH26 and in the Al488 RTX channel (Fig. 9B). In comparison, the Al647-RTX-opsonized PKH26-dyed 38C13-CD20+ B cells show fluorescent signals in excess of 600 U for both Al647 and PKH26 (Fig. 9E, 9F). After incubation of these two cell types for 20 min, a fraction of the recovered macrophages (R2 gate) have acquired substantial amounts of both PKH26 and Al487 RTX signals (Fig. 9C, 9D). For some of these cells, the intensity of the signals corresponds to that of whole 38C13-CD20+ B cells, suggesting that such events likely correspond to phagocytosis or to B cell-macrophage conjugates (Fig. 9D, upper right quadrant), whereas some 38C13-CD20+ cells are still found in the peritoneal macrophage sample, despite the washing steps, and can clearly be identified in the R3 gate (Fig. 9C). In Fig. 9D (upper left quadrant), one can also identify a population of peritoneal macrophages that take up considerably less PKH26 and Al487 RTX (trogocytosis), strongly suggesting that these cells have engaged in trogocytosis rather than phagocytosis. As early as after 10 min, approximately one quarter of the macrophages are found to have taken up amounts of the dyes that correspond to less than a whole cell (Fig. 9D, upper left quadrant). Remarkably, this proportion remains relatively stable for the duration of the experiment while the proportion of macrophages having engulfed whole B cells increases with time. The intensity of fluorescence of Al647 does not decrease at the acid pH of a lysosome (41); therefore, the reduced Al647 signal observed in the upper left
The slides were examined for signals associated with Al488 RTX, PKH26, and Al647 anti-mAbId. Bound 38C13-CD20+ B cells were then washed to remove loosely adherent J774 macrophages. After the 38C13-CD20+ B cells were removed, the adherent J774 cells were stained with Al647 anti-mouse IgM and then analyzed by fluorescence microscopy for Al488 RTX, PKH26, and Al647 anti-mouse IgM. Based on the images (see Results), selected regions in which the B cells were still adhered (AD) or in which the B cells had been either phagocytosed (PH) or trogocytosed (TR) by the J774 cells are identified (original magnification ×40). Similar results were obtained in four independent experiments. B. 38C13-CD20+ B cells were opsonized with Al488 RTX and cocultured with peritoneal macrophages for the indicated times. The chamber slides were then washed to remove loosely bound 38C13-CD20+ B cells. To distinguish residual bound B cells from B cells that were phagocytosed, the slides were then stained with Al647 mAb 11G6, specific for the BCR (Id) on the 38C13-CD20+ B cell. As in Fig. 7A, the slides were examined for signals associated with Al488 RTX, PKH26, and Al647 anti-Id (original magnification ×40). Similar results were obtained in two independent experiments.

FIGURE 8. Fluorescence microscopy analyses of simultaneous trogocytosis and phagocytosis performed by macrophages cocultured with RTX-opsonized 38C13-CD20+ B cells. A. 38C13-CD20+ B cells opsonized with Al488-RTX and dyed with PKH-26 were cocultured for 30 min with adhered J774 macrophages. After the 38C13-CD20+ B cells were removed, the adherent J774 cells were stained with Al647 anti-mouse IgM and then analyzed by fluorescence microscopy for Al488 RTX, PKH26, and for Al647 anti-mouse IgM. Based on the images (see Results), selected regions in which the B cells were still adhered (AD) or in which the B cells had been either phagocytosed (PH) or trogocytosed (TR) by the J774 cells are identified (original magnification ×40). Similar results were obtained in four independent experiments. B. 38C13-CD20+ B cells were opsonized with Al488 RTX and cocultured with peritoneal macrophages for the indicated times. The chamber slides were then washed to remove loosely bound 38C13-CD20+ B cells. To distinguish residual bound B cells from B cells that were phagocytosed, the slides were then stained with Al647 mAb 11G6, specific for the BCR (Id) on the 38C13-CD20+ B cell. As in Fig. 7A, the slides were examined for signals associated with Al488 RTX, PKH26, and Al647 anti-Id (original magnification ×40). Similar results were obtained in two independent experiments.

quadrant of Fig. 9D cannot be attributed to acid pH quenching of phagocytosed Al647 RTX. Combined, the results in Fig. 9G strongly support our interpretation that events that fall in the upper left quadrant correspond to trogocytosis, whereas phagocytosis events fall in the upper right quadrant. If the former corresponded to digestion products after phagocytosis, their proportion should increase with time (Fig. 9G). Alternatively, flow cytometric analyses of the 38C13-CD20+ cells that had been recovered by washing and reopsonization with Al647 RTX reveal that more CD20 was removed from the cells with increasing incubation times, indicating that, in addition to phagocytosis, trogocytosis can proceed for the entire 90-min incubation period (Fig. 9H). Thus, combined flow cytometric and fluorescence microscopic studies provide evidence for the occurrence of both trogocytosis and phagocytosis events during coculture between opsonized B cells and macrophages. Finally, in agreement with our previous reports with human B cells, reaction of naive 38C13-CD20+ B cells with peritoneal macrophages led to no loss of CD20 from the B cells (not shown) (34, 35).

Discussion

The exchange of plasma membrane components between immune cells is common during cellular encounters and could play important roles in physiologic, pathologic, or therapeutic situations (4, 27). For example, trogocytosis mediated by FcγR+ cells appears to be critically involved in antigenic modulation (i.e., the loss of mAb-targeted Ag at the surface of cells) (23, 25, 26). Indeed, FcγR+ monocyte/macrophages remove CD20-RTX complexes associated with other membrane components from the surface of leukemia cells in vitro, and it is likely that this reaction contributes to the escape of leukemia cells that have lost CD20 in vivo as a consequence of RTX treatment (24, 26, 27, 39, 40). In other studies, membrane fragments were shown to be captured by the mAb-targeted cells, making the direction of membrane movement somewhat uncertain (29). By varying the nature of the mAb-targeted cells, of the targeting mAb and of the FcγR-expressing cells, we found that the latter was the major factor that determined the direction of the trogocytosis reaction. With monocytes/macrophages or monocytic cell lines, which are prone to perform capture, exchange of plasma membrane with the mAb-targeted cells was bidirectional, but was quantitatively dominated by capture of membrane by the FcγR-expressing cells (Figs. 2, 4–7). In contrast, with other FcγR-expressing cells such as P815 cells, the exchange of plasma membrane components was almost exclusively mediated by the mAb-opsonized lymphocytes—that is, the Ab-opsonized B cells or T cells took up membrane fragments from the P815 cells (Figs. 4, 5).

In human systems, mainly RTX (and in one study, two other mAbs approved by the U.S. Food and Drug Administration) has been used to study the exchange of plasma membrane components and was found to induce antigenic modulation by shoving both in fluorescence microscopy and flow cytometric experiments (24, 26, 27, 39, 40). In contrast, with other FcγR-expressing cells such as P815 cells, the exchange of plasma membrane components was almost exclusively mediated by the mAb-opsonized lymphocytes (4, 27). For example, trogocytosis mediated by FcγR+ cells was specifically caused by these mAbs, we approached the question from a more general perspective and examined a large number of other unrelated mAbs for their capacity to trigger the exchange of plasma membrane components in human immune cells (Table I; Fig. 3). We identified a series of such mAbs acting in a cell-specific (e.g., anti-CD3, anti-CD8 mAb) or nonspecific manner (e.g., anti-MHC class I, anti-β2m mAbs). When compared with the results previously obtained in the mouse system (29), we found that large numbers of the determinants, shared by both human and murine cells, promote trogocytosis by B or T cells after these cells are opsonized with specific mAbs. For example, this was observed in T cells in the case of the TCR/CD3 complex, CD2, CD8 (in CD8+ T cells only), MHC class I, and β2m. Notable differences with our previous work on murine cells included the absence of trogocytosis-triggering activity in human cells after reaction with anti-CD27, CD28, and CD4 mAbs, but positive triggering by anti-CD43 and CD44 mAbs. In human B cells, trogocytosis was triggered by mAbs to the BCR, MHC class I, and β2m, as was observed in murine B cells. A difference in regard to murine B cells was the positive effect of anti-CD43 and -CD44 mAbs, and the absence of an effect mediated by the anti-MHC
class II mAb. As pointed out in our previous study (29), these differences could be due to the epitope recognized by the mAb (with multiple mAbs against the same determinant, some could trigger trogocytosis and others could not) or due to differences in the determinant itself, which could have different features in humans and mice. Furthermore, confirming a recently published paper (42), we found that anti-human MHC class II mAb triggered trogocytosis when loaded on Raji cells (not shown), although the same mAb did not when loaded on normal human B cells (Fig. 3), suggesting that triggering of trogocytosis by anti-MHC class II molecules can vary depending on the cellular environments. Many other surface determinants did not trigger trogocytosis either in humans or in murine cells when engaged by specific mAbs. For example, RTX-opsonized normal B cells only modestly took up membrane dye from P815 cells, and we have not yet been able to define the rules that govern whether a determinant targeted by a given mAb will promote the reaction.

Based on these findings, we evaluated the role of mAbs in dictating the direction of plasma membrane exchange. We found that the opsonizing mAb determined whether the exchange of membrane fragments could occur, but it did not appear to have any effect on the directionality of these exchanges (Figs. 4, 7). In contrast, the nature of the FcγR+ cells was critical; we found that P815 cells function effectively as donor cells and promote trogocytosis mediated by mAb-opsonized B or T acceptor cells. The P815 cells only weakly capture membrane fragments themselves, thereby confirming our previous results obtained with other cell lines (29) (Figs. 3–6). Thus with such cells, the capture of plasma membrane is largely unidirectional. Alternatively, when we examined J774 cells (Figs. 7, 8) or THP-1 cells (26, 39) (Figs. 2, 4, 5, 6), we found that both of these cell types were efficient at performing trogocytosis and taking up both opsonizing mAb and membrane fragments from donor cells. Interestingly, this ability to take up membrane fragments from targeted cells does not prevent...
these monocyte/macrophages from also serving as donor cells, thus allowing some of their membrane fragments to be taken up by the mAb-opsonized cells. However, on a quantitative basis, this membrane trafficking is dominated by trogocytosis mediated by these FcγR+ cells. Similar results were obtained with primary human monocytes (Fig. 7), and with mouse peritoneal macrophages (Figs. 8, 9), and these FcγR+ cells and tissue macrophages are likely to promote trogocytosis in vivo (24, 39, 40). Bi-directional exchanges of plasma membrane components are not, however, a unique feature of monocytes, and data present in the literature have shown that other myeloid cells do perform capture (14, 18, 22) or can be involved in bidirectional exchange (24, 39, 40). Finally, although we cannot exclude the possibility, we have never observed reactions in which mAbs (such as those tested in our study; Table I) could trigger unidirectional trogocytosis by FcγR+ cells, without some reciprocal transfer of membrane components to the mAb-opsonized cells (data not shown).

The occurrence of unidirectional versus bidirectional trogocytosis is not a matter of compatibility between the species of donor and acceptor cells. We found bidirectional trogocytosis with human donor cells and either human or murine recipient cells, provided that the latter were of monocytic origin (Figs. 4, 5, 7). Furthermore, we also saw trogocytosis by murine macrophages coincubated with murine donor cells (Figs. 8, 9). Thus, the cell type, not the cell species (i.e., human versus mouse), drives the directionality of trogocytosis. Most of our observations were made based on the transfer of lipophilic dyes incorporated in the plasma membrane of donor cells. Our findings were also confirmed based on using either the transfer of proteins endogenously and specifically expressed by the cells or based on studies of transfer of cell-surface biotin from biotinylated donor cells, or by following the transfer of labeled RTX from donor to acceptor cells (Figs. 5–9). Because most FcR-expressing cells perform some level of phagocytosis, and because macrophages, which preferentially perform bi-directional trogocytosis, have been shown to phagocytose RTX-opsonized cells (45 and references therein), we tested whether we could differentiate trogocytosis from phagocytosis in cocultures between RTX-opsonized B cells and macrophages. Based on fluorescence microscopy and flow cytometric analyses, we found that both these processes could occur simultaneously during the cocultures, but could be easily distinguished based on various criteria. Fluorescence microscopy analyses showed that both whole B cells and small fragments of lipophilic dye-labeled membranes and small amounts of A1488 RTX could be detected within J774 or peritoneal macrophages (Fig. 8). This finding is in agreement with a previous report in which we used high-resolution digital imaging in a flow cytometric environment (Amnis Technology, Seattle, WA) to show that individual THP-1 cells had taken up both A1488 RTX and PKH26 from donor B cells, thus eliminating any ambiguities owing to possible doublets (40).

Flow cytometry showed that macrophages exposed to fluorescently labeled B cells could exhibit either a level of staining corresponding to whole B cells, as expected for phagocytosis (whole B cell capture), or inferior (below the threshold set for phagocytosis), as expected after trogocytosis (capture of B cells fragments; Fig. 9). A remarkable observation noted that the proportion of macrophages with low staining levels, suggesting the occurrence of trogocytosis, remained relatively stable over the course of the experiment, while the proportion of macrophages harboring levels of staining corresponding to at least one whole B cells increased with time (Fig. 9G). This finding strongly supports the interpretation that the weaker signals indeed correspond to trogocytosis and not to the aftermath of phagocytosis; the proportion of cells harboring weaker signals would also be expected to increase with time if they corresponded to events that followed phagocytosis. Rather, our observations that B cells were shaved of RTX-CD20 complexes (Fig. 9), a phenomenon that can be attributed to trogocytosis (26, 40) but not phagocytosis, are compatible with a scenario whereby, over time, more macrophages encounter opsonized cells and perform mostly trogocytosis and sometimes phagocytosis. When the latter occurs, however, the intensity of the signals will mask any weaker signals because of previous trogocytotic activity. This type of scenario probably also occurs in vivo, because in our previous clinical study we observed decrease of RTX staining owing to trogocytosis, as well as clearance of leukemia cells owing to phagocytosis (27).

The detailed mechanism of trogocytosis has yet to be elucidated (4), but likely requires contact between donor and acceptor cells and the formation of an immunologic synapse (24, 39, 40). In pathophysiological terms, the capture of membrane fragments including Ags or MHC molecules by myeloid cells has been shown to play a role in T cell development (46, 47) as well as in Ag capture and presentation to T cells (48, 49). We note that not all DCs can perform capture of membranes with a similar efficiency, and some of them might not perform capture at all (20, 50). In immunotherapeutic terms, the capture of mAb-targeted determinants present on tumor cells by myeloid cells has been shown to compromise the effectiveness of RTX-based treatment of CLL (24, 26, 39, 40); repeated administration of lower doses of RTX reduced the removal of CD20-RTX complexes by shaving and enhanced targeting of leukemia cells (27). Our results might lead to alternative approaches, based on manipulating either the effector cells or the targeting mAbs for limiting unwanted processes, such as shaving, that compromise immunotherapies.

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Disclosures
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References


