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Regulated Expression of FcγR in Human Dendritic Cells Controls Cross-Presentation of Antigen-Antibody Complexes

Yi Liu,* Xiaoni Gao,* Emi Masuda,* Patricia B. Redecha,* Marissa C. Blank,* and Luminita Pricop2*†‡

Receptors for IgG (FcγR) expressed in dendritic cells (DCs) influence the initiation of Ab-mediated immunity. Dynamic variations in FcγR expression allow DCs to adjust their capacity to capture Ab-opsonized Ag. The current paradigm predicts a progressive decline in FcγR-mediated phagocytic function upon DC maturation. Surprisingly, we find that expression of the phagocytic receptor FcγRIIa is preserved in immature and mature DCs at comparable levels with macrophages. Moreover, phagocytosis of antigenic peptides directed to FcγRIIa on DCs leads to dramatic increases in Ag cross-presentation and T cell activation. In immature DCs, high expression of inhibitory FcγRIIb correlates with decreased uptake and cross-presentation of Ab-Ag complexes. In contrast, engagement of FcγRIIb is not associated with changes in cross-presentation in mature DCs. We provide evidence that FcγRIIb expression is patently reduced in mature DCs, an effect that is modulated by treatment with cytokines. The regulated expression of activating and inhibitory FcγRs in DCs emerges as a critical checkpoint in the process of Ag uptake and cross-presentation.

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In view of the role of FcγR as regulators of DC function, we analyzed the kinetics of expression of activating and inhibitory FcγRs during DC maturation. FcγRI, which are highly expressed in monocytes and macrophages (Ma), were down-regulated at early stages of DC differentiation. Low levels of FcγRI expression persisted on both immature (iDCs) and mature DCs (mDCs). FcγRIIa were expressed at comparable levels throughout DC differentiation, including the mature stages. FcγRIIb were found to be highly expressed in iDCs and were down-regulated following maturation of DCs with either TNF-α or a mixture containing inflammatory cytokines TNF-α, IL-1β, IL-6, and PGE2. The expression of FcγRIIa and FcγRIIb was induced in mDCs by treatment with IL-10 and IL-13, indicating that the expression of FcγRII isosforms in these cells can be modulated by cytokine treatment.

DCs acquire exogenous Ag by phagocytosis and cross-present antigenic peptides in association with MHC class I molecules to cytotoxic CD8+ T lymphocytes (6, 7). Interaction of Ab-Ag complexes with FcγR influences the ability of DCs to present Ag to T lymphocytes (8, 9). Selective engagement of activating or inhibitory FcγRs has the potential to induce differences in the efficiency of Ag cross-presentation. Targeting influenza antigenic peptides in DCs through activating FcγRs overcame the need for full DC maturation, and induced dramatic increases in IFN-γ-producing influenza-specific CD8+ T cells. Engagement of inhibitory FcγRIIb limited phagocytosis and decreased TNF-α production by DCs, leading to reduced expansion of memory T lymphocytes. The present study characterizes the dynamic regulation of FcγRs during DC maturation and identifies factors that change the balance of FcγR in human DCs subsequently affecting the initiation of adaptive immune responses.

Materials and Methods

Peptides and Abs

The HLA-A*0201-restricted immunodominant influenza A matrix peptide (MP) system was used to stimulate influenza-specific memory CD8+ T cells (10, 11). The 9-mer core MP (GILGFVFTL) and a 17-mer peptide (TGKILGFVFTLVTPSER) containing N- and C-terminal flanking residues were used as antigenic peptides. MP-specific polyclonal antiserum
was obtained by immunization of rabbits with 17-mer and showed reactivity with 9-mer and 17-mer MP in immunoblots (see Fig. 3Aa). Fab(‘)2 of MP-specific rabbit IgG was obtained by pepsin digestion using ImmuNoPure Fab(‘)2 preparation kit (Pierce). Fab of mouse anti-FcRIIa mAb, clone IV.3, and F(ab’)2 of anti-FcRIIa, clone 22.2, and anti-FcRII, clone 7.3 were purchased from Medarex and Research Diagnostics, respectively. Mouse anti-FcRIIa mAb, clone FLI.28 was purchased from Research Diagnostics. The rabbit polyclonal Abs reacting specifically with FcRIIa (260) or FcRRIIb (Fc(κ)RIIB/IFC) used for Western blotting were previously described (12, 13).

Isolation and culture of DCs
Leukopheresed PBMC (8–10 × 10⁶/sample) were isolated from HLA-A*0201-positive healthy donors by sedimentation over Ficoll-Hypaque (Pharmacia Biotech). T cell-enriched and monocyte-enriched fractions were separated by rosetting with sheep RBC treated with a 1–2,3,6,8-neuraminidase (Calbiochem). T cells were frozen in liquid nitrogen and thawed after 6 days and incubated at autologous DC in ELISOPT and Pro5 pentamer-staining assays. DCs were differentiated from monocyte-enriched fractions in culture by treatment with cytokines (all purchased from R&D Systems unless otherwise specified). iDC were differentiated from monocytes in 6 day cultures in complete medium (RPMI 1640 medium, 1% HEPES, 1% glutamax, and 1% penicillin-streptomycin) supplemented with 1% human plasma, 1000 U/ml GM-CSF, and 10 ng/ml IL-4. mDC were obtained from iDC cultured on day 5 with cytokine mixture consisting of 10 ng/ml TNF-a, 10 ng/ml IL-1β, 1000 U/ml IFN-g, and 1 μg/ml LPS. Macrophages were obtained by culturing adherent monocytes (6 × 10⁶ cells/well) in 6-well plates with complete medium plus 5% pooled AB human serum PHS (Pel-Freeze Clinical Systems) supplemented with 1000 μg/ml recombinant human GM-CSF. For modulation of FcγR expression, iDC and mDC cultures were supplemented with 50 ng/ml IL-13, 25 ng/ml IL-10 (PeproTech), or IL-13 plus IL-10 for 24 h.

Evaluation of receptor expression and FcγR-mediated phagocytosis by flow cytometry
For evaluation of FcγR expression, DCs were incubated with FITC-labeled anti-FcγR mAbs (clones 22.2, IV.3, and 7.3). Membrane expression of CD14, MHC class I (MHC-I), HLA-DR, CD83, CD86, CD40 was detected with FITC-labeled specific mAbs (BD Pharmingen).

A flow cytometric assay using erythrocytes labeled with the lipophilic dye PKH26 (Sigma-Aldrich) and coupled with biotin/avidin to F(ab’)2 of anti-FcγR mAb was used to determine the attachment and phagocytosis of specific probes through FcγR (14). Briefly, Fab of anti-FcγRII clone IV.3, F(ab’)2 of anti-FcγR mAb (clones 22.2 and 7.3), and F(ab’)2 of anti-MP rabbit polyclonal Abs were biotinylated with EZlink-sulfo-NHS-LC-biotin (Sigma-Aldrich). Ox erythrocytes (E) were biotinylated with sulfo-N-hydroxysuccinimide-biotin (Sigma-Aldrich) and saturated with streptavidin (Roche Diagnostics). Biotin-streptavidin-coated erythrocytes were opsonized with biotinylated anti-FcγR mAb and with biotinylated Fab(‘)2 of anti-MP rabbit polyclonal Abs, followed by incubation with 100 μg/ml 9-mer or 17-mer MP for 1 h. MP-FcγR-specific probes were labeled with PKH26. iDCs and mDCs (5 × 10⁵ cells/ml) were incubated with MP-FcγR-specific fluorescent probes (1.25 × 10⁶ erythrocytes/ml) at 37°C for 20 min. For assessment of phagocytosis, noninternalized probes were then lysed and the uptake of PKH26-labeled MP-FcγR-specific probes was measured by flow cytometry using a FACScan (BD Immunocytometry Systems) equipped with a standard optical filter set. The PKH26 fluorescence was detected in the FL2 channel and displayed on a logarithmic scale. The phagocytic index (PI) was calculated by multiplying the percentage of DCs containing PKH26+ probes and the mean fluorescence intensity (MFI) of PKH26+ probes/100 cells.

For assessment of attachment, 5 × 10⁶ DCs/ml and 1 × 10⁶ PKH26-labeled erythrocytes/ml coated with biotinylated fragments of IV.3 (E-IV.3) or 7.3 (E-7.3) were centrifuged at 44 × g for 3 min at 4°C, followed by incubation on ice for 5 min (14). The percentage of cells having attached PKH26-labeled probes on the surface was determined by flow cytometry.

Immunofluorescence microscopy
Following phagocytosis of MP-FcγR-specific probes, DC (50,000 cells/condition) were cytospun at 800 rpm for 5 min with Cytospin 2 (Shandon) onto charged Superfrost slides (VWR Scientific). Slides were fixed with cold acetone for 5 min at −20°C and permeabilized with 0.1% saponin for 15 min. Slides were blocked with 2% BSA-PBS for 15 min, incubated with rabbit anti-MP Abs and Alexa-546-conjugated goat anti-rabbit and costained with 7.3-FITC or IV.3-FITC for 1 h at room temperature. Slides were examined with a fluorescent microscope (Leica).

Real-time PCR
Total RNA was isolated with the NReasy kit (Qiagen). cDNA was synthesized from 1 μg of total RNA with random hexamers (Invitrogen Life Technologies). Real-time PCR was conducted with the SYBR Green PCR Supermix (PerkinElmer) and the iQ MultiColor Real-Time PCR Detection System (Bio-Rad), according to manufacturer’s instructions. The PCR consisted of one cycle at 94°C for 3 min, followed by 40 cycles at 94°C for 30 s and 54°C for 30 s. The following primer pairs were used for amplification: FcγRIIa forward, 5′-GACTACCGATTTAAGCTC-3′ and FcγRIIa reverse, 5′-AAAGGCAGCAAGGAAAAG-3′; FcγRIIB2 forward, 5′-GGATTGATTGGCTGCTG-3′ and FcγRIIB2 reverse, 5′-ATTTGAGTGTTCGCTA-3′; and GAPDH forward, 5′-GATGCGACAAATATC CACTT-3′. During amplification, absorption readings measured the relative amount of amplification produced in each cycle. This data was used to make a relative determination of gene expression under each experimental condition. All PCR assays were done in triplicate and the data were pooled.

Immunoprecipitation and Western blotting
DC (5 × 10⁶/ml) were solubilized in 1% Nonidet P-40 lysis buffer and immunoprecipitated for 2 h at 4°C with anti-FcγRII mAb (FL18.26) coupled to protein G-Sepharose beads. The immunoprecipitates (20 μl/sample) were separated by SDS-PAGE on 10% polyacrylamide gels and subsequently transferred to nitrocellulose. Blots were incubated for 1 h at room temperature with the polyclonal rabbit anti-FcγRIIb (FcγRIIB/c) or anti-FcγRIIa (Ab 260) Abs, washed, and incubated with HRP-conjugated donkey anti-rabbit Abs (final dilution 1/3000). The reaction was developed using the ECL Enhanced Western blotting detection kit (Amersham Biosciences).

Production of TNF-a by DC after incubation with FcγR-specific probes
DC (5 × 10⁵/ml) were cocultured in 5 ml of polypropylene tubes with probes opsonized with mAbs IV.3, 7.3, or no Ab, at 1:25 E:T ratios for 18 h at 37°C. Cell-free supernatants were collected and samples were frozen at −70°C. Production of TNF-a was determined by sandwich ELISA (R&D Systems). OD values were determined by spectrophotometer at 405 nm.

IFN-γ production measured by ELISPOT assay
Following uptake of MP-FcγR-specific probes or direct infection with influenza virus, iDC and DC (4 × 10⁵/well) were incubated with autologous T cells (2 × 10⁵/well) for 40–44 h at 37°C in 96-well ELISPOT plates (Millipore) coated overnight with 5 μg/ml of the anti-IFN-γ mAb clone 7B26-1 (Mabtech). DCs infected with influenza virus A (PR8/34; Charles River Laboratories; SPAFAS) was pulsed with 100 μM soluble influenza MPs were used as control. Wells without T cells served as negative control. Wells were washed four times with PBS containing 0.05% Tween 20 (Sigma) followed by incubation for 2 h with 1 μg/ml biotin conjugated anti-IFN-γ mAb clone 7B6-1 (Mabtech). Plates were washed four times in PBS with 0.1% Tween 20, stained with the Vectastain Elite kit (Vector Laboratories), and developed with stable diaminoenzidine (Invitrogen Life Technologies). Spots representing the IFN-γ-releasing T cells were counted with a stereomicroscope and reported as spot-forming cells (SFC)/10⁵ cells.

Detection of MP-specific T cells by Pro5 pentamer staining
MP-specific T cells were stained with Pro5 Pentamer (Proimmune), following the manufacturer’s instruction. DC probed with MP-FcγR-specific erythrocytes were incubated with autologous T cells for 40–44 h at 37°C as described for the ELISPOT assay. Cells were harvested and resuspended in 50 μl of PBS, incubated with 2 μl of unlabeled MP-specific Pro5 pentamer for 15 min at room temperature, and then stained with 8 μl of Pro5 Fluorotag and FITC-conjugated anti-human CD8 mAb for 20 min. The cells were washed, fixed in 2.5% paraformaldehyde, and analyzed by flow cytometry.

Results
Dynamic variations in FcγR expression occur during DC maturation
Down-regulation of phagocytic receptors upon DC maturation supports the view that phagocytic function is abandoned following Ag uptake, while Ag-presenting function is gained (15). Indeed, a
decline in FcγR expression in DCs was found to be associated with reduced ability to engulf IgG-coated particles (1). Yet, phagocytosis of particulate Ag through FcγR is known to occur in DCs and is associated with increased Ag presentation (16, 17).

To address this apparent contradiction, we revisited the expression and function of FcγR in DCs. We analyzed the pattern of FcγR expression during the process of maturation of monocyte-derived human DCs. Flow cytometric analysis using anti-FcγRI mAb (22.2-FITC) indicated that iDC and mDCs have decreased levels of FcγRI expression compared with Ma (5.8 ± 0.9 and 5.4 ± 0.9 vs 52.9 ± 2.3) (Fig. 1A). FcγRII expression was assessed with two anti-FcγRII mAbs (IV.3 and 7.3). mAb IV.3 exhibits high binding to activating FcγRIIa and has low interaction with FcγRIIb (black histograms, Fig. 1A, inset). In contrast, mAb 7.3 exhibits preferential binding to FcγRIIb, while maintaining interaction with FcγRIIa (gray histograms, Fig. 1A, inset) (18). Staining with IV.3-FITC resulted in similar MFIs on iDC (51.8 ± 2.9) and mDC (56.7 ± 3.9) suggesting that, FcγRIIa expression is sustained at comparable levels at early and advanced stages of DC maturation (Fig. 1A). The staining intensity of IV.3-FITC was moderately higher on Ma (80.4 ± 4.4) than on DCs. The expression of FcγRIIb on circulating DCs (4, 5) and cultured DCs (5) assessed with specific anti-FcγRIIb mAbs was recently reported. We performed surface staining with 7.3-FITC mAb on DCs and explored potential differences in binding at distinct DC maturation stages. The intensity of staining with 7.3-FITC was higher on iDC compared with mDCs matured with the cytokine mixture (112.8 ± 32.7 vs 51.7 ± 10.6) (Fig. 1A). The reduced binding of 7.3 mAb to mDC was associated with increased expression of HLA-DR, CD86, CD83, and CD40 (Fig. 1B), suggesting that FcγRIIb expression is down-regulated concomitantly with the induction of phenotypic markers of DC maturation.

We analyzed the expression of FcγRIIA and FcγRIIB RNA transcripts in human DCs at distinct maturation stages by RT-PCR (Fig. 1C, left panel) (12) and real-time PCR (Fig. 1C, right panel), iDCs generated in culture with GM-CSF and IL-4 predominantly expressed FcγRIIB transcripts (Fig. 1C, left and right panel). FcγRIIB transcripts were undetectable or low in DCs matured with either TNF-α alone (DC_{TNFα}) or with the mixture of inflammatory cytokines (mDC). FcγRIIA transcripts were down-regulated following maturation of DCs with TNF-α (Fig. 1C, left panel), but persisted in mDCs cultured with inflammatory cytokine mixture (Fig. 1C, right panel). Semiquantitative real-time PCR indicated skewing in the ratio of FcγRIIA/FcγRIIB in favor of FcγRIIB transcripts in iDCs (Fig. 1C, right panel). In contrast, the ratio of FcγRIIA/FcγRIIB transcripts was increased in mDCs as a result of reduced FcγRIIB expression (Fig. 1C, right panel).

To determine whether the differential expression of FcγRIIA and FcγRIIB transcripts during DC maturation is reflected at the protein level, we analyzed FcγRIIA and FcγRIIB by Western blotting (Fig. 1D). Lysates obtained from Ma, iDCs, and mDCs were immunoprecipitated with pan-FcγRII mAb clone FL1B.26 that binds both FcγRIIA and FcγRIIB (12, 19). The immunoprecipitated proteins were run on SDS-PAGE followed by immunoblotting with specific Abs raised against the intracellular domain of FcγRIIA (260) and FcγRIIB (FcγRIIB/IC), respectively (18). Lysates obtained from A375 melanoma cells transfected with rFcγRIIA (IIA), FcγRIIB2 (IIIB), or vector only (NT) were used for control.

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Phagocytosis and TNF-α production mediated by activating and inhibitory FcγR in human DCs. A, Phagocytosis of PKH26-labeled probes E-22.2, E-IV.3, and E-7.3 was measured by flow cytometry. The PI is presented as mean ± SEM of five to eight experiments (*, p < 0.05 and **, p < 0.01). B, Membrane expression of CD86 on DC following internalization of FcγRI- and FcγRII-specific probes (E-22.2, E-IV.3). iDCs and mDCs were cultured for 24–48 h following phagocytosis and were stained with FITC-conjugated anti-CD86 mAb. Cells that were not incubated with phagocytic probes were used as control (the MFI was set as 100). Staining with CD86-FITC was expressed as a percentage of control incubated with phagocytic probes were used as control (the MFI was set as 100). Results are representative of four individual experiments (107.3 ± 10.7 and 96.2 ± 13.3 vs 127.0 ± 13.4), indicating that phagocytosis via FcγRIIIa is preserved during DC maturation. Both iDCs and mDCs showed higher FcγRIIIa-mediated phagocytosis of E-IV.3 probes compared with phagocytosis of E-22.2 probes mediated through FcγRI (Fig. 2A). Interestingly, despite low FcγRI expression in DCs, CD86 expression was higher after phagocytosis of E-22.2 probes compared with E-IV.3 probes in both iDCs and mDCs (Fig. 2B). This finding is in agreement with other reports indicating that signaling through the FcγRII-associated γ-chain induces a mature phenotype in DCs (6).

Cross-linking of activating and inhibitory FcγR initiates the inhibitory signaling cascade leading to down-regulation of cellular functions (22). With the identification of FcγRIIb expression on DCs (23, 4, 5), we sought to dissect the role of this receptor in phagocytosis. We analyzed attachment and phagocytosis of PKH26-labeled E-IV.3 and E-7.3 by flow cytometry (Fig. 2C). The level of IV.3 and 7.3 mAb on the probes was verified with FITC-conjugated rabbit anti-mouse IgG. In all experiments, the E-7.3 probe was matched to E-IV.3 probe as far as amount of Ab coated onto the probes. At equivalent concentrations of IV.3 and 7.3 mAb used to coat the probe (example using 4 ng mAb/probe), the percentage of iDCs with E-7.3 probes attached to the surface was similar to the percentage of iDCs with attached E-IV.3 probes, while the percentage of iDCs that had internalized E-7.3 probes was lower than the percentage of iDCs that had internalized E-IV.3 probes (Fig. 2C). The internalization of E-7.3 probes was lower than the internalization of E-IV.3 probes in Ma, iDCs, and mDCs (36.8 ± 11.6 vs 127.0 ± 13.4, 43.2 ± 15.7 vs 107.3 ± 10.7, and 41.3 ± 22.6 vs 96.2 ± 13.3), suggesting that engagement of FcγRIIb is associated with decreased phagocytosis (Fig. 2, A, C, and D). In prior studies, we found that cross-linking of surface receptors on monocytes with 7.3 mAbs was associated with phosphorylation of the ITIM motif of FcγRIIb, suggesting that 7.3 mAb mediates inhibitory signaling (18). The marked decrease in phagocytosis of E-7.3 probes compared with E-IV.3 probes suggests that phagocytosis of Ab-opsonized particles can be altered by cross-linking of FcγRIIIa and FcγRIIb.

TNF-α is an essential cytokine required for the maturation of DCs. Maturation of DCs following autocrine production of TNF-α has been described following viral infection (24). In human and mouse monocytes/Ma, the production of TNF-α is differentially regulated by activating and inhibitory FcγR (18, 25). We investigated the production of TNF-α by iDCs following phagocytosis of E-IV.3 and E-7.3 probes. TNF-α production was measured by ELISA in iDC placed in culture for 48 h with E-IV.3, E-7.3, and erythrocytes with no Ab as control (Fig. 2E). There was a marked induction in TNF-α production by iDCs incubated with IV.3 probes as compared with erythrocytes not coated with Abs (3.2 ± 2.1-fold induction). The production of TNF-α was significantly lower after incubation of iDCs with E-7.3 probes (1.25 ± 0.9-fold induction, p < 0.02) in comparison with E-IV.3 probes. We did not detect major differences in the membrane expression of MHC-I, HLA-DR, CD80, CD86, CD40, and CD83 following incubation of E-IV.3 probes compared with E-7.3 probes, suggesting that the differences in TNF-α production were not associated with major phenotypic changes.

We assessed the roles of FcγRIIIa and FcγRIIb in the internalization of antigenic probes coupled with the immunodominant influenza MP and opsonized with anti-FcγRII mAbs. E-MP-IV.3 and E-MP-7.3 probes were made with biotinylated MP and with fragments of IV.3 or 7.3 mAbs, as described in Materials and Methods (Fig. 3A, a–c). Phagocytosis assessed by flow cytometry revealed reduced internalization of PKH26-labeled E-MP-7.3 probes by iDCs and mDCs compared with PKH26-labeled E-MP-IV.3 probes (Fig. 3B, a–d). Internalization of MP probes was detected by immunofluorescence staining with MP-specific polyclonal rabbit antiserum. Higher numbers of MP-positive particles were detected in immunofluorescence microscopy when MP was delivered by E-MP-IV.3 probes as compared with E-MP-7.3 probes in mDCs (Fig. 3B, e and f), suggesting that FcγRIIb acts as a negative regulator of Ag uptake in mature DCs.
FcγRIIb deficiency is associated with increased phagocytosis and increased proinflammatory cytokine production, suggesting a role for FcγRIIb as a negative regulator of innate immune functions (25, 26). Our results indicate that by establishing molecular constraints that hamper internalization of IgG-opsonized Ag and the autocrine action of TNF-α in DCs, FcγRIIb has the potential to restrain adaptive immune responses.

Cross-presentation of Ab-Ag complexes is dependent on FcγR expression on DCs

Peptides derived from phagocytosed Ag are presented by DCs with increased efficiency (20, 27). DCs that acquire Ag by phagocytosis elicit Ag-specific CD8⁺ T cell immunity (7, 10, 28). Phagocytically active FcγR are expressed in blood DCs and targeting Ag to these receptors increases the efficiency of T cell activation (29). As DCs express both activating and inhibitory FcγR, their relative expression likely influences the cross-presentation of Ab-complexed Ag (4, 9).

We tested the ability of human DCs from HLA-A*0201 healthy donors to elicit IFN-γ production by influenza-specific memory T cells in a well-characterized antigenic system for Ag presentation and CD8⁺ T cell activation (11). The HLA-A*0201-restricted 9-aa MP (GILGFVFTL) and a 17-aa peptide (TKGILGFVFLTVPSER) containing additional N- and C-terminal flanking residues present in the influenza virus sequence were used as antigenic peptides. The 9-mer and 17-mer peptides were coupled to FcγRIIa (E-MP-IV.3) phagocytic probes (middle and right panels). DCs were subsequently used to stimulate autologous T cells at a 1:5 ratio. B, ELISPOT assay for IFN-γ-producing influenza memory T cells cultured with iDCs (left panel) and mDCs (right panel) following uptake of 9-mer and 17-mer coupled to IV.3 probes (E-MP-IV.3) and 7.3 probes (E-MP-7.3). Significant differences are marked *, p < 0.05. C, Detection of MP-specific CD8⁺ T cells by Pro5 pentamer staining in flow cytometry. T cells isolated from three independent donors were cultured with autologous iDCs and mDCs probed with E-MP-IV.3 and E-MP-7.3, and control probes with no Ab. The percentage of MP-specific pentamer binding CD8⁺ cells from one representative experiment is shown.
E-MP-IV.3 probes (Fig. 4C). IL-4/IL-13 and IL-10 leads to the up-regulation of inhibitory regulation by cytokines. Prior work showed that treatment with soluble MP (355.5 ± 70.0 and 400.3 ± 97.1 vs 55.7 ± 8.1 and 54.3 ± 4.3 SFC/10^5 T cells, p < 0.05). The cross-presentation of MP probes directed through either FcγRI or FcγRIIa in mDCs was similar (Fig. 4A). These results point out the novel observation that cross-presentation by mDCs can be enhanced when Ag is given by the route of activating FcγR.

To determine the role of inhibitory FcγRIIb in cross-presentation, we quantified IFN-γ-producing T cells stimulated with DCs incubated with E-MP-7.3 probes. Cross-linking of FcγRIIb in iDC was associated with a 50% reduction in the number of IFN-γ-producing T cells compared with E-MP-IV.3 probes (Fig. 4B, left panel). Interestingly, no decrease in cross-presentation was detected following cross-linking of FcγRIIb by E-MP-7.3 probes in mDC (Fig. 4B, right panel). The percentages of influenza-specific memory CD8^+ T cells after incubation with DCs loaded with E-MP-IV.3 probes and E-MP-7.3 probes evaluated by MP-specific HLA-A*0201 pentamer binding were analogous with the results obtained in ELISPOT (Fig. 4C). The delivery of E-MP-IV.3 probes compared with soluble MP into iDCs and mDCs determined clonal expansion illustrated by a marked increase in the percentage of Pro5^+ MHC pentamer-binding CD8^+ T cells (Fig. 4C). The percentage of double-positive CD8^+ and Pro5^+ MHC pentamer-binding T cells was reduced by 34.7 ± 4.2% p < 0.01 when FcγRIIb was cross-linked with E-MP-7.3 probes compared with E-MP-IV.3 in iDCs (Fig. 4C). In mDCs, phagocytosis of E-MP-7.3 probes was associated with reduced expansion of MP-specific CD8^+ cells by 15.8 ± 2.1%, p < 0.01 as compared with E-MP-IV.3 probes (Fig. 4C).

The regulation of FcγR expression by cytokines enables the adaptation of FcγR-bearing cells to changes in the microenvironment. Induction of FcγRI by IFN-γ in iDCs leading to augmented uptake of Ab-opsonized antigenic particles has been documented (30). Likewise, inhibitory FcγRIIb are subject to regulation by cytokines. Prior work showed that treatment with IL-4/IL-13 and IL-10 leads to the up-regulation of inhibitory FcγRIIb in human monocytes (12, 18). Moreover, IL-10 mediates transcriptional activation of the FcγRIIb promoter in monocytic cells (18).

We tested whether IL-13 and IL-10 have the ability to modulate the expression of FcγRIIa and FcγRIIb isofoms in mDCs. The expression of both FcγRIIa and FcγRIIB RNA transcripts was up-regulated in mDCs by overnight treatment with IL-10, whereas IL-13 did not significantly change the expression of either transcript (Fig. 5A). The combined treatment of IL-13 plus IL-10 significantly increased FcγRIIa transcript expression (Fig. 5A). Elevated expression of FcγRIIa protein in Western blots using FcγRIIa-specific Abs was observed following IL-10 treatment (Fig. 5B). Up-regulation of FcγRIIb in mDCs by IL-13 plus IL-10 was detected at protein level in Western blots using FcγRIIb-specific Abs (Fig. 5B). The FcγRIIa/FcγRIIb protein ratio evaluated by densitometry was increased after IL-10 treatment, whereas the combined treatment of IL-13 plus IL-10 decreased the FcγRIIa/FcγRIIb protein ratio (Fig. 5B). Increased IV.3-FITC binding to mDCs suggested higher FcγRIIa surface expression following treatment with IL-10 (Fig. 5C). Increased 7.3-FITC binding to mDCs suggested higher FcγRIIb surface expression following treatment with IL-10 and IL-13 plus IL-10 (Fig. 5C). Reduced expression of MHC-I and -II, CD80, and CD86 after treatment of mDCs with IL-10 and IL-13 suggested reversal to a less mature phenotype (Fig. 5D).

We investigated whether the phagocytosis of E-IV.3 and E-7.3 probes by mDCs was altered by the treatment with IL-10 and IL-13. The phagocytosis of E-IV.3 probes was increased in mDCs treated with IL-10 (167.7 ± 26.7%, p = 0.05) compared with control mDCs (Fig. 6A). IL-13-treated mDCs had similar phagocytosis of E-IV.3 probes (113.1% ± 4.1) with control mDCs (Fig. 6A). Treatment of mDCs with IL-13 plus IL-10 increased phagocytosis of E-IV.3 probes compared with control mDCs (160.9 ± 25.4%, p = 0.06) (Fig. 6A). We assessed whether the uptake of E-7.3 vs E-IV.3 was altered by the treatment of mDCs with IL-10.

FIGURE 5. Cytokine regulation of FcγR in mDCs. A. Real-time PCR analysis of FcγRIIA and FcγRIIB RNA transcripts in mDCs cultured for 24 h in complete medium, or in medium supplemented with IL-10 (25 ng/ml), IL-13 (50 ng/ml), or a combination of IL-10 plus IL13 (n = 4–5, *, p < 0.05). B. FcγRIII and FcγRIIb protein levels in mDCs cultured with medium, IL-10, IL-13, and IL-10 plus IL-13 assessed in Western blotting. Cell lysates were immunoprecipitated with pan-FcγR mAb FLl8.26 and blotted with rabbit polyclonal Abs specific for the intracellular domain of FcγRIIA (260) and FcγRIIb (FcγRIIb/IC) (upper panels). Cytokine-induced changes in the FcγRIIa/FcγRIIb protein ratio were determined by densitometry (lower panel). C. Surface binding of IV.3-FITC and 7.3-FITC was assessed by flow cytometry following mDC treatment with medium, IL-10, IL-13, and IL-10 plus IL-13 (n = 5–7, *, p < 0.05). D. Expression of maturation markers and costimulatory molecules on mDCs after treatment with IL-10 and IL-13. Membrane expression of MHC-I, HLA-DR, CD80, CD40, and CD86 on mDCs cultured for 24 h in complete medium, medium supplemented with IL-10 (50 ng/ml), IL-13 (25 ng/ml), or a combination of IL-10 plus IL-13. Results from five to seven individual experiments are shown.

(141.1 ± 15.6 vs 65.1 ± 13.9 SFC/10^5 T cells). In mDCs, internalization of Ag through activating FcγR further amplified cross-presentation and activation of Ag-specific T cells. There was a marked increase (up to 25-fold for 9-mer and up to 7-fold for 17-mer) in IFN-γ-spot-forming T cells by mDCs when the antigenic peptides were coupled to FcγRIIa- and FcγRIIb-specific probes, as compared with soluble MP (355.5 ± 70.0 and 400.3 ± 97.1 vs 55.7 ± 8.1 and 54.3 ± 4.3 SFC/10^5 T cells, p < 0.05). The cross-presentation of MP probes directed through either FcγRI or FcγRIIa in mDCs was similar (Fig. 4A). These results point out the novel observation that cross-presentation by mDCs can be enhanced when Ag is given by the route of activating FcγR.
IgG-opsonized Ag in tissues and at sites of immune complex property may enable mature DCs to boost T cell responses against far superior ability to activate Ag-specific CD8+ T cell immunity and verify the role of inhibitory FcR during DC maturation critically influences the presentation of Ag-specific T cell responses (31). Our results suggest an essential function for FcγRIIa expressed on monocyte-derived DCs in the cross-presentation of Ab-opsonized exogenous Ag to CD8+ T cells. These findings underscore the role of FcγRIIa on DCs in the regulation of adaptive immune responses driven by self or foreign Ag-specific T cells.

FcγRIIb receptors were recently defined as important negative regulators of DC activation and function (4, 5). Interestingly, we detected high expression of FcγRIIb in iDC, whereas in mDCs FcγRIIb were markedly down-regulated. Differences in expression levels of FcγRIIb at various stages of DC maturation point out the versatile nature of Ab-Ag complex interactions with DCs. The efficient delivery of Ag coupled to FcγRII-specific Abs is expected to enhance naive and recall responses by T cells. Our study shows that, when FcγRIIb is expressed, its engagement by Ab-Ag complexes can result in inhibition of DC activation and Ag uptake. Various targeting Abs directed against the extracellular domain of FcγRII display differential binding to FcγRIIa and FcγRIIb, thus influencing the functional outcome (4, 5). As targeting Ags to phagocytic receptors on DCs is regarded as a promising approach to modulate immunity or tolerance, our findings shed new light on the function of FcγR in DC function.

In search of factors that modify FcγR expression, we found that IL-10 modulated the expression of FcγRIIa and FcγRIIb in DCs. Our results suggest that IL-10 increased the ratio of FcγRIIa vs FcγRIIb on mDCs, an effect that could lead to enhanced uptake of Ag-Ab complexes. IL-10 is regarded as an immunoregulatory cytokine that limits autoimmune reactions (33, 34). Unexpectedly, patients with rheumatoid arthritis showed enhanced responsiveness to immune complex stimulation in vivo (35), and an effect that could be explained by increased FcγRIIa-mediated uptake of immune complexes. In autoimmune diseases associated with elevated IL-10 serum levels, such as systemic lupus erythematosus, increased uptake of DNA-Ab complexes through FcγRIIa by DCs could stimulate autoreactive T cells and lead to amplification of the autoimmune reaction (31).

Genetic differences in the binding affinity of IgG subclasses by DCs from donors bearing the FcγRIIa H/H131 and FcγRIIa R/R131 variants (36–38) associate with changes in the activation and function of DCs (5). Genetic alterations in the promoter of the human FCGR2B gene that correlate with altered gene expression (39, 40) may influence the activation state of DCs and their responsiveness to immune complex stimulation. Given that FcγR with opposing function are often coexpressed on DCs, selective targeting of Ag to activating or inhibitory FcγR is a complex task. Further dissection of acquired and genetic factors involved in the regulation of FcγR will add to our understanding of Ab-mediated Ag uptake in human DCs and will advance current strategies to reprogram the activation of T cells.

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**Disclosures**

The authors have no financial conflict of interest.
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