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Type 1 and Type 2 Cytokine Regulation of Macrophage Endocytosis: Differential Activation by IL-4/IL-13 as Opposed to IFN-γ or IL-10

Luis J. Montaner,† Rosangela P. da Silva,‡ Junwei Sun,* Shaheen Sutterwala,* Michael Hollinshead,† David Vaux,† and Sianon Gordon†

Cytokine regulation of endocytic activity in primary human macrophages was studied to define ultrastructural changes and mechanisms of pinocytic regulation associated with cytokines secreted by activated T cells. The effects of IFN-γ (type 1) and IL-4/IL-13 and IL-10 (type 2) cytokines on fluid phase and mannose receptor-mediated endocytosis were assessed by horseradish peroxidase and colloidal gold-BSA uptake and computer-assisted morphometric analysis. IL-4 and IL-13 enhanced fluid phase pinocytosis and mannose receptor-mediated uptake by activation of phosphatidylinositol 3-kinase. Inhibition of actin assembly showed that both cytokines exerted actin-dependent and -independent effects. Ultrastructurally, IL-4 and IL-13 increased tubular vesicle formation underneath the plasma membrane and at pericentriolar sites, concurrent with decreased particle sorting to lysosomes. By contrast, IL-10 or IFN-γ decreased both fluid phase pinocytosis and mannose receptor-mediated uptake. IFN-γ stimulated increased particle sorting to perinuclear lysosomes, while IL-10 decreased this activity. In summary, our data document differential effects on macrophage endocytic functions by type 1 or type 2 cytokines associated with induction and effector pathways in immunity. The Journal of Immunology, 1999, 162: 4606–4613.
Materials and Methods

Media and reagents

The medium used throughout was RPMI 1640 supplemented with 2 mM L-glutamine, 100 μg/ml Pen/Strep (Life Technologies, Grand Island, NY), and 5% autologous or pooled AB+ (Sigma, St. Louis, MO) human serum. Human recombinant (r) IL-4 and IL-13 were gifts from Kevin Moore (Sigma), human recombinant (r) IL-10 was purchased from Becton Dickinson (Lincoln Park, NJ), and were cultured in 5% autologous or pooled AB+ (Sigma) human serum for 48 h at 37°C. Cytokine concentrations were maintained at <25 pg endotoxin/mg protein. Chemicals (cytochalasin D and wortmannin), BSA-colloidal gold conjugates (20 nm), endotoxin standards, and the E-Toxate Kit were purchased from Sigma Scientific (Poole, U.K.). HRP (1000 U/mg) was purchased from Serva Feinbiochimica (Heidelberg, Germany) and Sigma. Texas Red 70-kDa dextran (TR-70DX) was purchased from Molecular Probes (Eugene, OR). Zymosan particles were purchased from Sigma and fluoresceinated as previously described (24).

Monocyte-derived macrophages: isolation, cultivation, and treatment

Human PBMC were isolated from healthy donors as previously described (25); in short, Ficoll-Hypaque (Pharmacia, Uppsala, Sweden)-isolated mononuclear cells were incubated for 1 h in 2% gelatin (Difco, Detroit, MI)-coated plates. Adherent cells (>94% CD14+ by FACS analysis) were cultivated in 5% autologous or pooled AB+ (Sigma) human serum for 48 h before transfer to 48-well plates (Nunc, Naperville, IL) at a density of 2.5 × 10^5 cells per well (500 μl total volume), 96-well plates (Nunc) at 10^5 cells/well (200 μl total volume), or 30-mm tissue culture plastic dishes at 3 × 10^6 (2-ml total volume). IL-4, IL-13, IL-10, and IFN-γ (all 20 ng/ml, except for IFN-γ at 100 U/ml) were added to day 6 postisolation differentiated macrophages for 72 or 144 h (specified for each assay) before measurement of endocytic uptake. Subsequent studies with selected inhibitors (concentrations described in Results) were performed by titration on cytokine-treated and control monocyte-derived macrophages (MDMs) for 4 h before measurement of HRP uptake as described below.

Quantitation of dextran and zymosan uptake

Macrophage cultures were treated with cytokines in triplicate in 48-well plates for 72 h as specified above. Texas Red 70-kDa dextran (TR-70DX) was added at a final concentration of 1 mg/ml for 60 min at 37°C, while fluoresceinated zymosan was added at 50 particles/cell. The cells were then washed four times with cold PBS and lysed in 1% Nonidet P-40 buffer (total volume, 100 μl) by thorough scraping of the wells. The amount of fluorescent probe accumulated was calculated by fluorometer plate readings (Fluorocan II, Labsystems, Chicago, IL) at the following settings for fluoresceinated zymosan and TR-70DX, respectively: 495 and 591 nm for excitation, and 520 and 612 nm for emission. Results from multiple experiments were analyzed by subtracting background fluorescence intensity obtained by the bicinchoninic acid assay (Bio-Rad, Richmond, CA).

HRP uptake

Macrophages were cultured in 96-well plates (Becton Dickinson, Lincoln Park, NJ) at a density of 1 × 10^5 cells/well. Cytokines were added as indicated above, with fresh media and cytokines replaced after 3 days. Following treatment, macrophages were incubated with HRP (1000 U/mg; Sigma) at a concentration of 1 mg/ml for 10, 20, 30, 45, and 60 min followed by washing three times with 1% FCS in PBS and three times with PBS alone. Wells were lysed at each indicated time in 100 μl of 0.05% Triton-X 100. The amount of HRP in the lysate was quantified by adding substrate (o-dianisidine and H₂O₂ in 0.05 M phosphate-citrate buffer) and measuring the rate at which oxidized o-dianisidine (absorbing at 460 nm) accumulates with reference to an HRP standard curve. Kinetic absorbance readings were performed with a Spectra Rainbow Reader (Salzburg, Austria), and data were analyzed with Delta Soft 3 software (Biometrical, Princeton, NJ). Values were expressed as nanograms of HRP per million MDM.

Electron microscopy

Three pulse-chase experiments were performed in which IL-4, IL-10, and IL-13, or IFN-γ-treated macrophages were cultivated in 30-mm tissue culture dishes and treated with cytokines for 72 h in RPMI 1640 and 5% autologous serum or medium. Cells were either incubated with 20-nm colloidal gold particles coupled to BSA (OD₅₂₀ of 10) for 30 min at 37°C, washed with PBS, fixed, and processed in Epon, as described below, or pulsed for 120 min with 20-nm colloidal gold-BSA at 37°C, after which cells were washed with warm PBS and cultured further for 8 h. In the latter pulse-chase experiments, HRP was incubated with pretreated cells at 10 mg/ml in normal medium for 40 min. Cultures were then washed with ice-cold PBS and fixed with 0.5% glutaraldehyde in 200 mM sodium cacodylate, pH 7.2, for 30 min at room temperature as described by Toozée and Hollinshead (26). To make electron microscopic sections, samples were immediately reacted with the HRP substrate DAB (1 mg/ml) for 30 min in dark. Samples were postfixed for 1 h in 1% OsO₄ plus 1.5% potassium ferricyanide, stained en bloc with 0.5% magnesium uranyl acetate overnight, dehydrated, and processed for flat embedding in Epon. Cell monolayers were separated from the culture dish using liquid nitrogen, and the blocks were trimmed to a similar area using a Reichert Ultratrim milling device. Sections were obtained using a Reichert Ultracut E ultramicrotome set at a section thickness of 100 nm to give a gold interference color and were collected onto Formvar-coated grids in a serial section series. Pulse-chase sections were immediately examined in the electron microscope (EM 912 Omega electron microscope (Zeiss, New York, NY) at 80 kV and elastic imaging) to avoid noticeable loss of DAB reaction product upon storage.

Single-cell quantitation of endocytic compartments

DAB product was used to quantify endocytic area, while colloidal gold is naturally electron dense. Samples were analyzed by computer-generated thresholded images from sections of cytoplasm viewed at a constant magnification (×4000). Representative areas were recorded as HRP-early sections by their position relative to the plasma membrane (immediately beneath the plasma membrane with no other organelles or nuclei between the organelle and the plasma membrane), while representative HRP-containing areas were recorded in perinuclear and Golgi-rich regions. At least 15 random representative sections per analysis were acquired in a double-blind format using a ProScan 1024×1024 CCD camera and the EsiVision proprietary software package (SIS, Munster, Germany). A database was initiated for each treatment group, and images were stored on a magneto-optical disk. Each treatment group was then decoded and analyzed using thresholded and density slice functions of the EsiVision analytical software. The software employs a digital version of point counting, a well-established quantitative immunocytochemical technique (27). Computer analysis of the images and quantitation of endocytic area were performed by establishing a threshold of electron density for every image, above which only the HRP and/or HRP-gold compartment was highlighted. The resulting computer thresholded image identified endosomal electron-dense areas by pseudo-color representation. Images were analyzed for the density of pixels, and the endocytic area for the section was calculated. Data for endocytic area were converted to relative image values by dividing pixels occupying the endocytic compartment by total pixels in cell profile per section.

Statistical analysis

Each group of data was analyzed for normal distribution, and all subsequent comparisons between groups were two tailed. Significance in the text is noted as differences at an α level of 0.05 (p < 0.05). All descriptive analysis and statistical tests were performed with JMP 3.2.1 (SAS Institute, Cary, NC).

Results

Cytokine regulation of macrophage HRP fluid-phase and MRM uptake

The effects of IL-4, IL-13, IL-10, and IFN-γ on HRP uptake by MDM (28) were examined in the absence or the presence of mann, to block MR-dependent uptake. IL-4 and IL-13 induced both MR-dependent and independent uptake, whereas IFN-γ and IL-10 shared suppressive effects. IL-4 and IL-13 induced a 280% increase in fluid phase HRP uptake, while corresponding MR-dependent uptake increased sixfold (n = 3; Fig. 1). Similar effects were observed on uptake of Texas Red-labeled dextran (n = 4; data not shown).

In addition to total uptake over a 1-h pulse, the kinetics of pinocytic activity in cytokine-treated MDM were determined over 1 h (expressed as nanograms per h). Rate determination was possible due to the steady rate at which HRP is accumulated and delivered to lysosomes without detectable exocytosis (28). Untreated macrophages internalized an average of 18 ng of HRP/ million cells/h, as shown in Fig. 2. Consistent with a sustained
increase in fluid phase and MRM uptake, IL-4 or IL-13 increased the rate of endosomal uptake to 41.4 and 52.2 ng HRP/10^6 macrophages/h, respectively. IFN-γ or IL-10-treated macrophages showed similar decreases in HRP rates of uptake, consistent with previous observations. Interestingly, IL-10 and IFN-γ decreases were associated with the lack of accumulation of HRP over time rather than with a decrease in total uptake within the first timed samples. Statistical differences between control and IL-10- or IFN-γ-treated samples were observed at the 45 min point, whereas IL-4 and IL-13 showed significant differences at the first time point.

Functional consequences of cytokine regulation of MR-mediated uptake were tested by quantifying phagocytosis of FITC-zymosan, which depends in part on the MR (29). IL-4 and IL-13 treatment increased zymosan uptake by 363 and 330%, respectively, whereas uptake was reduced by IL-10 (62%) or IFN-γ (40%; n = 3; data not shown). Taken together, the results showed that type 2 cytokines activated endocytosis differentially (IL-4 and IL-13 vs IL-10), while type 1 cytokine IFN-γ decreased fluid phase and MRM uptake.

Role of PI3-kinase and actin polymerization in induction of endocytosis by IL-4 and IL-13

Wortmannin was used as a PI3-kinase inhibitor (30, 31) and was studied for its role in HRP uptake in the presence or the absence of IL-4 or IL-13. Wortmannin at 25 μM significantly reduced IL-4- or IL-13-mediated uptake compared with that in control cytokine-treated macrophages (Fig. 3). The sustained increase in uptake in the presence of 25 μM wortmannin suggested a contribution by additional PI3-independent mechanisms in IL-3- or IL-4-induced pinocytosis. While higher concentrations of wortmannin enhanced the inhibition of uptake in both cytokine-treated and control cells, cellular functions other than PI3-kinase could be affected at these concentrations (31).

The role of actin in IL-4 and IL-13 induction of pinocytic uptake was tested by cytochalasin D blockade of actin polymerization. The results showed a decrease in uptake by both untreated and cytokine-treated macrophages, indicating a role for actin in internalization of HRP, in the presence or the absence of IL-4 or IL-13 (IL-4 results shown in Fig. 4). Interestingly, the ratio between control and cytokine-treated uptake levels remained similar at the highest concentrations of cytochalasin D tested, suggesting the presence of an enhanced uptake mechanism in cytokine-treated macrophages that is independent of actin.

Heterogeneity in HRP endosome morphology and colloidal gold sorting to lysosome-like compartments

Results from cytokine-treated macrophage cultures pulsed with HRP served as the basis of single cell ultrastructural analysis to 1) define the intracellular distribution of HRP-containing endosomes associated with cytokine regulation and 2) evaluate the relationship of fluid phase uptake and substrate sorting following uptake over an 8-h period. Cytokine (IL-4, IL-13, IL-10, or IFN-γ)-treated macrophages were analyzed by electron microscopy for changes in HRP endosome morphology and substrate sorting by incorporating a pulse chase with colloidal gold (26). HRP was added as a short, additional pulse following an 8-h previous timed incubation with colloidal gold-BSA to identify vesicles containing previously sorted colloidal gold from those taking up HRP within the subsequent shorter period. A schematic representation of the experimental design and expected results within untreated MDM is shown in Fig. 5.

HRP-containing endosomes in untreated macrophages were observed underneath the plasma membrane and in areas proximal to the nucleus and Golgi apparatus. Tubular vesicles, outlined by the electron density of HRP product, were found underneath the plasma membrane without colloidal gold (Fig. 6A), compatible with early endosome ultrastructure (26). Rounded HRP-containing
vesicles were also within Golgi-rich or perinuclear areas. The presence of colloidal gold aggregates sorted to a common vesicle distal from the surface plasma membrane was consistent with sorting of colloidal gold to later lysosome-like vesicles over the 8 h before fixation (Fig. 7A). The presence of HRP in perinuclear vesicles, both singly and colocalized with colloidal gold, indicated progression of HRP-only vesicles to these areas as well as a shared sorting route for HRP and colloidal gold in a subset of vesicles. Computer-assisted quantification of electron-dense areas within MDM serial sections showed a greater concentration of substrate within perinuclear and peri-Golgi areas, indicating active transport and accumulation of colloidal gold and HRP substrates (Fig. 8).

Differential cytokine-induced endosome formation and colloidal gold sorting to perinuclear/peri-Golgi vesicles
Cytokine-treated macrophages showed significant alterations in HRP and colloidal gold vesicle formation and associated ultrastructure. IL-4 and IL-13 increased the appearance of tubular HRP-containing vesicles in areas underneath the plasma membrane (Fig. 6, D and F), significantly increasing the ratio in substrate relative to that in perinuclear/peri-Golgi areas (Fig. 8). Quantitative analysis of tracer electron-dense areas containing HRP and/or colloidal gold confirmed that IL-4 and IL-13 significantly increased the endosomal area underneath the plasma membrane by 297% (p = 0.0005) and 229% (p = 0.029), respectively (Fig. 8). Although no significant change in perinuclear/peri-Golgi area was present in IL-13- or IL-4-treated macrophages, the morphology of this compartment was different from that in untreated cells. Specifically, an increased density of tubular vesicles with minimal amounts of colloidal gold particles indicated a decrease in lysosome-like vesicles containing colloidal gold aggregates, as in untreated controls (Fig. 7, D and F). Taken together, both IL-13 and IL-4 showed a similar induction of endosome formation underneath the plasma membrane, consistent with the increase in total uptake observed in previous experiments.

Morphological changes in IL-10-treated macrophages indicated a striking difference compared with those induced by IL-4 and IL-13. IL-10 treatment increased colloidal gold in areas underneath the plasma membrane, while perinuclear/peri-Golgi areas contained reduced HRP and colloidal gold compared with untreated controls (Fig. 8). Colloidal gold-laden vesicles proximal to the plasma membrane varied in size, with the occasional distinctive
observation of single enlarged vesicles that contained both colloidal gold and HRP substrate (Figs. 6G and 9C). A decrease in substrate within perinuclear/peri-Golgi areas was consistent with reduced endocytic uptake as observed in Figs. 1 and 2. The presence of colloidal gold in vesicles close to the plasma membrane suggested that the uptake that does take place may reach a common sorting vesicle that is able to accumulate substrate over time.

In contrast to IL-10, IFN-γ treatment reduced total substrate within perinuclear/peri-Golgi areas (Fig. 8), but increased the formation of lysosome-like vesicles containing distinct aggregates of colloidal gold (Fig. 10D). In contrast to untreated controls, a reduced amount of HRP was present at perinuclear/peri-Golgi areas. The latter finding is relevant to the observed reduction in uptake observed in IFN-γ-treated macrophages (Figs. 1 and 2); this may reflect a reduced accumulation of substrates over time concurrent with an increased capacity to sort contents to later vesicles with higher efficiency. Overall, cytokine-induced changes in endosomal morphology and substrate localization establish the distinct regulation of endosome formation and substrate sorting by different cytokines.

Discussion

We provide a comparative study in primary human macrophages of type 1 and type 2 cytokine effects on endocytosis. We document cytokine-mediated alterations of macrophage endocytosis, including route of uptake, mechanisms of action, ultrastructure of endosomes, and particle sorting to late lysosome-like vesicles. The differences observed between IFN-γ as opposed to IL-4 and IL-13 treatments show for the first time the consequences of type 1 and type 2 cytokine regulation of MR- and non-MR-mediated uptake in association with specific ultrastructural and functional changes.

Among T cell-derived cytokines, IFN-γ is a central type 1 cytokine involved in macrophage activation due to its induction of microbicidal mechanisms and Ag presentation, resulting in proteolytic degradation of internalized proteins or pathogens (16). Although type 2 cytokines are generally summarized as deactivateds
of IFN-γ-like effects and thus are seldom viewed as regulating an activated phenotype in macrophages. Cells were treated with cytokine for 72 h and processed as shown in Fig. 5. Values were obtained from computer-assisted analysis of sections for total cytoplasmic vs high electron-dense areas corresponding to HRP- and/or colloidal gold-containing vesicles. HRP/colloidal gold-containing vesicle areas are presented as a percentage (±SE) of the total cytoplasmic area within sections from untreated or cytokine-treated (IL-13, IL-4, IL-10, and IFN-γ) macrophages. Refer to the text for methods. Asterisks indicate significant differences determined by Student’s t test (p < 0.05).

In contrast to previous reports suggesting a predominant role for IL-4- or IL-13-mediated MRM uptake, we document a greater total, rather than only relative, increase in non-MRM uptake by both cytokines. Non-MRM HRP uptake includes micro- and macropinosome formation. The latter has been associated with activation of PI3-kinase and actin polymerization (20). Direct involvement of PI3-kinase in IL-13- and IL-4-mediated induction of both MRM and non-MRM uptake was shown by inhibition of uptake in the presence of wortmannin (Fig. 3). Involvement of PI3-kinase in the signal transduction pathway of IL-13 is further supported by its activation in epithelial cells expressing the IL-13R (23). The role of PI3-kinase in macropinosome formation in M-CSF-differentiated macrophages together with its role in the induction of IL-13
and IL-4-stimulated pinocytosis suggest a common signal transduction mechanism for endocytic enhancement in macrophages. Experiments with cytochalasin D showed that the effects of IL-13 and IL-4 were partly dependent on actin (Fig. 4).

Activation of pinosome formation by IL-4 and IL-13 was also demonstrated by an increased density of tubular vesicles underneath the plasma membrane, suggestive of expansion of the early endosomal compartment (Fig. 6). This conclusion was supported by the localization of a large density of tubular endosomes at pericentral regions that are associated with trafficking of recycling early endosomes, such as the mannose and transferrin receptors (6, 38, 39) (Fig. 10). An increase in transferrin-FITC uptake by IL-4- or IL-13-treated macrophages is consistent with activation of both fluid phase and recycling receptor-mediated uptake (L. J. Montaner, unpublished observations). In addition to endocytosis, IL-13 and IL-4 also increase gene expression of both the transferrin and mannose receptors (40, 41). Definitive characterization of cytokine effects on endosomal trafficking awaits the combined use of endocytic tracers with specific mAbs against early or late vesicle-associated proteins.

Although the relationship between pinocytosis and enhancement of Ag presentation capacity is well recognized in dendritic and related cells (8, 9, 42), our data suggest that T cell secretion of IL-4 or IL-13, in contrast to that of IFN-γ or IL-10, is able to induce dendritic cell-like properties in differentiated macrophages; IL-4/IL-13 could therefore enhance acquisition of soluble or mannosylated Ag for continued restimulation of memory T cells.

The selectivity of IL-4 and IL-13 effects among type 2 cytokines regulating macrophage function was best exemplified by the decrease in fluid phase and mannose-mediated uptake observed with IL-10 (Figs. 1 and 2). The latter was consistent with the described role of IL-10 as a general deactivator of immune function, including down-regulation of MHC class II expression by macrophages (32). The decreased HRP uptake induced by IL-10 together with its regulation of HRP endosome ultrastructure suggest a general effect on uptake associated with a decreased accumulation of substrates within later vesicles. Surprisingly, this analysis also showed an accumulation of colloidal gold particles within vesicles underneath the plasma membrane, which was not observed with other cytokine treatments (Fig. 9). It is of interest to contrast this latter observation with the recent characterization of an IL-10-induced decrease in membrane trafficking of MHC class II, since IL-10 inhibited re-expression of recycling molecules by promoting their accumulation within distended intracellular vesicles (12). Further analysis is needed to determine whether fluid phase substrates internalized in the presence of IL-10 are sorted to high MHC class II-containing vesicles. Taken together, our data suggest that IL-10 induces a distinct endocytic phenotype, consistent with a decreased capacity to take up and present Ag.

In marked contrast to the IL-10-induced phenotype, IFN-γ increased compartmentalization and sorting of colloidal gold aggregates (Fig. 10) consistent with the enhanced formation of late endosomes or lysosomes (43). The latter is also in accordance with its activation of aspartyl protease activity, cathepsin D (active at pH 3.5), and late endosomal Ag processing (13, 14, 44). We show for the first time that this activity is associated with a relative shut-down of fluid phase and MRM pinocytosis, further supporting a potential inverse relationship between the induction of pinocytosis (fluid phase and MR uptake) and lysosome formation. Additional experiments are needed to determine whether IL-4 and IL-13 decrease lysosomal maturation in contrast to IFN-γ, as predicted by our morphological observations.

Our data support a differential role for IL-4 or IL-13 compared with IFN-γ in suppressing colloidal gold aggregate formation; this may bear on the inability of macrophages to clear intracellular pathogens in immune environments associated with type 2 responses (45). On the other hand, the endosomal stimulation by IL-4 or IL-13 may support increased Ag uptake and presentation of soluble extracellular Ags, providing a mechanism for T cell-dependent enhancement of Ag uptake by bystander macrophages. Additional studies will explore whether differential regulation of endosome ultrastructure and sorting capacity influences Ag processing (7, 46) and MHC class I-associated presentation by macrophages (1, 47).

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References


