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Differential mechanisms of microparticle transfer to B cells and monocytes: anti-inflammatory properties of microparticles

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Microparticles are small vesicles released from the plasma membrane of various cell types independently of apoptosis or cell death, are transferred between cells, and carry membrane proteins from one cell to another. We have studied the mechanism of uptake of microparticles by monocytes and B cells. The transfer of microparticles to B cells was almost completely dependent on complement. Incubation of microparticles with serum resulted in opsonization of microparticles with the complement cleavage product iC3b. The subsequent transfer to B cells was mediated by the complement receptor CR2. The interaction between iC3b-opsonized microparticles and B cells reduced the activation of B cells as measured by expression of MHC class II, CD86 and CD25. In contrast, transfer of microparticles to monocytes was only partially complement dependent, but involved calcium and annexin V, and was found to change the cytokine profile of monocytes towards a reduced release of the pro-inflammatory cytokines GM-CSF and TNF-α and an increased release of the anti-inflammatory cytokine IL-10. These data show that microparticles are taken up by B cells and monocytes by different mechanisms and modulate the activation of monocytes and B cells towards an anti-inflammatory phenotype. Microparticles might be involved in counterbalancing pro-inflammatory signals arising from tissue injury or inflammation.

Key words: Microparticles · Complement · B cells · Monocytes

Introduction

Under physiological conditions, small membrane vesicles are spontaneously released from the plasma membrane of a variety of cell types [1, 2]. Shedding of microparticles can occur independently of apoptosis or cell death, and seems to be part of a normal cellular function. Microparticles typically range in size from 0.1 to 2 μm, and include cytoplasmic components and cell surface-derived elements, such as membrane receptors [3–6]. Microparticles are defined by their size and the presence of negatively charged phospholipids like phosphatidylserine in the outer membrane leaflet [7]. In most cases stimulation of cells increases the release of microparticles. The shedding of microparticles was first described from activated platelets [8] that resulted in activation of endothelial cells and an increased binding of monocytes to endothelial cells by up-regulation of adhesion molecules [9]. Megakaryocytes and platelets express high amounts of CXCR4 that is also found on microparticles derived from these cells [10]. Micro-

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Abbreviations: **CM-DiI:** chloromethylbenzamido-dialkylcarbocyanines · **DiI:** dialkylcarbocyanines · **EpCAM:** epithelial cell adhesion molecule

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particles can also transfer molecules from one cell to another, as we and others have shown for the chemokine receptor CCR5, CXCR4 and platelet-derived molecules [10–12]. It is also known that, upon T cell receptor stimulation, T cells release microvesicles with morphological characteristics of exosomes [13]. Exosomes derived from dendritic cells and B cells have also been shown to mediate antigen presentation and might also be involved in intercellular transfer of membrane material [14, 15]. B cell-derived vesicles are found in great quantities on the surface of follicular dendritic cells in human tonsils and render them MHC class II positive [16]. Microparticles are able to act locally, but are also detectable in the peripheral blood and might thereby act at distant sites. The appearance of microparticles in the peripheral blood is associated with a variety of clinical conditions [5, 6, 17].

The aim of our study was to define in more detail the mechanisms of transfer of microparticles to monocytes and B cells. We report that serum markedly increases the uptake of phosphatidylserine-containing microparticles by human B lymphocytes. Incubation with serum results in a deposition of iC3b on microparticles, which enables their transfer to B cells in a CD21-dependent fashion. Transfer of microparticles to monocytes is largely complement independent, but dependent on calcium and annexin V. Microparticle transfer influences both B cell and monocyte function, resulting in a reduced activation of B cells and induction of an anti-inflammatory cytokine profile in monocytes. Microparticles preferentially released from stressed or activated cells might help to control a potentially harmful immune response during conditions of tissue injury.

Results

Microparticles are transferred to PBMC and endothelial cells

To investigate whether microparticles derived from Kato cells express phosphatidylserine on their outer membrane leaflet, we stained the microparticles with the calcium-dependent ligand of phosphatidylserine, annexin V-FITC. Kato cells were incubated in medium for 90 min at 37° C to generate microparticles. After centrifugation for 5 min at 200 × *g* cell-free supernatant was recovered and the microparticles were pelleted by centrifugation for 10 min at 16 000 × *g*. Flow cytometry revealed that the majority of microparticles stained positive for annexin V-FITC (Fig. 1A). To further characterize the outer membrane leaflet of the microparticles, we tested for the presence of specific membrane proteins derived from the donor cells. Epithelial cell adhesion molecule (EpCAM) is a transmembrane glycoprotein, strongly expressed on the human gastric carcinoma cell line Kato. Microparticles released from Kato cells showed a strong expression of EpCAM (Fig. 1A). When Kato cells were stained with a fluorescent lipophilic membrane dye dialkylcarbocyanine (DiI), microparticles released from the surface of these cells were clearly DiI positive, and were used to determine microparticle transfer in most of the experiments (Fig. 1A).

Next, we examined the uptake of microparticles by different cell types. After incubation of Kato cells with PBMC for 90 min in medium, distinct staining for EpCAM was detectable on previously EpCAM-negative monocytes (Fig. 1B). In contrast to monocytes, little transfer of EpCAM to $CD19^+$ B cells or $CD19^-$ lymphocytes was detectable. In the absence of microparticles, there was no binding of EpCAM antibodies to lymphocytes or monocytes (Fig. 1B). Fig. 1C shows the transfer of microparticles derived from DiI-labeled Kato cells. During a 90-min co-incubation with PBMC, DiI-stained microparticles were transferred to monocytes and to a much lesser extend to B cells. To exclude that the membrane dye DiI is released from stained cells, microparticle-free supernatant from DiI-stained cells was generated by ultracentrifugation (15 000 × *g*) and incubated with PBMC. Microparticle-free supernatant (SN 15 000 × *g*) did not result in a DiI-positive staining of monocytes, while the microparticle fraction (pellet 15 000 × *g*) generated by ultracentrifugation of cell-free supernatant of DiI-stained Kato cells is transferred to monocytes (Fig. 1E). The transfer of microparticles to monocytes is temperature and time dependent. At 4°C no transfer of microparticles is observed even after prolonged incubation. At 37°C microparticle transfer becomes clearly visible after 30 min (Fig. 1D).

The transfer of microparticles from Kato cells to monocytes was also analyzed by fluorescence microscopy. Kato cells were stained with chloromethylbenzamido-dialkylcarbocyanines (CM-DiI) and co-incubated with PBMC for 90 min. After co-incubation $CD14^+$ monocytes were enriched by magnetic beads, resulting in 90% monocytes and 10% Kato cells present after enrichment. Fig. 1F reveals a brightly stained Kato cell and several significantly smaller monocytes that show an uptake of DiI-labeled microparticles along the cell membrane and also in intracellular compartments.

Differential involvement of actin polymerization, calcium and annexin V on microparticle transfer to monocytes and B cells

To explore the role of phagocytosis in this transfer model, we pretreated acceptor PBMC with cytochalasin D, a potent inhibitor of actin polymerization and phagocytosis (Fig. 2A). After cytochalasin D incubation, monocytes took up fewer Kato-derived microparticles,

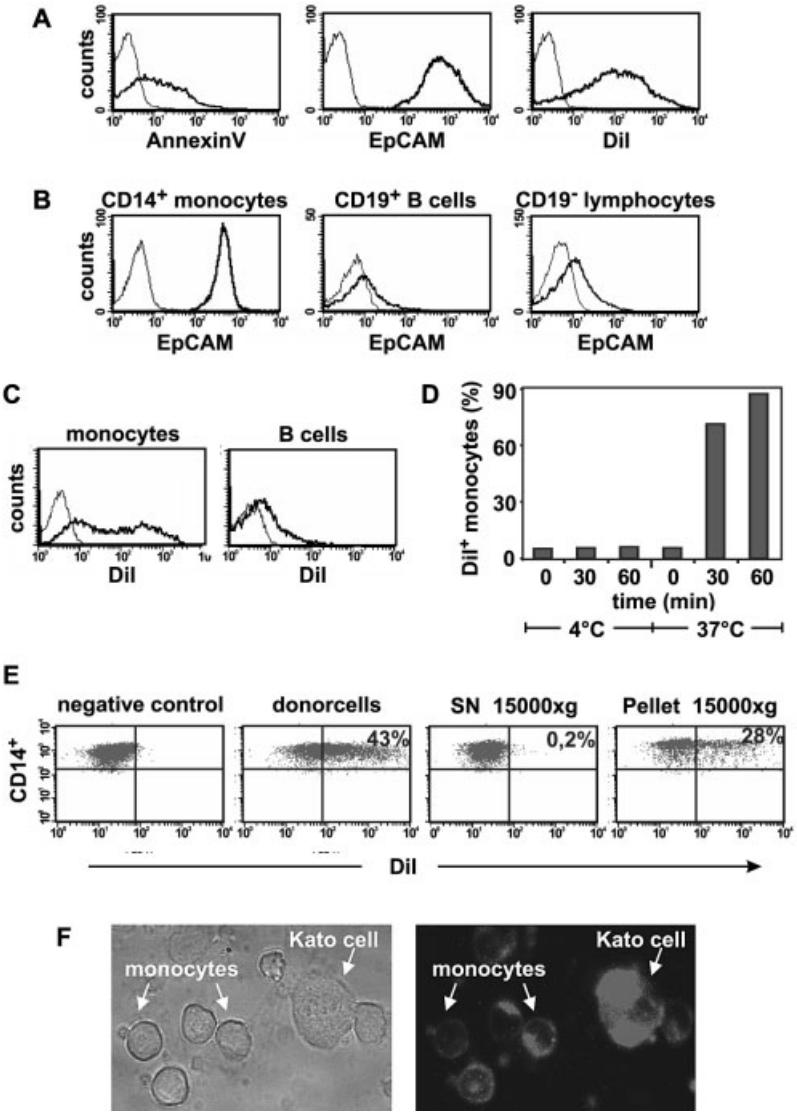

Figure 1. Characterization and transfer of microparticles. (A) Isolated Kato cell-derived microparticles were stained with annexin V-FITC (thick line) or binding buffer (thin line) (left). Staining with an mAb against EpCAM (thick line) or an isotype control antibody (thin line) is shown in the middle. The right panel shows microparticles derived from DiI-stained Kato cells (thick line) compared to microparticles from unstained Kato cells (thin line). (B) Transfer of EpCAM from Kato cells to monocytes, $CD19^+$ B cells and $CD19^-$ lymphocytes (thick lines). Thin lines show EpCAM expression before transfer of microparticles. (C) Transfer of microparticles derived from DiI-stained (thick line) or unstained (thin line) Kato cells to PBMC as detected by DiI staining on monocytes and B cells (D) Transfer of microparticles from DiI-stained Kato cells to monocytes is only seen at 37°C after 30 min. (E) PBMC were incubated with unstained (negative control) or DiI-stained (donor cells) Kato cells. Cell-free supernatant of DiI-stained Kato cells was centrifuged at 15 000 × *g* to generate microparticle-free supernatant (SN 15 000 × g) and the microparticle fraction (pellet 15 000 × g). After co-incubation only the microparticle fraction and not the microparticle-free supernatant resulted in DiI-positive monocytes. $CD14^+$ monocytes were gated. (F) Fluorescence microscopy of microparticle transfer. Monocytes were incubated with CM-DiI-stained Kato cells. Monocytes were enriched with magnetic beads and inspected by bright field light microscopy (left) and fluorescence microscopy (right) under 40× magnification.

whereas the transfer of microparticles to B cells was not altered. We also determined the influence of calcium on the transfer of microparticles. While microparticle transfer to monocytes was completely dependent on calcium, the transfer to B cells was not altered by removal of calcium with EDTA (Fig. 2B).

Annexins, including annexin V, have been shown to be involved in processes of membrane fusion [18]. Interestingly, annexin V that is primarily an intracellular molecule is readily detectable on the surface of monocytes by flow cytometry, while lymphocytes and Kato cells do not express annexin V on their surface (Fig. 2C). As microparticles express phosphatidylserine on their surface (see Fig. 1A) and thereby have the ability to bind annexin V, we analyzed the role of annexin V on the transfer of microparticles. An mAb

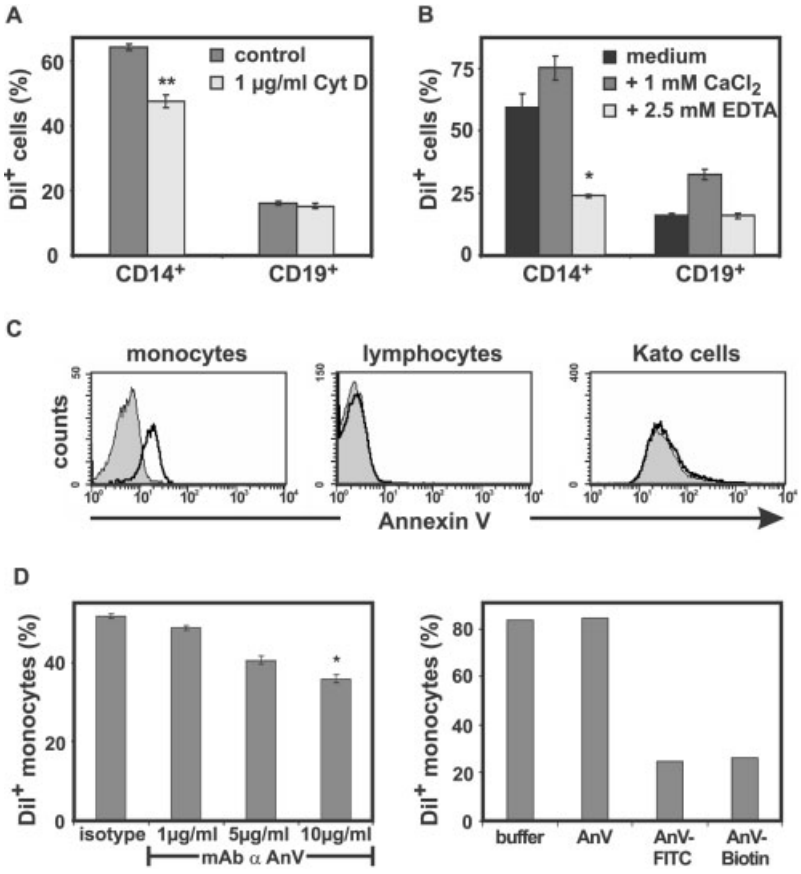

Figure 2. Effect of actin polymerization, calcium and annexin V on transfer of microparticles to monocytes and B cells. (A) PBMC were pre-incubated with cytochalasin D or dimethylsulfoxide (control), washed and then incubated with DiI-stained Kato cells. Cytochalasin D decreased the transfer of microparticles to monocytes but had no effect on the uptake of microparticles by B cells. Error bars represent ± SEM (**$p<0.01$ *vs.* control, $n=4$). (B) Transfer of microparticles from Kato cells to PBMC in the presence of RPMI medium (containing 1 mM Ca^{2+}) or in RPMI medium with additional $CaCl_2$ (+ 1 mM) or EDTA (2.5 mM). EDTA substantially decreased the microparticle transfer to monocytes, but not to B cells. Error bars represent ± SEM (*$p<0.05$ *vs.* control, $n=4$). (C) Expression of annexin V on the surface of monocytes, lymphocytes and Kato cells as determined by flow cytometry using the monoclonal annexin V antibody UMC01 (10 µg/mL, thick line) or IgG1 isotype control antibody (10 µg/mL, gray area) followed by PE-labeled rabbit anti mouse secondary antibodies. (D) The mAb against annexin V (clone UMC01) present during co-incubation of PBMC and DiI-stained Kato cells significantly reduces the transfer of microparticles to monocytes (left panel). IgG1 isotype control antibody was used at 10 µg/mL (left panel). FITC- and biotin-labeled annexin V (5 µg/mL) (AnV-FITC, AnV-biotin) also markedly reduce the microparticle transfer to monocytes, while unlabeled annexin V (AnV) has no effect (right panel). The calcium containing annexin V binding buffer alone (BD Bioscience) was used as control.

against annexin V significantly reduced the transfer of microparticles to monocytes (Fig. 2D, left panel). Also FITC- or biotin-labeled annexin V markedly reduced the microparticle transfer to monocytes, while unmodified annexin V had no effect. We assume that the annexin V antibody interferes with the ability of annexin V present on monocytes to interact with phosphatidylserine on microparticles. FITC- or biotin-labeled annexin V might cover phosphatidylserine on microparticles and, in contrast to unmodified annexin V, fail to interact with monocytes. Neither the annexin V antibody nor modified annexin V had any influence on microparticle transfer to lymphocytes or B cells (data not shown).

Preincubation of acceptor PBMC with a blocking mAb against the phosphatidylserine receptor (mAb 217) [19] did not result in a diminished uptake of microparticles by monocytes or lymphocytes (data not shown).

Influence of serum and complement components on the uptake of microparticles by human PBMC

Circulating microparticles exist in the peripheral blood and are associated with certain diseases [5, 6, 17]. To analyze the transfer of microparticles under more physiological conditions, we studied the transfer of Kato-derived microparticles in the presence of human serum or plasma. Kato cells were preincubated for

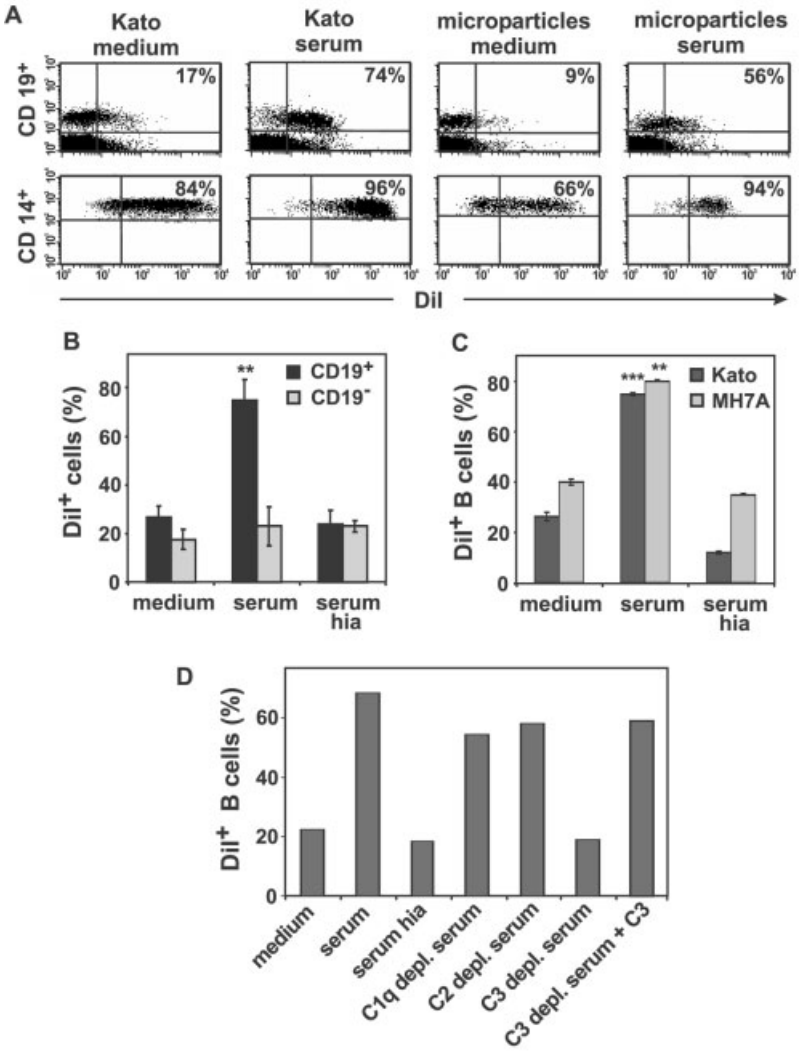

Figure 3. Influence of complement on microparticle transfer to monocytes and B cells. (A) DiI-stained Kato cells or isolated microparticles were preincubated with medium or serum, washed and then incubated with PBMC. Preincubation with serum markedly enhanced the transfer of microparticles to B cells (upper panels) and to a lower extent to monocytes (lower panels). Numbers in the upper right quadrants give the fractions of DiI-positive B cells and monocytes cells. Gating was performed on the lymphocyte fraction in the upper panels and on $CD14^+$ monocytes in the lower panels. (B) Isolated Kato cell-derived microparticles were preincubated with medium, serum or heat-inactivated serum. Serum in contrast to heat-inactivated serum enabled the microparticle transfer to $CD19^+$ B cells but not $CD19^-$ lymphocytes. Data represent mean ± SEM (*$p<0.0001$ *vs.* medium control, $n=4$). (C) Uptake of microparticles derived from Kato cells (Kato) and human fibroblasts (MH7A) by B cells. Isolated microparticles were preincubated with medium, human serum or heat-inactivated (hia) serum. (D) Microparticles were preincubated with medium, serum, heat-inactivated serum, C1q-, C2- or C3-depleted serum and co-incubated with PBMC. The transfer of microparticles to $CD19^+$ B cells is reduced with C3-depleted serum and restored by add-back of C3. Data are representative of four independent experiments.

30 min with serum, washed and then incubated with PBMC. Preincubation of donor cells with serum markedly increased the transfer of microparticles to B cells from 17% to 74%. The transfer of microparticles to monocytes was already 84% in the absence of serum and increased to 96% with serum (Fig. 3A). To exclude the possibility that preincubation of Kato cells with serum only increases the release of microparticles by the donor cells, we performed the same experiments with isolated microparticles. Preincubation of isolated microparticles with serum also clearly enhanced their transfer to B cells and to a lesser extent to monocytes (Fig. 3A).

As a coarse indicator of a potential contribution of complement for the observed serum effect, we heat inactivated serum prior to incubation with microparticles. Heat-inactivated serum, in contrast to untreated serum, was completely unable to enhance the transfer of microparticles to B cells (Fig. 3B). The transfer of microparticles to $CD19^-$ lymphocytes that primarily consist of T cells and NK cells was not enhanced by

preincubation of microparticles with serum (Fig. 3B). In addition to Kato cells, we used a second cell line (human fibroblast cell line MH7A) to confirm the increased uptake of microparticles by B cells in the presence of human serum (Fig. 3C). Again, heat-inactivated serum did not influence the microparticle uptake by B cells (Fig. 3C). Heat inactivation of serum suggested that complement components might be responsible for the enhanced transfer with serum. To identify the responsible complement components, serum depleted of distinct complement components was analyzed. Serum depleted of C1q or C2 was almost as effective as undepleted serum, while no enhanced transfer of microparticles was observed with C3-deficient serum (Fig. 3D). Moreover, to verify the specificity of these observations, we performed add-back experiments with the purified complement component C3. When the complement component C3 was added to C3-deficient serum, the uptake of microparticles by B cells was restored and returned to the value observed with undepleted serum. However, preincubation of microparticles with the complement component C3 alone (1 mg/mL) did not result in an enhanced uptake by B cells, suggesting that other yet unidentified serum components are also required (data not shown).

These data were confirmed by fluorescence microscopy using the lipophilic membrane dye CM-DiI to stain microparticles. Preincubation of microparticles with serum greatly enhanced the transfer of microparticles to B cells, while no enhancement was observed with medium or heat-inactivated serum (Fig. 4).

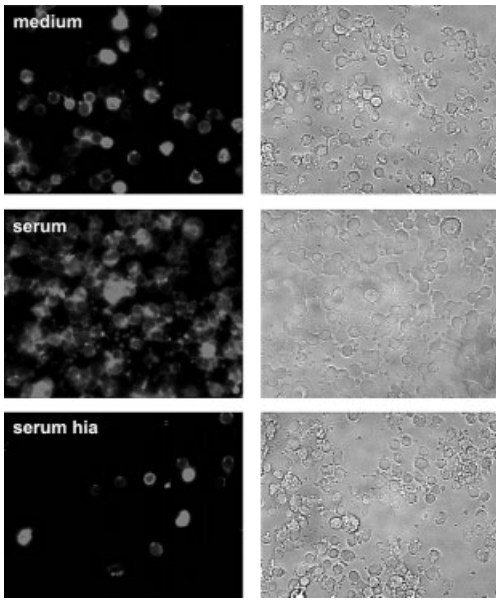

Figure 4. Fluorescence microscopy of transferred microparticles. Kato cell-derived microparticles were stained with CM-DiI and pre-treated with medium, serum or heat-inactivated serum, washed and then incubated with isolated B cells. Fluorescence microscopy (left) showed an increased uptake of microparticles with serum compared to heat-inactivated serum or medium. On the right side the cells are shown by bright field light microscopy under 40× magnification.

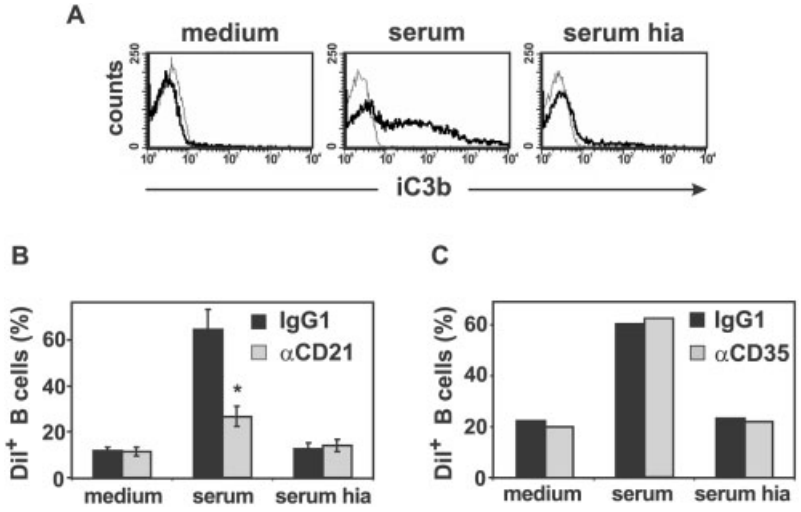

Figure 5. Complement and complement receptor dependent transfer of microparticles. (A) Histogram analysis showing Kato cell-derived microparticles stained for iC3b after pre-incubation with medium, serum or heat-inactivated (hia) serum. Thick lines show staining with an antibody against iC3b, thin lines are the isotype controls. (B) Transfer of microparticles to B cells in the presence of a blocking antibody against CD21 (αCD21) or an isotype control antibody (mouse IgG1). Microparticles were preincubated with medium, serum or heat-inactivated (hia) serum as indicated. Error bars represent ± SEM (*$p<0.05$ *vs.* control, $n=3$). (C) B cells were preincubated with a blocking antibody against CD35 or an isotype control antibody. Transfer of microparticles pretreated with medium, serum or heat-inactivated (hia) serum was analyzed by flow cytometry. The graph is representative of three independent experiments.

Complement receptor CR2 is required for efficient uptake of iC3b-coated microparticles by B cells

To further investigate how complement might be involved in the transfer of microparticles, we examined whether the major C3 breakdown product, iC3b, was deposited on the microparticle surface (Fig. 5A). Flow cytometry revealed that the majority of isolated microparticles treated with serum were coated with iC3b, whereas microparticles incubated with medium or heat-inactivated serum did not show any binding of iC3b (Fig. 5A). These data show that microparticles have the ability to at least partially activate the complement pathway resulting in deposition of iC3b on their surface.

Human B cells express two receptors, CR1 and CR2, which bind complement components or their degradation products. CR1 (CD35) binds mainly C3b, C4b and C1q [20, 21], whereas CR2 (CD21) binds the natural proteolytic fragments iC3b, C3dg and C3d [22, 23]. Flow cytometry with mAb to CR1 and CR2 confirmed that B cells are positive for CR1 and CR2 (data not shown). Since Kato cell-derived microparticles were coated with iC3b after serum exposure, we performed blocking experiments with mAb specific to the complement receptors CR1 and CR2 to evaluate their role in ligand binding. The transfer of microparticles pretreated with serum was inhibited by preincubation of B cells with a blocking antibody against CR2 (Fig. 5B) but not with an antibody against CR1 (Fig. 5C).

Uptake of microparticles shifts the cytokine release of monocytes towards an anti-inflammatory profile and negatively affects B cell activation

To determine if the transfer of microparticles alters cellular function of monocytes, we analyzed their release of cytokines after stimulation with LPS. Monocytes were exposed to Kato cell-derived isolated microparticles that were preincubated with or without

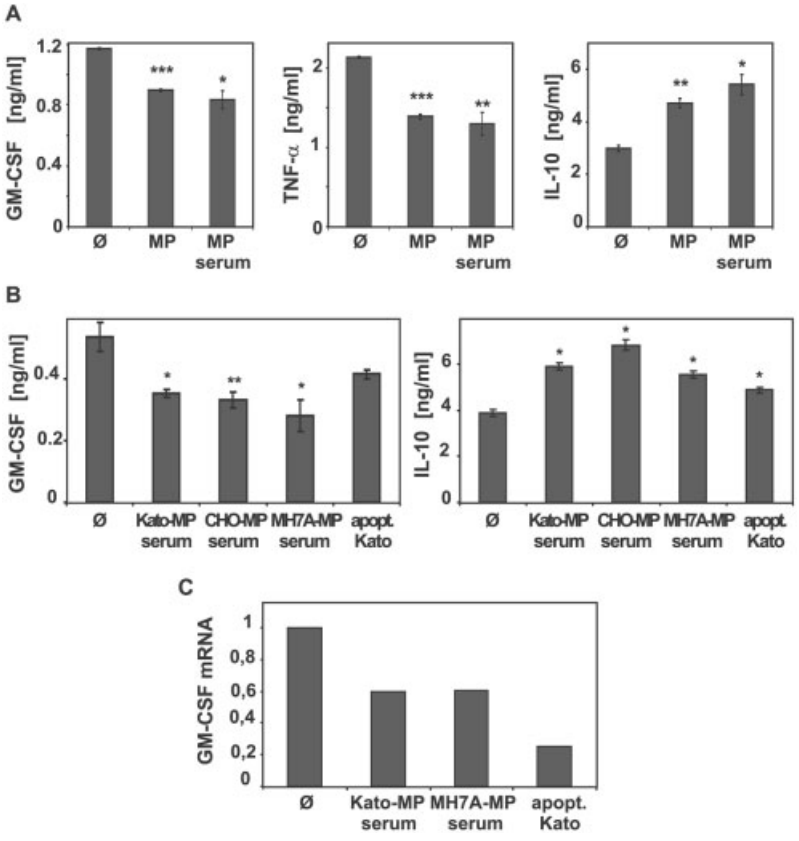

Figure 6. Transfer of microparticles modulates the cytokine release by monocytes. Isolated monocytes were incubated with microparticles (MP) or serum-treated microparticles (MP/serum) and then stimulated with 100 ng/mL LPS. After 48 h, the release of GM-CSF, TNF-α and IL-10 into the supernatant was measured by ELISA. (A) Microparticles derived from Kato cells were used. (B) Microparticles derived from Kato cells, CHO cells and human fibroblasts (MH7A) as well as apoptotic Kato cells were used. Data represent the mean for three experiments ± SEM; (*$p<0.05$, **$p<0.01$ and ***$p<0.001$ *vs.* untreated monocytes). (C) Preincubation of monocytes with microparticles derived from Kato cells or fibroblasts and apoptotic Kato cells down-regulate GM-CSF expression on mRNA level as measured by real-time PCR. Target gene expression was normalized to two housekeeping genes (GAPDH, 18S rRNA) with comparable results. Here target gene expression normalized to 18S rRNA is shown, untreated $CD14^+$ cells were set as 1.

human serum for 30 min at 37°C and subsequently washed to remove the serum. Monocytes were washed to remove unbound microparticles and then stimulated for 48 h with LPS (1 ng/mL). Supernatants were analyzed for GM-CSF, TNF-α, IL-10 and TGF-β (Fig. 6A). Monocytes that have taken up microparticles showed a significantly decreased production of GM-CSF and TNF-α in comparison to monocytes that had not taken up microparticles. In contrast, the transfer of microparticles to monocytes clearly increased the release of IL-10 ($p<0.01$). When microparticles pre-incubated with serum were used, the production of GM-CSF further decreased, and the release of IL-10 further increased (Fig. 6A). The effect of microparticles on the IL-10 release was dose dependent, as reduced amounts of microparticles (25%) resulted in a lower increase in IL-10 (data not shown). No enhanced release of TGF-β was observed after exposure of monocytes to microparticles (data not shown). Apart from Kato cells, we used Chinese hamster ovary cells (CHO cells) and a human fibroblast cell line (MH7A) to generate microparticles (Fig. 6B). Microparticles derived from all three cell lines were clearly able to reduce the GM-CSF release and to increase the IL-10 release from monocytes. Apoptotic Kato cells were somewhat less effective than Kat cell-derived microparticles in altering the cytokine profile of monocytes (Fig. 6B). Also on the mRNA level microparticles and apoptotic cells reduced the expression of GM-CSF by monocytes (Fig. 6C).

Next we analyzed how the transfer of iC3b opsonized microparticles to B cells affects B cell activation. Isolated $CD19^+$ B cells were first exposed to serum-treated microparticles and then incubated with medium or stimulated with PMA (10 ng/mL). After 48 h, cell size and expression of MHC class II, CD25 and CD86 was measured by flow cytometry. Under basal conditions (in the absence of PMA) transfer of iC3b-coated microparticles resulted in a marked down-regulation of MHC class II and CD86 expression in B cells, while CD25 expression and cell size remained unchanged (Fig. 7A). Stimulation of B cells with PMA in the absence of microparticles induced, as expected, the expression of CD25 and CD86 and resulted in an increased cell size. Preincubation of B cells with serum-treated microparticles significantly reduced the PMA-induced increase in cell size and CD25 expression (Fig. 7A, B). We also compared the ability of microparticles and apoptotic cells to negatively affect B cell activation. Apoptotic Kato cells as well as microparticles derived from Kato cells or human fibroblasts were equally effective in reducing PMA-induced CD25 expression as well as basal and PMA-induced CD86 expression in B cells (Fig. 7B).

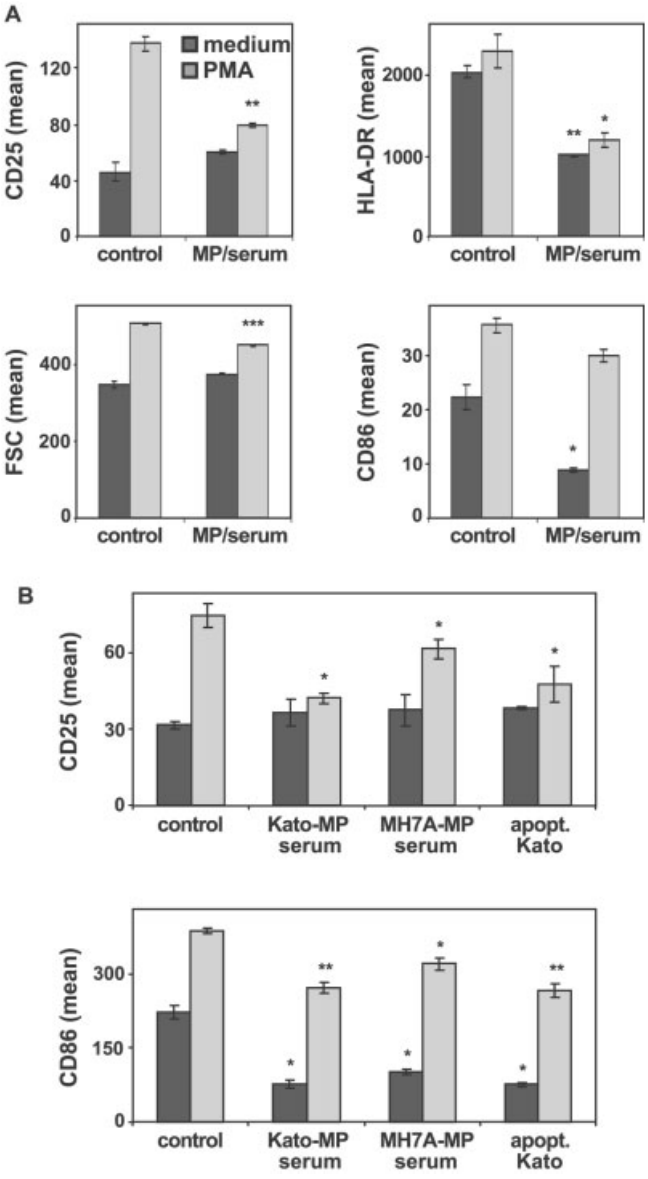

Figure 7. Transfer of microparticles negatively affects the activation of B cells. (A) Isolated B cells were incubated with or without serum-treated microparticles (MP) and incubated with medium (black bars) or stimulated with PMA (10 ng/mL) (gray bars). After 48 h, expression of CD25, MHC class II (HLA-DR), CD86 and cell size (FSC) was measured by flow cytometry. (A) Microparticles derived from Kato cells were used for the assay. (B) Microparticles derived from Kato cells and human fibroblasts (MH7A) as well as apoptotic Kato cells were used. Data represent the mean for three experiments. Error bars represent ± SEM (*$p<0.05$, **$p<0.01$ and ***$p<0.001$ *vs.* untreated control B cells).

Discussion

Under physiological conditions microparticles are released from different cell types and carry membrane proteins from their donor cells. Microparticles and their membrane receptors are transferred to acceptor cells and can be detected by flow cytometry and fluorescence

microscopy. We now show that the mechanism of microparticle transfer differs between various acceptor cell types. Microparticles exposed to serum activate complement resulting in a deposition of iC3b on microparticles. This enables the uptake of microparticles by human B cells. Transfer of microparticles to B cells is almost completely mediated by the complement receptor CD21. In contrast, monocytes efficiently take up microparticles in the absence of serum, but in a manner depend on the presence of calcium and require annexin V. The transfer of microparticles alters the cellular functions of acceptor cells towards an anti-inflammatory phenotype. Microparticle transfer to monocytes decreased the release of pro-inflammatory cytokines and increased the release of IL-10, while transfer to B cells interfered with B cell activation.

Although there is a large body of evidence that microparticles can be transferred between cells, and even carry membrane proteins and other membrane components from one cell to another, little is known about the mechanisms by which microparticles are taken up by different acceptor cells. In the present study, microparticles released from Kato cells could be stained with annexin V, indicating the presence of phosphatidylserine on their surface. Exposure of large amounts of phosphatidylserine on the outer membrane leaflet is a well-known feature of apoptotic cells. Phosphatidylserine on apoptotic cells is recognized by a specific receptor that together with other receptors allows the recognition and removal of apoptotic cells by macrophages [19, 24–26]. In the case of microparticles, however, a blocking antibody against the recently described phosphatidylserine receptor (mAb 217) [19] did not inhibit the microparticles uptake by lymphocytes or monocytes. However, we were able to show that the transfer of microparticles to monocytes is dependent on the presence of calcium and also requires an intact cytoskeleton as shown with cytochalasin D, an inhibitor of actin polymerization. In contrast to monocytes, neither calcium nor actin polymerization were required for the transfer of microparticles to lymphocytes. Annexins, including annexin V, are involved in membrane fusion [18]. We demonstrate that annexin V is present on the surface of monocytes, while it is not detectable on lymphocytes or Kato cells. Blockade of annexin V with an mAb or presence of FITC-/biotin-modified annexin V during microparticle transfer significantly reduces the transfer to monocytes without influencing the transfer to lymphocytes or B cells. One could imagine that annexin V acts as adaptor molecule that binds to both microparticles via phosphatidylserine and to monocytes via a so far unidentified structure. The binding of annexin V to monocytes appears to not involve phosphatidylserine, as removal of calcium does not result in a loss of surface annexin V on monocytes (data not shown). FITC- or biotin-modified annexin V appear to have lost this adaptor function.

Microparticles or apoptotic cells generated within tissues could primarily be taken up by macrophages. However, microparticles derived from various cell types are also present in the peripheral blood. There they would encounter monocytes and lymphocytes and also be exposed to plasma. For this reason we studied the uptake of microparticles by monocytes and lymphocytes and also analyzed the influence of plasma or serum on microparticle transfer. Given the efficient uptake of microparticles by monocytes in the absence of serum, it may not be unexpected that serum only marginally increased microparticles uptake by monocytes. In contrast, exposure of microparticles to serum resulted in marked enhancement of microparticle uptake by a subpopulation of lymphocytes. These lymphocytes could be identified as B cells by costaining with the B cell marker CD19. We were then interested in identifying the serum components responsible for the enhanced transfer to B cells. Heat-inactivated serum completely lost the ability to mediate the transfer of microparticles to B cells, pointing towards the involvement of complement components. Use of serum, depleted of distinct complement components, and re-addition of complement components clearly demonstrated that the complement component C3 is essential for the transfer of microparticles to B cells. However, treatment of microparticles with the complement component C3 alone did not allow microparticle uptake by B cells. This indicates that other yet-unidentified serum components (*e.g.*, Factor B, D or I) are also required for C3 activation. Serum depleted of the complement components C1q and C2 is still able to fully support the uptake of microparticles by B cells, suggesting that the classical and lectin pathway that depend on these two components for conversion of C3 are not involved. We assume that surface structures of microparticles activate C3 by the alternative pathway. Microparticles exposed to serum are able to activate complement, resulting in a deposition of iC3b on their surface. It has previously been shown for apoptotic cells that complement activation plays an important role for their uptake by macrophages and immature dendritic cells [27–29]. Phosphatidylserine on apoptotic cells is at least partially involved in the activation of complement and contributes to the deposition of complement components like iC3b on the membrane surface [27, 30, 31]. Phosphatidylserine-containing liposomes induce the activation of complement through the alternative pathway, and their clearance by the liver is complement dependent [32]. Apart from phosphatidylserine, other phospholipids such as cardiolipin and phosphatidylethanolamine have been shown to activate complement in an antibody-independent manner [31, 33, 34].

We further demonstrate that the complement receptor CR2 is crucially involved in the uptake of iC3b-coated microparticles by B cells. B cells express two complement receptors, CR1 (CD35) and CR2 (CD21) that can interact with C3. CR1, also expressed on monocytes, granulocytes and erythrocytes, mediates the removal of C3-coated immune complexes from the circulation by binding to C3b. CR2 is predominantly expressed on mature B cells and follicular dendritic cells [35, 36] and serves as the primary receptor for C3dg and iC3b [37, 38]. Blockade of CR2 with an mAb almost completely prevented the uptake of iC3b-coated microparticles by B cells. In contrast, blockade of the complement receptor CR1 had no effect, indicating that either most of the C3 is converted on microparticles to iC3b or that the interaction of C3b and CR1 plays no role for uptake of microparticles by B cells.

We further analyzed if the uptake of microparticles alters the cellular function of monocytes and B cells. Transfer of microparticles to isolated monocytes clearly reduced the LPS induced release of pro-inflammatory cytokines, such as GM-CSF and TNF-α and increased the release of the anti-inflammatory cytokine IL-10. Induction of an anti-inflammatory phenotype in macrophages has extensively been analyzed with apoptotic cells. In the presence of apoptotic lymphocytes, macrophages produce more IL-10 and lower levels of pro-inflammatory cytokines [39]. After contact with apoptotic neutrophils LPS-stimulated macrophages increase their IL-10 and TGF-β production with only little production of TNF-α and IL-1β [40]. In addition, uptake of iC3b-coated apoptotic cells by dendritic cells markedly suppresses the secretion of pro-inflammatory cytokines [41]. *In vivo*, interaction of phosphatidylserine-containing liposomes with the 'phosphatidylserine-receptor' suppresses inflammation in tissues by mediating the release of the anti-inflammatory cytokine TGF-β [42]. Recently, microparticles from neutrophils have been described to shift the cytokine profile of macrophages towards an anti-inflammatory one with increased TGF-β release but decreased IL-10 release [43]. In the present study we noted an increased release of IL-10 by monocytes after microparticle uptake but did not detect changes in TGF-β release. These differences might be explained by the fact that we used monocytes instead of macrophages and microparticles derived from Kato cells instead of neutrophils. The influence of microparticles on monocytes activation seems to be largely independent of the cellular source of microparticles, as microparticles derived from three different cell lines (Kato cells, CHO cells and human fibroblasts) were equally effective in altering the cytokine release form monocytes. The efficacy of microparticles was even somewhat more pronounced than that of apoptotic cells. The inhibitory effect of microparticles on monocytes was dose dependent. This suggests that *in vivo* the local production and concentration of microparticles within tissues will determine the strength of the inhibitory effects of microparticles. *In vivo* the release of microparticles by cells might largely be influenced by cellular activation or cell stress.

Transfer of iC3b-opsonized microparticles to B cells markedly down-regulated the expression of MHC class II and CD86 under basal conditions. Moreover, the PMA-induced increase in cell size and CD25 expression was significantly reduced after uptake of serum-treated microparticles. Again microparticles were at least as effective as apoptotic cells. In this context, it is of interest that a reduced expression of MHC class II and CD86 has also been described with dendritic cells that were exposed to iC3b-coated apoptotic cells [29].

In conclusion, our data show that different mechanisms are involved in the transfer of microparticles to different acceptor cells. Microparticle transfer is complement dependent in B cells, and calcium and annexin V dependent in monocytes. In addition, microparticles have inhibitory effects on B cells and monocytes. We hypothesize that microparticle transfer to leukocytes may be involved in dampening an immune response against tissue antigens under physiological conditions and during tissue injury, where an enhanced generation of microparticles might counterbalance endogenous and exogenous proinflammatory signals.

Materials and methods

Reagents and antibodies

Cytochalasin D, LPS, PMA, cisplatin and human standard complement serum were obtained from Sigma (St Louis, MO). Complement components and complement-depleted sera were purchased from Calbiochem (La Jolla, CA). The cell labeling solutions Vybrant DiI and CM-DiI were from Molecular Probes (Eugene, OR). The mAb to EpCAM (clone 3B10C9, IgG1) was from Micromet AG (Munich, Germany), mAb to iC3b (IgG2b) was from Quidel (San Diego, CA). Blocking mAb to CR2 (CD21, clone FE8, IgG1) and CR1 (CD35, clone J3D3, IgG1) were from Upstate (New York) and from Beckman Coulter Immunotech (Krefeld, Germany), respectively. Unlabeled and biotin- or FITC-conjugated annexin V, annexin V binding buffer, and directly labeled antibodies against human CD14, CD19, CD25, CD86, HLA-DR and azide-free anti-human CD3 were obtained from BD Bioscience (San Jose, CA). The annexin V mAb (clone UMC01, previously RUU-WAC2A) was obtained from Sanbio Deutschland GmbH (Germany). The secondary PE-conjugated rabbit anti-mouse IgG antibody (R439) was from Dako (Hamburg, Germany). The antibody (mAb 217) against the phosphatidylserine receptor was kindly provided by Dr. Fadok [19].

Cell culture and cell preparation

The human carcinoma cell line Kato III and the human fibroblast cell line MH7A [44] were grown in RPMI 1640 medium containing 10% heat-inactivated FCS (Life Technologies, Paisley, UK), 100 μg/mL streptomycin and 100 U/mL penicillin. CHO cells were grown in alpha-MEM medium containing 10% heat-inactivated, 100 μg/mL streptomycin and 100 U/mL penicillin. PBMC were isolated from fresh blood of healthy donors by Ficoll density gradient centrifugation and cultured for 12 h in RPMI 1640 medium containing 10% heat-inactivated FCS. Monocytes and B cells were isolated with magnetic beads against CD14 and CD19 (MACS, Miltenyi, Bergisch Gladbach, Germany) according to the manufacturer's instructions.

Generation of microparticles and transfer

Kato cells, CHO cells and MH7A cells were removed from the culture flasks with PBS containing 1 mM EDTA and washed twice in cell culture medium. After centrifugation, the cell pellet was resuspended in culture medium and incubated for 90 min at 37°C. Cell-free supernatant was obtained by centrifugation at 200 × *g* for 5 min. Microparticles were isolated from the supernatant by centrifugation at 16 000 × *g* for 10 min. By electron microscopy, these microparticles have the typical appearance of small vesicles with a lipid bilayer membrane and have a variable size ranging from 0.2 to 1.0 μm, with most microparticles being between 0.4 and 0.8 μm.

Isolated Kato cell-derived microparticles were stained with annexin V-FITC diluted 1:5 in annexin V binding buffer for 20 min at room temperature or with 10 μg/mL mAb to EpCAM.

For transfer experiments 2×10^6 donor cells or isolated microparticles from the same number of donor cells were co-incubated with 2×10^6 acceptor cells for 90 min at 37°C in 100 μL culture medium. To visualize the microparticle transfer for FACS analysis, donor cells were stained with Vybrant DiI cell labeling solution (Molecular Probes, Eugene, OR) according to the manufacturer's instructions. To block the uptake of microparticles, acceptor cells were preincubated with 1 μg/mL cytochalasin D for 30 min or control medium containing 1% dimethylsulfoxide. To analyze if the microparticle transfer is dependent on serum, Kato cells or Kato cell-derived microparticles were preincubated for 30 min at 37°C with serum from healthy donors, human standard serum, complement-depleted serum, or the complement component C3 (1 mg/mL). Where indicated, C3 (1 mg/mL) was re-added to C3-deficient serum before incubation with microparticles. Before co-incubation with acceptor cells serum was removed by several washing steps. Where indicated acceptor cells were preincubated with mAb to CR2 (CD21, clone FE8), CR1 (CD35, clone J3D3) or isotype control antibodies at a concentration of 10 μg/mL for 40 min at 4°C. Blocking antibodies were present during subsequent co-incubation with microparticles. Labeled or unlabeled annexin V and annexin V antibodies were preincubated with donor and acceptor cells and also present during co-incubation of donor and acceptor cells.

Generation and use of apoptotic Kato cells

To induce apoptosis in Kato cells, we incubated the cells for 24 h with 100 μg/mL cisplatin according to a protocol established by Buttiglieri *et al*. [45]. The nonadherent apoptotic Kato cells were recovered and washed three times to remove the cisplatin, and approximately 500 000 apoptotic Kato cells were incubated with 100 000 monocytes or 50 000 B cells. Activation of monocytes and B cells was performed as described below with microparticles.

Flow cytometry

Flow cytometric analysis was performed using a FACSCalibur cytometer with CellQuest analysis software (Becton Dickinson, Heidelberg, Germany). Lymphocytes were identified by their light-scatter properties, while B cells and monocytes were identified by labeling with antibodies against CD19 and CD14, respectively.

Fluorescence microscopy

Unstained PBMC, isolated $CD14^+$ monocytes or $CD19^+$ B cells were co-incubated with CM-DiI stained Kato cells or microparticles. Analysis of microparticle transfer was performed on a fluorescence microscope (DM RBE) and a DC camera 300F from Leica (Solms, Germany).

Analysis of cytokine production

Monocytes (100 000/well) were allowed to take up isolated microparticles and were then stimulated with 1 ng/mL LPS. After 48 h, culture supernatants were collected and centrifuged at 400 × *g*. The amount of cytokines (GM-CSF, IL-10, TNF-α and TGF-β) released into the culture supernatant was quantified by ELISA according to manufacturer's recommendations (DuoSet Development Kit, R&D Systems, Minneapolis, MN).

Real time PCR

Total RNA was isolated from $CD14^+$ cells using silica-gel columns (RNeasy-Mini, Qiagen, Germany). Total RNA underwent random-primed reverse transcription for 1 h at 42°C using reverse transcriptase (Superscript; Life Technologies, Germany). Real-time RT-PCR was performed on a 7500 Real-Time PCR System (Applied Biosystems, Germany) using Taq DNA polymerase (Amplitaq Gold; Applied Biosystems). Thermal cycler conditions contained holds at 50°C for 2 min and 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. Sequence-specific primers and fluorescence probes were obtained from Applied Biosystems. GAPDH and 18S rRNA were used as reference genes and gave comparable results. Messenger RNA expression for each target was calculated following the ΔΔCt procedure. Controls consisting of double-distilled H_2O were negative for target and housekeepers.

Stimulation of B cells with PMA

Isolated $CD19^+$ B cells (50 000/well) were allowed to take up serum treated microparticles and then stimulated with 10 ng/mL PMA in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 100 μg/mL streptomycin, 100 U/mL penicillin, 1% sodium pyruvate, 1% nonessential amino acids and 50 μM β-mercaptoethanol. After 48 h, the expression of CD25, HLA-DR and CD86 was evaluated by FACS analysis, as well as the changes in B cell size and granularity.

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