Immune Complexes Inhibit Differentiation, Maturation, and Function of Human Monocyte-Derived Dendritic Cells[1]

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The interaction between immune complexes (IC) and the receptors for the Fc portion of IgG (FcγRs) triggers regulatory and effector functions in the immune system. In this study, we investigated the effects of IC on differentiation, maturation, and functions of human monocyte-derived dendritic cells (DC). When IC were added on day 0, DC generated on day 6 (IC-DC) showed lower levels of CD1a and increased expression of CD14, MHC class II, and the macrophage marker CD68, as compared with normally differentiated DC. The use of specific blocking FcγR mAbs indicated that the effect of IC was exerted mainly through their interaction with FcγRI and to a lesser extend with FcγRII. Immature IC-DC also expressed higher levels of CD83, CD86, and CD40 and the expression of these maturation markers was not further regulated by LPS. The apparent lack of maturation following TLR stimulation was associated with a decreased production of IL-12, normal secretion of IL-10 and CCL22, and increased production of CXCL8 and CCL2. IC-DC displayed low endocytic activity and a reduced ability to induce allogeneic T cell proliferation both at basal and LPS-stimulated conditions. Altogether, these data reveal that IC strongly affect DC differentiation and maturation. Skewing of DC function from Ag presentation to a proinflammatory phenotype by IC resembles the state of activation observed in DC obtained from patients with chronic inflammatory autoimmune disorders, such as systemic lupus erythematosus disease and arthritis. Therefore, the altered maturation of DC induced by IC may be involved in the pathogenesis of autoimmune diseases. *The Journal of Immunology,* **2007, 179: 673–681.**

The formation of immune complexes (IC)[3] occurs as the physiological consequence of the encounter of Abs with their cognate foreign Ags or as the result of autoimmune disorders (1, 2). IC interact with receptors for the Fc portion of Igs (FcRs) which are expressed by many cells of the immune system (3). Ligation of FcRs, specific for IgG, termed FcγRs, on myeloid cells induces cell activation including phagocytosis of opsonized pathogens, Ab-dependent cell-mediated cytotoxicity, release of proinflammatory mediators and reactive oxygen intermediates, and production of several cytokines and chemokines (4–7).

FcγRs are important effector molecules of humoral immunity and are implicated in the pathogenesis of inflammatory diseases characterized by the accumulation of IC, such as rheumatoid arthritis, vasculitis, and systemic lupus erythematosus (SLE) (8, 9). In humans, three different classes of FcγRs have been described: FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16) that differ in cell distribution, function, and affinity for IgG isotypes (3, 10, 11). FcγRI is a high-affinity receptor for IgG exclusively expressed on myeloid cells such as monocytes, macrophages, and granulocytes (upon IFN-γ activation). FcγRII is the most widely expressed FcγR being present on virtually all hemopoietic cells. This receptor exists as two major isoforms, FcγRIIA and -B, which exert divergent functions. FcγRIIA contains an ITAM in its cytoplasmatic tail which mediates positive signaling (12). Activation of FcγRIIA results in IC internalization as well as in initiation of the immune response. By contrast, FcγRIIB presents a cytoplasmatic ITIM which mediates inhibitory functions (12–14). FcγRIII is expressed either as an intrinsic (FcγRIIIA) or glycosphingolipid-linked protein (FcγRIIIB). Although FcγRI is able to bind monomeric IgG; FcγRII and FcγRIII bind mostly IgG forming IC. The balanced signaling through activating and inhibitory FcγRs regulates the activity of various immune effector cells, thus determining the magnitude of the response in IC-driven inflammation and autoimmune diseases. In fact, in noninflamed tissues, the ratio of activating to inhibitory FcγRs is low, whereas it becomes increased under inflammatory conditions (15).

Dendritic cells (DC) are highly specialized APCs that play a crucial role in the regulation of innate responses and in the initiation of adaptive immunity (16–18). Bone marrow DC progenitors enter the blood stream and home to nonlymphoid tissues where they reside as immature cells exerting a sentinel function for incomings Ags (16–18). Immature DC actively capture and process

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Received for publication June 28, 2006. Accepted for publication April 4, 2007.

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[1] This work was supported by grants from Fundación Antorchas, Buenos Aires, Argentina and Consejo Nacional de Investigaciones Científicas y Técnicas (PIP 6117), Buenos Aires, Argentina (to M.V.); from the Agencia Nacional de Promoción Científica y Tecnológica (SeCyT), Buenos Aires, Argentina (to M.A.I.); and from Ministero dell'Università e della Ricerca Scientifica (to S.S.).

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[3] Abbreviations used in this paper: IC, immune complex; SLE, systemic lupus erythematosus; DC, dendritic cell; aggIgG, heat-aggregated IgG; MHCII, MHC class II; mono-DC, monocyte-derived DC.

Ags and in response to direct stimulation by specific pathogens or inflammatory cytokines they undergo a terminal process of maturation that promotes their migration to T cell-dependent areas of secondary lymphoid organs where they present processed Ags to resting T cells (18–22).

Monocytes represent important DC precursor cells both in vitro and in vivo (23). Culture of monocytes with GM-CSF and IL-4 (24, 25) or IL-13 (26) have been very useful for the in vitro generation of large quantities of DC, providing a model to investigate the effect of self or environmental agents on the differentiation pathway.

The aim of this study was to investigate the effects of IC during monocyte differentiation and maturation to DC. The results reported herein show that the presence of IC during the differentiation process gives rise to a subset of DC with an altered phenotype profile and impaired APC functions.

Several aspects of mono-derived DC generated in vitro in the presence of IC resemble those reported in DC obtained from patients with chronic inflammatory autoimmune disorders characterized by the presence of large amounts of circulating IC. Therefore, the data presented in this study may help to better understand the consequences of FcγRs activation during DC differentiation in autoimmune pathologies.

Materials and Methods

Cell culture media and reagents

The following solutions and reagents were used: pyrogen-free saline (Rivero), RPMI 1640, and aseptically collected heat-inactivated FCS (Invitrogen Life Technologies). LPS from *Escherichia coli* strain 055:B5 (LPS) and OVA were obtained from Sigma-Aldrich. Human recombinant GM-CSF was obtained from Novartis. Purified recombinant human IL-4 was from PeproTech.

IC and heat-aggregated IgG (aggIgG)

IC were prepared using anti-OVA rabbit IgG, isolated from heat-inactivated serum by affinity chromatography as previously described (27). Briefly, precipitating rabbit anti-OVA IgG IC were prepared at 3-fold Ag excess, based on equivalence points determined by quantitative precipitation curves. Ag and Abs were incubated at 37°C for 30 min and subsequently at 4°C for 1 h. IC were then centrifuged at 10,000 × *g* for 5 min, the supernatant was discarded and the precipitated IC were suspended in saline at 1 mg of Ab/ml. The soluble human aggIgG was prepared by heating purified human IgG (Sigma-Aldrich) at a concentration of 5 mg/ml at 63°C for 12 min. Then, aggIgG was centrifuged at 10,000 × *g* for 5 min and the precipitate was then discarded. The supernatant containing soluble aggIgG was diluted with saline to the concentration of 1 mg/ml. Both IC and aggIgG preparations contained <0.125 endotoxin units/ml evaluated by the *Limulus* amebocyte assay (Microbiological Associates).

Monocyte-derived DC (mono-DC) preparation

DC were generated as previously described (28, 29). Briefly, highly enriched blood monocytes (>95% $CD14^+$) were obtained from buffy coats (through the courtesy of Hemocentro, Buenos Aires, Argentina) by Ficoll-Hypaque (Ficoll, Pharmacia; Hypaque, Winthrop Products) and Percoll (Amersham Pharmacia Biotech) gradient centrifugation. Monocytes were cultured for 6 days at 1×10^6/ml in 6-well tissue culture plates (Falcon; BD Biosciences) in RPMI 1640 supplemented with 10% FCS, 50 ng/ml GM-CSF, and 20 ng/ml IL-4 in the absence or presence of 150 μg/ml IC added at the beginning of the culture or as otherwise specified. Where indicated, DC were extensively washed and further cultured in the presence of 200 ng/ml LPS for 24 h.

In some experiments, DC were generated from monocytes obtained from SLE patients. A total of 20–30 ml of heparinized whole blood from 10 patients was collected after the informed consent of the donors and with the approval of the local ethics committee. Purified monocytes were differentiated into DC as described above. All patients were under treatment with nonsteroidal immunosuppressive drugs and fulfilled the American College of Rheumatology criteria for SLE (30).

To block individual FcγRs, purified monocytes (1×10^6/ml) were incubated at 4°C for 45 min with 1 μg/ml $F(ab')_2$ anti-FcγRI (clone 10.1), $F(ab')_2$ anti-FcγRII (clone 7.3), or $F(ab')_2$ anti-FcγRIII (clone 3G8) (Ancell). For cross-linking experiments, cells were then incubated with 500 ng/ml $F(ab')_2$ goat-anti-mouse IgG (Jackson ImmunoResearch Laboratories) at 4°C for 45 min. Afterward, monocytes were washed and incubated for 6 days with GM-CSF and IL-4 in the absence or presence of IC.

Flow cytometric analysis

Cells were washed with PBS supplemented with 1% FCS and incubated with saturating concentrations of the following mAbs followed by FITC-conjugated $F(ab')_2$ goat anti-mouse IgG (Fc-specific) (Sigma-Aldrich): anti-CD1a (IgG2a, NA1/34), anti-MHC class II (MHCII; IgG2a, L243), anti-CD16 (IgG1, 3G8), anti-CD32 (IgG2b, IV.3) (American Type Culture Collection), PAM-1 (IgG1, anti-mannose receptor). FITC- and PE-conjugated mAbs used were: FITC-anti-CD83 (IgG1κ, HB1/5e), PE-anti-CD86 (IgG1κ, B70/B72), PE-anti-CD11c (BD Pharmingen); PE-anti-CD14 (IgG2aκ, RM052), FITC-anti-CD64 (clone 22, IgG1), PE-anti-CD40 (IgG1, MAB89) (Immunotech). Staining with FITC-anti-CD68 (KP1; DakoCytomation) was done in previously permeabilized cells (Cytofix/CytoPerm kit; BD Pharmingen). Isotype-matched mAbs were used as controls. Cells were analyzed with a FACScan flow cytometer (BD Immunocytometry Systems) using CellQuest software.

Endocytosis assays

Mannose receptor-mediated endocytosis was measured as the cellular uptake of FITC-dextran (Sigma-Aldrich) and quantified by flow cytometry. Briefly, DC (2×10^5 cells/sample) were incubated in medium containing FITC-dextran (1 mg/ml; m.w. 40,000) for different time points. Afterward, cells were washed twice in cold PBS, fixed in 1% paraformaldehyde, and analyzed by flow cytometry.

Mixed leukocyte reaction

Irradiated immature or LPS-stimulated DC were added in graded doses to 2×10^5 purified allogeneic T cells in 96-well round-bottom microtest plates. Each group was performed in triplicate. [^{3}H]Thymidine incorporation was measured on day 5 after a 16-h pulse (5 Ci/μm; Amersham Biosciences).

Measurement of cytokines and chemokines

Supernatants from DC cultures were stored at −80°C until they were tested for the presence of cytokines and chemokines. Specific ELISA were used to quantify the production of IL-12p75 (PeproTech), IL-10, CXCL8 (BD Pharmingen), CCL3 (PeproTech), CCL22 (R&D Systems). CCL2 was measured as described elsewhere (28).

Immunocytochemistry

Cytosmears were rinsed in water and counterstained with hematoxylin. For immunochemistry, slides were incubated for 30 min with primary mAbs to CD1a and CD68 (DakoCytomation) followed by biotinylated secondary Abs and then streptavidin-HRP (DakoCytomation). The slides were finally incubated in 0.03% H_2O_2 and 0.06% 3,3′diaminobenzidine (DakoCytomation) for 3–5 min.

Statistical analysis

Statistical significance was determined using the paired Student *t* test ($p < 0.05$).

Results

IC inhibit DC differentiation

To determine the effect of IC on DC differentiation, peripheral blood monocytes (1×10^6/ml) were cultured in medium containing the appropriate differentiation cytokines (IL-4 and GM-CSF) in the absence or presence of an optimal concentration of IC (150 μg/ml) added at the beginning of the culture. On day 6, nonadherent cells were harvested and phenotype characterization was conducted by flow cytometric analysis. The presence of IC decreased total cell recovery (41.7 ± 9.3 and 61.0 ± 7.0% of input cells, for cells cultured with or without IC, respectively; mean ± SD; $n = 20$, $p < 0.001$). As shown in Fig. 1*A*, cells differentiated in the absence of IC (DC) presented the typical phenotype profile of mono-DC defined by high CD1a and MHCII expression and low levels of CD14. On the contrary, cells cultured in the presence of IC (IC-DC) expressed significantly lower levels of CD1a and an

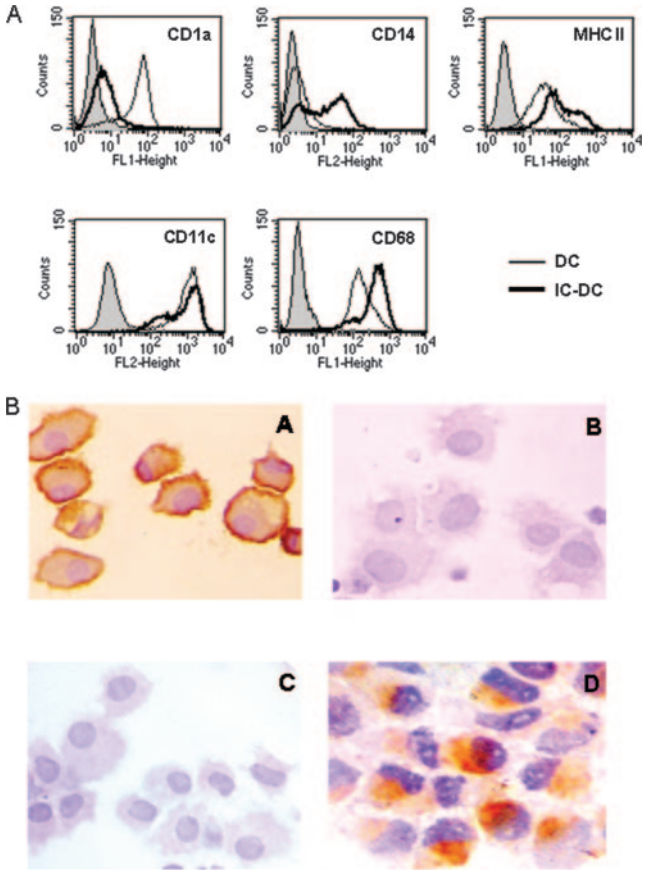

FIGURE 1. Phenotype analysis of DC differentiated in the absence (DC) or presence of IC (IC-DC). DC were differentiated by culturing monocytes with GM-CSF plus IL-4 alone or in the presence of IC, added at the beginning of the culture (day 0). Phenotype analysis of harvested cells was done on day 6. *A*, Phenotype profile of DC and IC-DC, obtained by flow cytometric analysis, of 1 representative experiment of 20 performed with similar results. Filled histograms correspond to isotype control Abs. *B*, Cytosmears immunostaining show CD1a expression on DC (*A*) but not on IC-DC (*C*). CD68 is weak or absent on DC (*B*) but strongly expressed in the cytoplasm of IC-DC (*D*). Morphological differences between DC and IC-DC reveal that IC-DC exhibit abundant cytoplasm with eccentrical nucleus location and less cytoplasmatic protrusions and membrane ruffling than DC. *A* and *B*, ×100; *C* and *D*, ×400.

increased expression of CD14, CD68, and MHCII. No differences in CD11c expression were observed between DC and IC-DC. Similar results were observed when human aggIgG (100 μg/ml) were used instead of IC (data not shown). For instance, CD1a and CD14 expression was 16.5 ± 7.6% and 53.4 ± 9.2%, and 21.3 ± 6.0% and 62.4 ± 8.4% (mean ± SD; $n = 10$) for IC and aggIgG, respectively. Controls performed using rabbit IgG or OVA alone had no effect on DC differentiation (data not shown). Cytosmear immunostaining of DC and IC-DC confirmed the differential expression of CD1a and CD68 in DC cultured under the two different conditions and revealed differences in their morphology (Fig. 1*B*). Although conventional differentiated DC presented abundant cytoplasmatic protrusions and membrane ruffling, cells differentiated in the presence of IC appeared as large rounded cells with abundant vacuoles, eccentrical nucleus location, and few cytoplasmatic protrusions.

The effect of IC is dose and time dependent

In previous experiments, different concentrations of IC (range: 15–200 μg/ml) were tested for their ability to affect DC differentiation.

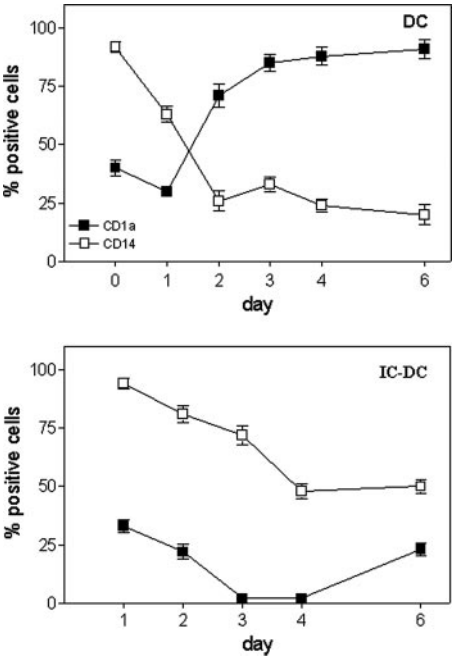

FIGURE 2. Kinetics of CD1a and CD14 expression during the differentiation of DC and IC-DC. DC differentiation was conducted in the absence (DC) or presence of IC (IC-DC) added at the beginning of the culture. CD1a and CD14 expression were analyzed by flow cytometry at different time points. Results are shown as mean values (±SD) of the percentage of positive cells obtained from three independent experiments.

It was observed that the IC effect was dose dependent: at 15, 50, 100, 150, and 200 μg/ml; CD1a expression was inhibited by 2 ± 0.5%, 42 ± 3.5%, 73 ± 8.2%, 85 ± 5.2%, and 83 ± 6.8%, respectively (mean ± SD; $n = 3$). In accordance with these results,

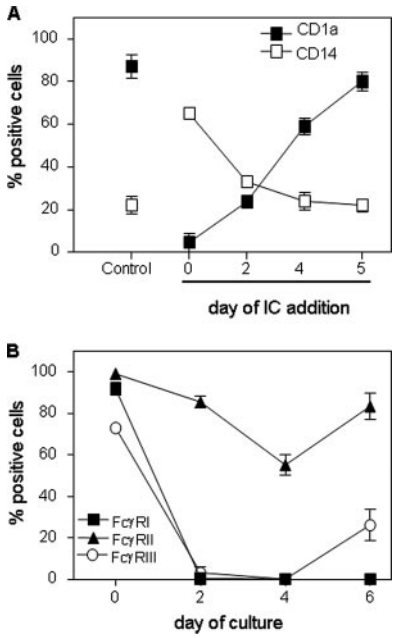

FIGURE 3. Influence of the timing of IC addition during DC differentiation. *A*, Monocytes were differentiated into DC in the absence (control) or presence of IC added at different times of the culture. On day 6, the expression of CD1a and CD14 was determined by flow cytometric analysis. *B*, FcγRs expression was determined at different time points during the differentiation of DC. Results are shown as mean values (±SD) of the percentage of positive cells obtained in three independent experiments.

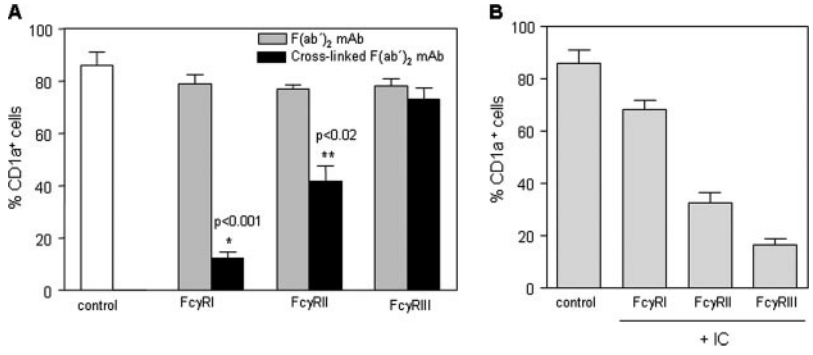

FIGURE 4. IC action is mediated through the interaction with FcγRI and FcγRII. *A*, FcγR cross-linking: monocytes (1×10^6/ml) were incubated at 4°C for 45 min with 1 μg/ml of the indicated murine $F(ab')_2$ anti-human FcγR followed by incubation with 500 ng/ml $F(ab')_2$ goat-anti-mouse IgG and then cultured in medium with GM-CSF plus IL-4. *B*, FcγR blocking: Monocytes were incubated with the indicated murine $F(ab')_2$ anti-human FcγR and then cultured in medium containing GM-CSF plus IL-4 in the presence of IC. Results are shown as mean values (±SD) of the percentage of $CD1a^+$ DC obtained after 6 days of culture in three independent experiments.

the concentration of 150 μg/ml IC was chosen and used throughout the study.

As shown previously (Fig. 1), the addition of IC at the beginning of the culture (day 0) inhibited the expression of CD1a and increased CD14 levels. Therefore, it was interesting to investigate the kinetics of expression of these two markers during the whole differentiation time. In normal differentiated DC, the up-regulation of CD1a expression (from day 2 of culture) was accompanied by a progressive down-regulation of CD14 levels (Fig. 2). On the contrary, during the differentiation of IC-DC the basal expression of CD1a decreased throughout the days of culture and was only partially restored to the initial levels at the end of the 6-day culture. Although CD14 expression was also down-regulated, levels remained significantly higher compared with those obtained for DC (Fig. 2).

Next, the influence of the time of IC addition to the cultures was investigated. IC were added at different time points during monocyte differentiation and their effect was evaluated by analyzing the expression of CD1a and CD14 at the end of the 6-day culture. As shown in Fig. 3*A*, DC differentiated in the absence of IC (control) showed, as expected, high CD1a expression and low levels of CD14. As previously demonstrated (Fig. 1), addition of IC on day 0, resulted in the differentiation of $CD1a^{low}CD14^{high}$ cells. When IC were added on day 2, the percentage of $CD1a^+$ and $CD14^+$ cells harvested on day 6 was 24 and 33%, respectively. Increased numbers of $CD1a^+$ cells (~60%) with low CD14 expression (~24%) were obtained when IC were added to the culture at latter time points (day 4). No significant differences were observed when IC were added on day 5.

IC interact with specific FcγRs (31), human monocytes express three types of FcγRs while mono-DC express mainly FcγRII and FcγRIII (3, 32). To gain insight into IC action, it was important to investigate whether FcγR expression was modulated during monocyte differentiation to DC. For this purpose, monocytes were cultured for 6 days in the presence of IL-4 and GM-CSF and the expression of FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16) was evaluated at different times of the culture. Fig. 3*B* shows that the expression of the three types of FcγRs was high at day 0 and decreased after 2–4 days of culture. A complete down-regulation of FcγRI and FcγRIII was present at days 2 and 4. On day 6, the expression of FcγRII, and partially, of FcγR III was restored, whereas no expression of FcγRI was detectable.

Altogether, these results suggest that IC effect depends mainly on specific interaction with the FcγRs expressed on monocytes as their action was mostly observed when added at the beginning of the culture.

IC action is mostly mediated by FcγRI and FcγRII

Two different experiments were used to examine throughout which FcγR, IC could alter DC differentiation. In the first series of studies, purified monocytes (1×10^6/ml) were incubated at 4°C for 45 min with mAbs against individual FcγRs and then cross-linked by the addition of $F(ab')_2$ goat anti-mouse IgG for 45 min at 4°C. Afterward, monocytes were washed to remove unbound mAbs and incubated in the presence of GM-CSF and IL-4 to allow DC differentiation. As shown in Fig. 4*A*, the single ligation of individual FcγR (or the simultaneous ligation of diverse combinations of them, data not shown) did not alter DC differentiation. Instead, cross-linking of FcγRI significantly inhibited DC differentiation, evaluated as CD1a expression. Cross-linking of FcγRII also altered DC differentiation, although with a lower potency. In contrast, no alteration of DC differentiation was obtained by FcγRIII cross-linking. No synergistic interaction was observed when both FcγRI and FcγRII were simultaneously cross-linked (data not shown).

In a second set of experiments, monocytes were incubated at 4°C for 45 min with each blocking FcγRs mAb and then cultured for 6 days with GM-CSF and IL-4 in the presence of IC. Results presented in Fig. 4*B* show that the inhibitory effect of IC was significantly prevented through the block of FcγRI or FcγRII while FcγRIII blocking was irrelevant. Altogether,

Table I. *LPS-induced maturation of DC and IC-DC*[a]

	CD83		CD86		CD40	
	%	MFI	%	MFI	%	MFI
DC	36.6 ± 14.6	14 ± 3.2	87 ± 3.0	156.2 ± 49.0	99.5 ± 2.0	366.0 ± 91
DC plus LPS	80.4 ± 8.0*	30 ± 5.7	93 ± 2.5	505.3 ± 102**	99.7 ± 1.6	666.7 ± 81**
IC-DC	61.3 ± 18.0*	18 ± 2.4	91 ± 2.7	347.5 ± 56.8***	91.5 ± 3.8	561.0 ± 86***
IC-DC plus LPS	77.3 ± 12.2	22 ± 3.1	94 ± 3.3	477.8 ± 39.2	99.1 ± 2.7	683.2 ± 80

[a] Phenotype analysis of DC and IC-DC incubated in the absence or presence of LPS (200 ng/ml) for 24 h. Results are expressed as the mean values (±SD) of the percentage of positive cells (%) and the mean fluorescence intensity (MFI) of 7–12 independent experiments. Values of p vs DC: *, $p = 0.001$; **, $p = 0.02$; and ***, $p = 0.01$.

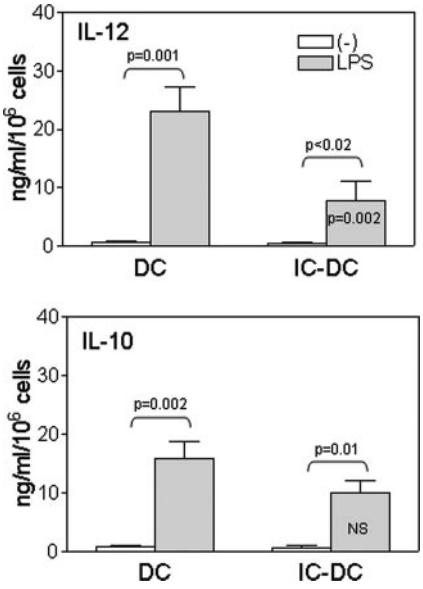

FIGURE 5. IL-12 and IL-10 production by DC and IC-DC. DC were generated in the absence (DC) or presence (IC-DC) of IC added at the beginning of the culture (day 0). On day 6, harvested cells were extensively washed and incubated alone (−) or with LPS (200 ng/ml) for additional 24 h. Supernatants were evaluated for the presence of IL-12 or IL-10 by ELISA. Results are shown as mean values (±SD) of five to eight independent experiments. Values of *p* indicated inside the bars reflect differences between DC and IC-DC under similar culture conditions.

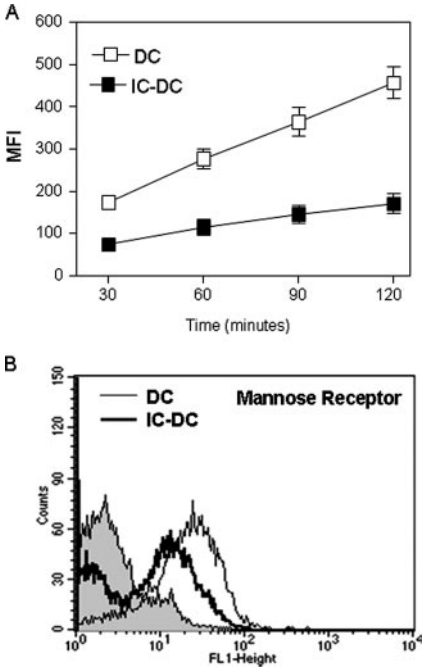

FIGURE 6. Endocytic activity of IC-DC. DC were generated in the absence (DC) or presence (IC-DC) of IC added at the beginning of the 6-day culture. *A*, Endocytosis was evaluated as FITC-dextran uptake at the indicated time points. Results are shown as mean values (±SD) of three independent experiments. *B*, Mannose receptor expression of DC and IC-DC. One representative profile of six performed with similar results is shown.

these data demonstrated that IC specifically interact with FcγRI and to a lesser extend with FcγRII to inhibit DC differentiation.

IC-DC have a defect of maturation

To evaluate the capacity of IC-DC to undergo maturation, both DC and IC-DC were stimulated with LPS. After 24 h, the expression of costimulatory molecules, such as CD83, CD86, and CD40, was determined as a measurement of DC maturation. Results presented in Table I indicate that, as expected, conventional immature DC expressed low levels of CD83, CD86, and CD40 which were up-regulated following LPS stimulation. On the contrary, the generation of DC in the presence of IC leads to the differentiation of cells with higher levels of the maturation markers, even in their immature state, which did not further increase after LPS stimulation.

Maturation of DC is associated with IL-12 secretion (16, 17, 28). Supernatants of immature and LPS-stimulated DC and IC-DC were assayed for IL-12 production. As expected, maturation of DC resulted in the secretion of IL-12 whereas LPS-stimulated IC-DC produced significantly lower levels (60.1 ± 5.2% inhibition, $n = 8$) of this cytokine (Fig. 5). Although, unstimulated IC-DC expressed high levels of maturation markers, they did not produce detectable levels of IL-12. Suppression of IL-12 is frequently associated with the production of IL-10 (33–35). However, as shown in Fig. 5, IL-10 production by IC-DC did not differ significantly from that obtained with DC.

To further investigate whether alterations in the phenotype of IC-DC correlated with biological altered APC functions, both Ag capture ability and the capacity of IC-DC to induce T cell proliferation were evaluated. Immature DC display potent endocytic activity, which decreases upon maturation (16). To study the endocytic capacity of IC-DC, FITC-dextran uptake, a marker of mannose receptor-mediated endocytosis, was examined. As shown in Fig. 6*A*, the kinetic of FITC-dextran uptake by IC-DC was lower at all time points tested compared with normal differentiated DC. In agreement with these results, surface expression of MR was also found defective in IC-DC with respect to DC (Fig. 6*B*).

Finally, the ability of IC-DC to induce allogeneic T cell proliferation was evaluated by MLR assays. Immature IC-DC showed reduced capacity to induce T cell proliferation, which was comparable to that exerted by immature DC, although at a high APC:T cell ratio (3%), this ability was strongly increased (Fig. 7). However, LPS-stimulated IC-DC, with respect to LPS-DC, showed a reduced ability in their capacity to stimulate T

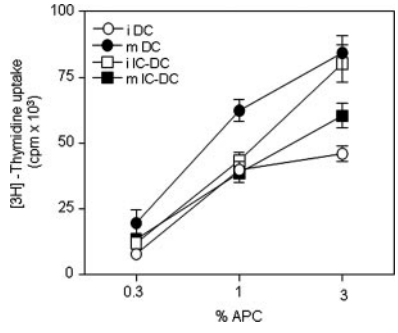

FIGURE 7. Effect of DC and IC-DC in MLRs. Different concentrations of irradiated immature (iDC; iIC-DC) and LPS-stimulated DC (mDC; mIC-DC) were cocultured with 2×10^5 purified allogeneic T lymphocytes. Proliferation was assayed as uptake of [^{3}H]thymidine added on the last 16 h of the 5-day culture assay. Results (triplicates ± SE) of one representative experiment are shown.

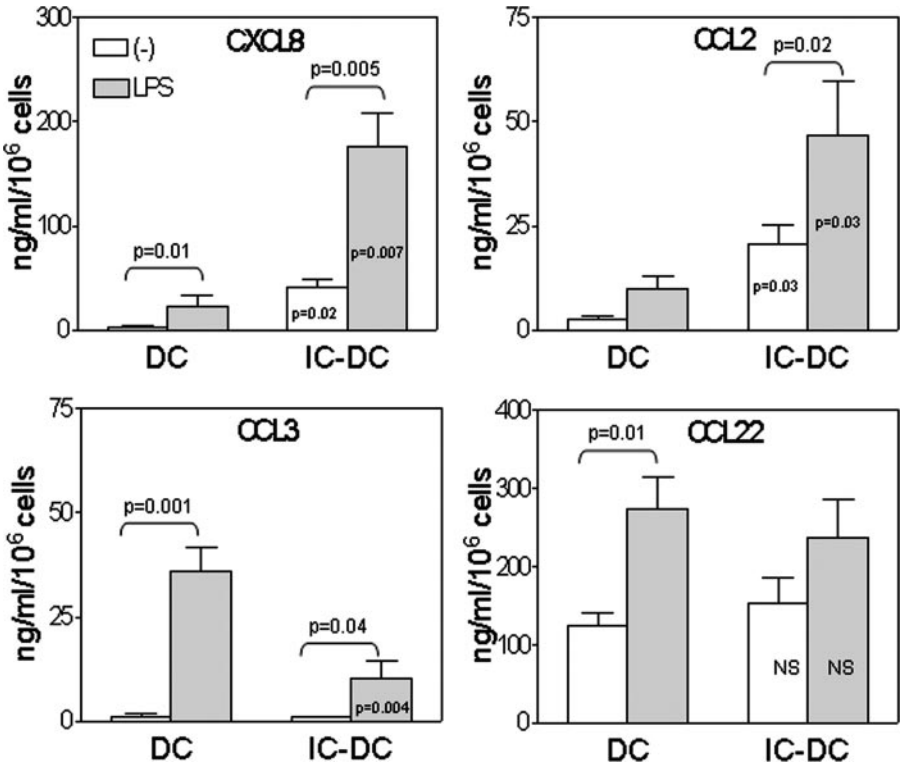

FIGURE 8. Chemokine production by IC-DC. DC and IC-DC were cultured in the absence or presence of 200 ng/ml LPS for 24 h. Supernatants were tested for chemokine production by using specific ELISA. Results are shown as mean values (±SD) of 6–12 independent experiments. Values of p indicated inside the bars reflect differences between DC and IC-DC under similar culture conditions.

cell proliferation that was evident at all the APC:T cell ratio tested.

IC-DC and DC express a different profile of chemokine production

DC produce high levels of several chemokines which are involved in the recruitment of precursor cells and immature DC to the peripheral sites of inflammation (18, 36–38) and within the lymph nodes, where they play a role in T and B cell localization and in the DC-T cell interaction (18, 37, 39). Fig. 8 shows that IC-DC presented an altered pattern of chemokine secretion. The levels of CXCL8 and CCL2 produced by immature IC-DC were higher than those secreted by DC and were further increased upon stimulation with LPS. Instead, CCL3 production was similar in both immature DC and IC-DC, but the secretion of this chemokine was only partially induced by LPS in IC-DC. No significant differences in CCL22 production between DC and IC-DC were observed.

Differentiated DC are refractory to the action of IC

Finally, it was interesting to analyze whether IC could act as maturation factors on differentiated DC. Results shown in Table II, indicate that neither IC nor aggIgG were able to induce DC maturation. Moreover, the simultaneous stimulation of DC with LPS plus IC did not modify the ability of LPS in inducing the expression of maturation markers or IL-12 secretion (data not shown). No detectable levels of IL-10 were observed upon DC stimulation with IC or aggIgG (data not shown).

DC differentiation of monocytes from SLE patients

SLE is an IC-mediated disease involving inflammation in multiple organs. Monocytes obtained from 10 SLE patients were tested for their ability to differentiate into DC in vitro (Table III). On day 6, DC from SLE patients presented the typical DC cytoplasmatic morphology, however, cell recovery was lower (25.7 ± 3.2% of input cells, mean ± SD; $n = 10$) compared with healthy donors (61.0 ± 7%; $n = 20$). Table III shows the phenotypic profile of SLE-DC. All the patients evaluated presented low levels of CD1a. Expression of CD14 and CD68 was higher in the majority of the patients tested compared with healthy donors, while MHCII expression was similar. Furthermore, the differentiation of SLE-DC in the presence of IC resulted in the production of cells expressing minimal or no levels of CD1a (data not shown).

Stimulation of SLE-DC with LPS for 24 h did not up-regulate the expression of CD83 (SLE-immature DC = 28.3 ± 3.2; SLE-mature DC = 31.6 ± 2.2%, mean ± SD; $n = 8$). This impairment

Table II. *Induction of DC maturation through FcγR ligation*[a]

	CD83		CD86		CD40	
	%	MFI	%	MFI	%	MFI
DC	23.4 ± 10.7	10 ± 3.1	86 ± 4.7	155.8 ± 48.6	99.2 ± 3.8	341.2 ± 54.4
DC plus LPS	84.0 ± 7.9*	26 ± 4.1	94 ± 3.4	505.3 ± 102**	99.1 ± 3.4	629.4 ± 76.4**
DC plus IC	27.9 ± 16.4	10 ± 2.7	88 ± 5.8	257.5 ± 60.1	97.2 ± 5.5	448.6 ± 65.9
DC plus aaIgG	31.8 ± 12.5	12 ± 3.6		nd		nd

[a] DC and IC-DC were incubated with LPS (200 ng/ml), IC (150 μg/ml), or aaIgG (200 μg/ml) for 24 h. Results are expressed as the mean values (±SD) of the percentage of positive cells (%) and the mean fluorescence intensity (MFI) of 7–12 independent experiments. Values of p vs DC: *, $p = 0.001$; **, $p = 0.02$; and ***, $p = 0.01$; nd, not done.

Table III. *Phenotype of DC generated from monocytes of SLE patients*[a]

SLE Patient	CD1a	CD14	MHCII	CD68
Control	86.2 ± 4.6 (120 ± 9.2)	22.4 ± 3.3 (15 ± 3.8)	98.2 ± 2.0 (168 ± 23)	96.4 ± 1.3 (211 ± 18)
SLE 1	12.0 (8.1)	10.5 (12)	86.3 (115)	91.0 (421)
SLE 2	18.8 (9.8)	53.2 (14)	72.6 (101)	90.3 (396)
SLE 3	31.0 (10)	44.3 (10)	83.3 (96)	93.0 (310)
SLE 4	14.2 (9.9)	37.5 (16)	92.4 (142)	nd
SLE 5	24.1 (12)	65.7 (10)	99.1 (126)	89.2 (566)
SLE 6	7.6 (10)	29.3 (15)	nd	nd
SLE 7	5.8 (9.5)	48.3	85.2 (121)	85.6 (498)
SLE 8	10.6 (11)	16.7 (12)	92.1 (96.0)	83.4 (499)
SLE 9	7.1 (9.2)	36.2 (12)	85.4 (111)	nd
SLE 10	26.5 (10)	40.8 (11)	nd	87.9 (478)

[a] Results are expressed as percentage of positive cells, mean fluorescence intensity values are shown in parentheses. For SLE patients results of single experiments are provided. For healthy donors (control) average values ± SD are shown ($n = 12$); nd, not done.

in maturation correlated with a decreased ability to produce IL-12 after LPS stimulation (SLE-immature DC = 2.1 ± 0.3; SLE-mature DC = 5.3 ± 0.1 ng/ml; mean ± SD; $n = 8$) while IL-10 levels did not differ significantly from those obtained for healthy donors (data not shown).

Discussion

This study reports that the differentiation in vitro of DC from monocytes in the presence of IC gives rise to a subset of DC (IC-DC) characterized by an altered phenotype profile and impaired functions. IC-DC exhibited significant lower levels of CD1a and increased expression of CD14 and MHCII than normal differentiated DC. Morphological differences were also observed, with IC-DC presenting a larger and rounded shape with few cytoplasmatic protrusions, abundant vacuoles and eccentrical nucleus location. Moreover, the increased expression of the macrophage marker CD68 suggests that IC promote the differentiation of monocytes into macrophage-like cells.

IC exert their biological actions through the interaction with membrane FcγRs. The use of blocking FcγR-specific mAbs could determine that the inhibitory effect of IC on DC differentiation was exerted mainly through the interaction with FcγRI and to lesser extend with FcγRII. Moreover, it was observed that cross-linking of FcγRI and FcγRII is required for the alteration of the normal DC differentiation pathway. Indeed, human soluble monomeric IgG do not have any effect on DC differentiation (data not shown). The main role of FcγRI in mediating IC effect confirms the crucial role of this receptor during the initiation of the immune response (3, 11). FcγRI has 10- to 100-fold higher affinity for IgG, compared with the low-affinity FcγRII and FcγRIII. Thus, FcγRI acts in the early phases of the immune response, through the internalization of IC, the processing and the subsequent presentation of the Ag, long before engagement of other low-affinity FcγRs (40, 41). Monocytes, the precursors of DC, express the three FcγRs. In line with the results obtained in the FcγR blocking and cross-linking studies, our data indicate that the effect is more potent when IC were added at the beginning of the differentiation process. This timing parallels the kinetics of FcγR expression during the differentiation of monocytes into DC. Indeed, at the beginning of the culture (day 0), monocytes express considerable high levels of the three types of FcγRs. After 2 days of culture, both FcγRI and FcγRIII were completely down-regulated and although the expression of FcγRIII is partially restored at the end of the 6-day culture, this receptor does not appear to mediate IC effect. The down-regulation of the FcγRs is likely to be due to the action of IL-4 (42, 43). Although also FcγRII was down-regulated its levels remained considerably high during the whole differentiation. However, the effect observed either by blocking or cross-linking this receptor was always of minor relevance compared with FcγRI suggesting that the interaction of IC with these receptors has a limited relevance during the DC differentiation process. Of interest, immature IC-DC, in the absence of maturation stimuli, express relatively higher levels of CD83, CD86, and CD40 than immature DC although the expression of these markers were not further increased by LPS.

Maturation of DC by TLR ligands or by CD40L leads to the secretion of IL-12, a cytokine that plays a crucial role in the initiation, amplification, and orientation of the immune response (44). The increased expression of costimulatory markers on immature IC-DC was not associated with IL-12 secretion; moreover, LPS-stimulated IC-DC secreted lower amounts of IL-12 than those produced by normal differentiated DC. The lack of IL-12 secretion by $CD1a^-$ was previously reported in another experimental system (45). Deficiencies in IL-12 secretion were associated with increased IL-10 production (34, 35, 46). However, this is not the case of fully differentiated IC-DC, because these cells secrete similar levels of IL-10 as compared with DC. Interestingly, two recent reports indicate that the balance between the expression of activating (FcγRIIA) and inhibiting (FcγRIIB) FcγRs by mono-DC and circulating DC enables IC to mediate opposing effects on DC maturation and function (47, 48). It was reported that the block of FcγRIIB on immature DC leads to IL-12 production and DC maturation even though without the involvement of microbial products or inflammatory signals (48). Our study extends these findings, suggesting that the interaction of IC with activating and inhibitory FcγRs during monocyte differentiation into DC is responsible for the generation of DC with an altered phenotype associated with impaired APC functions. In fact, DC generated in the presence of IC display low endocytic activity, poor capacity to induce allogeneic T cell proliferation, and an altered repertoire of chemokine secretion.

Taking into account that monocytes also differentiate into DC in vivo (49), our findings are relevant to better understand the state of DC deficiencies observed in patients with chronic inflammatory or autoimmune disorders. Indeed, in the course of these pathologies large amounts of IC are formed and remain in circulation and are responsible for altered immune responses and tissue damaged (8, 9). Phenotypic and functional deficiencies of mono-DC have been recently reported in patients with autoimmune diseases (50–52). In this study, we observed that monocytes from SLE patients differentiated into DC expressing low levels of CD1a and high amounts

of CD14 and CD68. Moreover, these cells presented impaired up-regulation of CD83 and IL-12 production after maturation with LPS. In agreement with our findings, it was shown that mature DC from SLE patients present impaired up-regulation of MHCII and are defective in MLRs (51–53). Also, DC from rheumatoid arthritis patients showed increased production of IL-1, IL-6, TNF-α, and IL-10 and an altered pattern of chemokine secretion (54, 55). In our study, we did not observe significant differences in the levels of IL-10, IL-6, and TNF-α produced by IC-DC, supporting that the complex inflammatory and autoimmune profile observed in these patients may have additional role on these cells.

In SLE patients, deposition of IC in tissues leads to the production of multiple proinflammatory chemokines. Elevated levels of CXCL8, CCL2, and CCL5 were reported in biological fluids from immune complex-related diseases (56–58). Because IC can cross-link leukocyte surface FcγRs, our finding that DC generated in the presence of IC secrete higher levels of CCL2 and CXCL8 could explain the elevated levels of these two chemokines in autoimmune disorders and their role in the recruitment of leukocytes to the involved tissues.

In several IC-related diseases, peripheral blood DC subsets are substantially decreased as compared with healthy controls (50–52). Several alterations could explain this low number in DC counts, including decreased output from the bone marrow, increased apoptosis rates, and deficiencies during the differentiation and activation pathway. Interestingly, in our study, the recovery of DC was strongly reduced when cultured in the presence of IC, suggesting an additional mechanism responsible for the decreased number of blood DC.

In conclusion, our findings reveal a new level in the regulation of the immune response through the interaction of IC with specific FcγRs during the differentiation and maturation of DC. These findings may help to better elucidate the emerging role of DC in autoimmune disease (59–62). Further clarification of alternative differentiation pathways of DC might help to develop novel immunotherapeutic strategies for the treatment of IC-related diseases.

Acknowledgments

We are grateful to the staff of the blood bank of Hemocentro Buenos Aires for supplying us with buffy coats.

Disclosures

The authors have no financial conflict of interest.

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