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Triggering of B7h by the ICOS Modulates Maturation and Migration of Monocyte-Derived Dendritic Cells

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B7h, expressed by several cell types, binds ICOS expressed by activated T cells. We have previously shown that B7h triggering by ICOS-Fc inhibits human endothelial cell adhesiveness. This work investigated the effect of ICOS-Fc on human monocyte-derived dendritic cells (DCs). We found that DCs matured with LPS in the presence of ICOS-Fc (mDCsICOS) produced greater amounts of IL-23 and IL-10, and promoted a higher secretion of IL-17A and IL-17F in MLCs than those DCs matured with LPS alone (mDCs). Moreover, mDCsICOS pulsed with the keyhole limpet hemocyanin Ag during the maturation phase were better stimulators of Ag-specific MHC class I-, but not class II-restricted T cells than mDCs. This was probably due to promotion of cross-presentation because it was not detected when the Flu-MA58–66 Ag was directly loaded on already matured DCs and mDCsICOS. Finally, ICOS-Fc inhibited the adhesion of both immature DCs and mDCs to vascular and lymphoid endothelial cells, their migratory activity, and the expression of the Rac-1 activator b-Pix involved in cell motility. These data suggest that B7h stimulation modulates DC function with effects on their maturation and recruitment into tissues. This opens a novel view on the use of interactors of the ICOS:B7h system as immunomodulatory drugs. The Journal of Immunology, 2013, 190: 000–000.

Full activation of naive T cells requires their receipt of three signals from APC (1). The first is delivered through the TCR upon recognition of the antigenic peptide presented by the appropriate MHC, the second is provided by T cell costimulatory molecules engaged by their ligands expressed on APC, and the third is delivered by cytokines in the microenvironment. In the absence of these signals, T cells become anergic or die by apoptosis.
cells or IL-10–secreting regulatory T cells, but it can also induce IFN-γ–secreting Th1 cells and support IL-17–secreting Th17 cells in some circumstances (28–30). In humans, it has been reported that ICOS-mediated costimulation of naive Th cells elicits different responses depending on the cytokine milieu, because it promotes IFN-γ secretion in the presence and IL-10 and TGF-β secretion in the absence of IL-2 (31). Moreover, ICOS triggering has been shown to induce IL-17 secretion in a subset of cord blood cells (32). The differences between the two species might be partly because of the B7h capacity to weakly interact with CD28 and CTLA-4 in humans but not in mice, using binding sites different from that interacting with ICOS (33).

The primary aim of this work was to assess a different aspect of ICOS function, that is, the reverse signaling, triggered through B7h, on human DCs, and it was prompted by our recent research showing that B7h ligation by ICOS-Fc significantly inhibits the capacity of HUVECs to adhere to several tumor cell lines and polymorphonuclear cells. Therefore, the B7h–ICOS interaction may modulate the spread of cancer metastases and the recruitment of polymorphonuclear cells in inflammatory sites (34). Moreover, other authors have shown that, in mice, B7h triggering induces partial maturation of immature DCs (iDCs) with prominent augmentation of IL-6 secretion (35).

In peripheral tissues, iDCs are specialized in Ag uptake and processing for MHC presentation (36, 37). In the presence of inflammatory mediators, such as TLR ligands and inflammatory cytokines, iDCs differentiate to DCs matured with LPS alone (mDCs) able to activate T cells and characterized by decreased endocytic activity, increased expression of costimulatory and MHC class II (MHC-II) molecules, and increased cytokine secretion (38–40). They are at least 100 times more potent than macrophages in activating naive T cells in vitro and are able to “cross-present” endocytosed proteins on MHC class I (MHC-I), in addition to the standard presentation on MHC-II (41, 42). iDCs are recruited to sites of inflammation by inducible inflammatory chemokines, such as CCL5, whereas mDCs are directed to draining secondary lymphoid tissues by constitutive chemokines, such as CCL19 and CCL21, via upregulation CCR7 (43). In this study, we show that the triggering of B7h substantially modulates LPS-induced maturation of DCs by influencing cytokine secretion and promoting cross-presentation. Moreover, it inhibits DC adhesiveness to ECs, their migratory activity, and expression of the Rac-1 activator β-Pix involved in cell motility.

Materials and Methods

**Cells**

PBMCs were obtained by density gradient centrifugation fromuffy coats provided by the local Blood Transfusion Service (Novara, Italy). iDCs were prepared from CD14+ monocytes, isolated from PBMCs with the Mono-Cult (CliniMACS, Miltenyi Biotec, Bergisch Gladbach, Germany) by culture for 5 d in culture medium, composed of RPMI 1640 (Invitrogen, Burlington, ON, Canada), 2 mM L-glutamine (Invitrogen), and 10% FBS (Invitrogen) or serum-free medium supplement (Lonza, Basel, Switzerland), with recombinant human GM-CSF (800 U/ml; Invitrogen) or serum-free medium supplement (Lonza, Basel, Switzerland), and expanded every 2 wk with 1 mg/ml PHA (Sigma-Aldrich, St. Louis, MO) or 1 mg/ml CTLA-4–Fc (R&D Systems). In some experiments, the soluble human IgG1 Fc was also used as a control (R&D Systems) and had no effect on DC maturation (data not shown).

The DC surface phenotype was assessed by immunofluorescence and flow cytometry using FITC- and PE-conjugated (mAb) to CD14, HLA-DR (Collag, Burlingame, CA), CD1a, CD80, CD83, CD86, HLA-A, -B, -C (Becton Dickinson Pharmingen, San Jose, CA), and B7h (BioLegend, San Diego, CA).

**Mannose receptor–mediated endocytosis**

Cell endocytosis was assessed by evaluating uptake of FITC-dextran (molecular mass 70 kDa; Invitrogen). In brief, each DC sample was split into two fractions of 1 × 10^6 cells that were incubated with 1 mg/ml FITC-dextran at either 37˚C or 0˚C for 60 min. Uptake was then stopped by adding ice-cold PBS followed by extensive washes in a refrigerated centrifuge, and cells were analyzed by flow cytometry. Ag uptake was expressed as the difference of mean fluorescence intensity between the test sample performed at 37˚C and the control sample performed at 4˚C.

**Mixed lymphocyte cultures**

DCs (5 × 10^5) were cocultured in 96-well round-bottom plates for 5 d at a 1:20 DC/lymphocyte ratio with allogeneic PBLs or CD8+ or CD4+ T cells purified with the Human CD8 or CD4 Microbeads kits (Miltenyi Biotec). Supernatants were then collected and used for cytokine analysis. In the final 6 h of culture, 0.5 μCi [3H]thymidine (Perkin Elmer, Waltham, MA) was added to each well; cells were then harvested with a semiautomatic cell harvester and their radioactivity was measured with a beta counter (Perkin Elmer).

**Cytokine analysis**

IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12p70, IL-17A, IL-17F, IL-21, IL-22, IL-23, CCL20, TNF-α, and IFN-γ were evaluated using standard ELISA (R&D Systems; BioLegend).

**Ag presentation assays**

iDCs were resuspended at 1 × 10^6/ml in serum-free RPMI 1640 and incubated with 10 μg/ml keyhole limpet hemocyanin (KLH; Calbiochem, Darmstadt, Germany) for 6 h at 37˚C. They were then washed and resuspended in RPMI 1640 supplemented with 5% heat-inactivated AB human serum (Lonza), and matured for 24 h with LPS in presence or not of ICOS-Fc. They were then cocultured with autologous T cells, purified with the Human PanT Microbeads Kit (Miltenyi Biotec) at a DC/T ratio of 1:10. On day 7, specific activation was evaluated by ELISPOT assay on total T cells or on CD8+ or CD4+ T cells purified with the Human CD8 or CD4 Microbeads kits (Miltenyi Biotec). Differentially mDCs from HLA-A2+ donors were resuspended at 1 × 10^6/ml in serum-free RPMI 1640, pulsed with the influenza matrix Flu-MA58–66 peptide (3 μg/ml; GILGFVFTL; Primm) for 4 h at 37˚C, and then cultured with autologous T cells at a 1:10 ratio, to assess whether ICOS-Fc modulated the ability to present HLA-A2–restricted peptides to T cells. On day 7, the recovered cells were restimulated in the same conditions by Flu-MA58–66-pulsed DCs and 1 mg/ml rIL-7 (Sigma-Aldrich). After another 7 d, specific activation was evaluated by ELISPOT assay.

**ELISPOT assay**

A total of 5 × 10^4 T cells were cultured, at a 30:1 ratio, with unpulsed or KLH-pulsed mDCs in a final volume of 100 μl AIM-V medium (Invitrogen) in 96-well ELISPOT assay plates (Millipore, Bedford, MA), previously coated with 100 μl human primary anti–IFN-γ mAb solution (BD Biosciences). In some experiments, 5 × 10^5 Flu-MA58–66+ T cells were cultured, at a 10:1 ratio, with unloaded and Flu-MA58–66–labeled mDCs. ELISPOT assays were performed following the manufacturer’s instructions. Substrate (AEC Staining Kit; Sigma-Aldrich) was added to each well to develop the spots. Reaction was stopped after 10–20 min with washing. The membrane was left to dry in the dark. The spots were counted by computer-assisted image analysis (Transect 1300 ELISPOT Reader; AMI Bioline, Buttigliera Alta, Italy). Data are expressed as number of specific spots/10^5 cells; specific spots were those obtained in
the presence of Ag-pulsed DCs minus those obtained in the presence of Ag-unpulsed DCs.

**Cells adhesion assay**

HUVECs were isolated from human umbilical veins by trypsin treatment (1%) and cultured in M199 medium (Lonza) with the addition of 20% FCS and 100 U/ml penicillin, 100 μg/ml streptomycin, 5 UI/ml heparin, 12 μg/ml bovine brain extract, and 200 mM glutamine. HUVECs were grown to confluence in flasks and used at the second to fifth passage. Informed consent was obtained from all donors. Human dermal lymphatic ECs (HDLECs) were purchased from PromoCell and cultured with Endothelial Cell Growth Medium MV2 (PromoCell, Heidelberg, Germany).

HUVECs and HDLECs were grown to confluence in 24-well plates and then treated or not with ICOS-Fc, or F119SICOS-Fc or CTLA-4–Fc (2 μg/ml) for 30 min, washed with fresh medium twice, and incubated for 1 h with the iDCs or mDCs (5 × 10⁴ cell/well). The 1-h incubation time was chosen to allow full sedimentation of the adhering cells, but similar results were obtained with a shorter incubation time (30 min). In some experiments, iDCs or mDCs and HUVECs or HDLECs were pretreated with ICOS-Fc, or F119SICOS-Fc, or CTLA-4–Fc (2 μg/ml) for 30 min, washed with fresh medium twice, and used in the adhesion assays. After incubation in the adhesion assay, nonadherent cells were removed by washing three times with M199. The center of each well was analyzed by fluorescence image analysis (33). Adherent cells were counted by the Image Pro Plus Software for microimaging (version 5.0; Media Cybernetics, Bethesda, MD). Single experimental points were assayed in triplicate, and the SE of the three replicates was always <10%. Data are shown as percentages of inhibition versus the control adhesion measured on untreated HUVECs or HDLECs; this control adhesion was 25 ± 2 cells/microscope fields (n = 6) for iDCs and in a similar range for mDCs, in the adhesion to both HUVECs and HDLECs.

**Migration assay**

To determine the effects of ICOS-Fc on iDCs or mDCs migration, we used the Boyden chamber (BD Biosciences) migration assay. iDCs or mDCs (5 × 10⁵ cells/well) were plated onto the apical side of 50 μg/ml Matrigel-coated filters in serum-free medium in the presence and absence of ICOS-Fc, or F119SICOS-Fc, or CTLA-4–Fc (2 μg/ml). Medium containing 10⁻⁶ M N-formyl-Met-Leu-Phe (nfMLP; Sigma-Aldrich) or 200 ng/ml CCL5 (R&D Systems) was placed in the basolateral chamber as chemoattractant for iDCs; 250 ng/ml CCL19 (R&D Systems) or 250 ng/ml CCL21 (R&D Systems) was used as chemoattractant for mDCs. After 6 h, the cells on
the apical side were wiped off with cotton swabs. Cells on the bottom of the filter were stained with crystal violet and counted (five fields of each triplicate filter) with an inverted microscope. The results are expressed as the number of migrated cells per high-power field.

**Western blot analysis**

Cells were treated or not with ICOS-Fc or F119FICOS-Fc (4 µg/ml) at 37°C for 30 min. Then they were lysed in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% v/v Triton X-100, phosphatase and protease (P2850, P8340; Sigma-Aldrich) inhibitors for 20 min at 4°C. The supernatants were collected after centrifugation at 14,000 rpm for 30 min, and 35 µg proteins was run in a 9.3% SDS-PAGE and transferred onto a nitrocellulose membrane. Filters were blocked with TBS (10 mM Tris HCl pH 7.9; 150 mM NaCl) containing 5% nonfat milk and 1% Tween 20 for 1 h at room temperature, and probes overnight with anti-β-Pix (SH3 domain) rabbit polyclonal Ab (Millipore, Billerica, MA) followed by the appropriate HRP-conjugated secondary reagent and detected by ECL. Densitometric analysis was performed by the Multi-Analyst (version 1.1; Bio-Rad Laboratories, Hercules, CA).

**Statistical analysis**

Statistical analysis was performed using the Friedman test followed by a Dunn post hoc analysis. Results are shown as medians and interquartile ranges (IQRs).

**Results**

**Effect of B7h stimulation on DC maturation**

Monocytes were induced to differentiate to iDCs by a 5-d culture with GM-CSF and IL-4. Because activation stimuli, such as LPS, induce iDCs maturation to mDCs, we assessed the effect of B7h stimulation in this process by using ICOS-Fc to trigger B7h. iDCs were treated for 2 d with control medium (iDCs), LPS (mDCs), immature DCs treated with ICOS-Fc (iDCsICOS), or LPS plus ICOS-Fc (mDCsICOS). Then we compared their surface marker expression and endocytic, cytokine secretion, allostimulatory, and Ag presentation activities.

Analysis of surface expression of B7h, B7.1, B7.2, CD83, HLA-DR, and class I HLA showed that treatment with LPS upregulated B7.1, B7.2, CD83, HLA-DR, and class I HLA as expected, whereas ICOS-Fc had no effect because no differences were found comparing iDCs with iDCsICOS and mDCs with mDCsICOS (data not shown). Endocytic activity was assessed by measuring uptake of FITC-conjugated dextran. Results showed that ICOS-Fc did not exert any effect on this activity because iDCs and ICOSICOS displayed the same ability to capture Ags, and that this activity was similarly downregulated in mDCs and mDCsICOS (data not shown).

Assessment of cytokine secretion by measuring levels of IL-1β, IL-6, IL-10, IL-12p70, IL-23, and TNF-α in the supernatants of the 2-d DCs cultures showed that secretion of all these cytokines was almost undetectable in iDCs and it was unchanged in ICOSICOS (data not shown). Treatment with LPS upregulated secretion of IL-6, IL-12p70, IL-23, IL-1β, and TNF-α, and intriguingly, mDCsICOS displayed a different cytokine secretion pattern from mDCs, with higher secretion of IL-23 and IL-10, and lower secretion of IL-6 and, to a lesser extent, TNF-α (Fig. 1). By contrast, secretion of IL-12p70 and IL-1β were unchanged (Fig. 1). To assess the specificity of the ICOS-Fc effect, we induced mDC maturation in the presence of either F119FICOS-Fc, which is a mutated form of ICOS incapable to bind B7h (44), or ICOS-msFc, in which the human ICOS was fused with a mouse Fcγ receptor to minimize interaction with the human Fcγ receptors, or CTLA-4–Fc, which binds B7.1/B7.2, but not B7h. Results showed that ICOS-msFc exerted the same effect as ICOS-Fc, whereas F119FICOS-Fc did not exert any effect. CTLA-4–Fc increased IL-10 decreased IL-6, and did not change IL-12p70 and IL-1β similarly to ICOS-Fc, but differently from ICOS-Fc, it had no effect on IL-23 and TNF-α (Fig. 1).

Evaluation of the mDCs allostimulatory activity, by assessing the lymphocyte proliferative response of PBLs, CD4+ T cells, and CD8+ T cells in allologeneic MLCs, showed that lymphocyte proliferation induced by mDCs was significantly higher than that induced by mDCsICOS in all lymphocyte preparations (Fig. 2). To assess whether differences in cytokine secretion displayed by mDCs and mDCsICOS paralleled differences in their ability to polarize the Th cell response, we evaluated secretion of IFN-γ, IL-2, IL-4, IL-10, IL-17A, IL-17F, IL-21, IL-22, and CCL20 in the supernatants from these mDCs. Compared with mDC-driven MLCs, MLCs driven by mDCsICOS displayed significant upmodulation of IL-17A and IL-17F, and downmodulation of IL-2 in PBLs and CD4+ T cells (Fig. 3). The effects induced by ICOS-Fc were not ascribable to binding of the Cγ1 portion of ICOS-Fc to DCs FcγRs, because they were also displayed by mDCs matured in the presence of ICOS-msFc, but not by those matured in the presence of F119FICOS-Fc (Fig. 3). By contrast, mDCs and mDCsICOS did not differ in their capacity to induce secretion of IFN-γ, IL-10, and CCL20 in all lymphocyte populations and IL-2 in CD8+ T cells (Supplementary Table I). IL-4, IL-21, and IL-22 were undetectable in every cell population and in all experimental conditions (data not shown).

Similar experiments performed using iDCs and ICOSICOS detected minimal MLC proliferation and cytokine secretion with no substantial differences between iDCs and ICOSICOS (data not shown).

In the MLCs, the ICOS-Fc effects were not due to decreased interaction between ICOS on T cells and B7h on DCs as a consequence of B7h downmodulation or blocking by the ICOS-Fc used to prepare mDCsICOS. Staining with anti-B7h mAb showed, in fact, similar expression levels in mDCs and mDCsICOS and staining of these cells with anti-human Ig Abs did not disclose any residual ICOS-Fc bound on the cell surface (data not shown).

Moreover, surface expression of ICOS was not different in T cells activated in the different MLC settings (data not shown). Hence, similar expression levels in mDCs and mDCsICOS and staining of these cells with anti-human Ig Abs did not disclose any residual ICOS-Fc bound on the cell surface (data not shown).

**Effect of B7h stimulation on Ag presentation**

iDCs were pulsed with KLH, matured with LPS in the presence and absence of ICOS-Fc, and then used as stimulators of autologous T cells, to assess whether B7h triggering modulates DCs Ag presentation.

**FIGURE 2.** Alloreactive proliferation induced in different lymphocyte populations by DCs treated with different stimuli, mDCs were prepared in the presence and absence of either ICOS-Fc, ICOS-msFc, or F119FICOS-Fc, and used as stimulators against allogeneic PBLs or purified CD4+ or CD8+ T cells at a stimulator/responder ratio of 1:20 for 5 d. [3H]thymidine (0.5 Ci/well) was added in the final 6 h of culture, and radioactivity uptake was measured by a beta counter; data are expressed as median and IQR of the results from six experiments performed in triplicate and marked with distinct symbols. *p < 0.05 versus mDCs.
presentation (45). After 7 d of primary culture, T cells were rechallenged with the same stimulators in the presence and absence of MHC-I or MHC-II blocking Abs; secretion of IFN-γ was then assessed by ELISpot to detect the MHC-II- and MHC-I-restricted response, respectively, because IFN-γ secretion can efficiently detect activation of both CD4+ and CD8+ T cells. The results showed that mDCsICOS induced higher proportions of IFN-γ-secreting, MHC-I-restricted cells than mDCs, whereas both stimulators elicited similar responses in the MHC-II-restricted population (Fig. 4A). Moreover, mDCsICOS displayed a similar stimulatory activity when matured in the presence of either ICOS-Fc or ICOS-msFc, whereas those matured in the presence of F119S ICOS-Fc displayed a similar activity as plain mDCs. By contrast, mDCs matured in the presence of CTLA-4–Fc displayed lower stimulatory activity in both MHC-I- and MHC-II-restricted T cells (Fig. 4A), which is in line with the notion that CTLA-4–Fc induces suppressive functions in DCs (6). To confirm these results, in a distinct set of experiments, we purified CD4+ and CD8+ cells from the 7-d primary culture, rechallenged them with the same stimulators, and analyzed them for IFN-γ secretion by ELISpot. The results obtained with the different ICOS-Fc variants on purified CD4+ and CD8+ cells strictly paralleled those obtained with whole T cells in the presence of anti–MHC-I and –MHC-II Abs, respectively. The inhibitory effect of CTLA-4–Fc also was still detectable, but it was significant in CD4+ cells only (Fig. 4B). Similar experiments performed in iDCs showed that iDCs and iDCsICOS displayed a similar low capacity to induce IFN-γ-secreting cells in both MHC-I- and MHC-II-restricted T cells (data not shown).

The increased ability of mDCsICOS to activate MHC-I-restricted T cells might be ascribed to potentiation of their capacity to cross-present endocytosed Ags in MHC-I or to increase of their intrinsic costimulatory activity. To distinguish between these possibilities, mDCs and mDCsICOS obtained from HLA-A2+ donors were

**FIGURE 3.** Cytokine secretion induced in different lymphocyte population by DCs treated with different stimuli. mDCs were prepared in the presence and absence of either ICOS-Fc, ICOS-msFc, or F119S ICOS-Fc, and used as stimulators as described in Fig. 2. Cytokine secretion was assessed in the supernatants by ELISA after 5 d of culture; data are expressed as median and IQR of results from six experiments performed in triplicate and marked with distinct symbols. *p < 0.05 versus mDCs.
pulsed with the influenza virus matrix peptide Flu-MA58–66 after their maturation in order directly to load the peptide on surface HLA-A2 molecules, skipping the Ag processing step, and then used to activate autologous T cells. ELISPOT analysis of IFN-γ–secreting cells showed similar proportions of positive cells using mDCs, mDCsICOS, or those matured in the presence of either ICOS-Fc, ICOS-msFc, F119SICOS-Fc, or CTLA-4–Fc, or F119SICOS-Fc, or CTLA-4–Fc (Fig. 4C). To confirm these results, we repeated the experiments using CD8+ T cell clones specific for Flu-MA58–66 presented on HLA-A2+, as responder cells. Also in this case, ELISPOT analysis did not detect substantial differences using the different stimulators (Fig. 4C). These data indicated that the increase of mDCsICOS ability to activate MHC-I–restricted T cells might be ascribed to promotion of Ag cross-presentation and not to an increase in their costimulatory activity toward CD8+ T cells.

**Effect of B7h stimulation on DC adhesiveness and migration**

In a previous work, we showed that ICOS-Fc inhibited adhesion of several tumor cell lines to HUVECs by triggering B7h expressed on either HUVECs or the tumor cell lines. To assess the effect of B7h triggering on iDC and mDC adhesion to ECs, we performed adhesion assays using both HUVECs and HDLECs. Either DCs or the EC lines were pretreated or not with ICOS-Fc for 30 min, washed, and incubated together in the adhesion assay; as a control, cells were treated with F119SICOS-Fc or CTLA-4–Fc. The results showed that pretreatment with ICOS-Fc significantly inhibited adhesion of both iDCs and mDCs to both HUVECs and HDLECs by ~50%. Inhibition was similar when B7h was triggered on either ECs or DCs, or both cell types. By contrast, pretreatment with F119SICOS-Fc or CTLA-4–Fc had no effect (Fig. 5).

Because adhesive mechanisms are also involved in DC migration, we used a Boyden chamber migration assay to investigate the effect of B7h triggering on spontaneous migration of iDCs and mDCs, migration of iDCs driven by mMLP or CCL5, and migration of mDCs driven by CCL19 or CCL21. iDCs or mDCs were seeded in the upper chamber of a Boyden chamber in the absence and presence of either ICOS-Fc, F119SICOS-Fc, or CTLA-4–Fc, and left to migrate toward the lower chamber loaded with medium.
in the absence or presence of the earlier indicated chemotactic agents. The results showed that iDCs and mDCs displayed a spontaneous migration that was increased by each chemoattractant and, in all conditions, substantially inhibited by ICOS-Fc, but not by F119SICOS-Fc and CTLA-4–Fc (Fig. 6).

ICOS-Fc alters β-Pix–mediated signaling

β-Pix is an Rac-1 activator involved in cell migration (46). Its downmodulation inhibits cell spreading, lamellipodial formation, and integrin-induced increase of Rac1 activity. To evaluate whether treatment with ICOS-Fc influences β-Pix expression, we treated iDCs and mDCs with ICOS-Fc or F119SICOS-Fc for 30 min and then analyzed β-Pix expression by Western blot. Results showed that treatment with ICOS-Fc decreased expression of β-Pix in both iDCs and mDCs, whereas no effect was exerted by F119SICOS-Fc (Fig. 7).

Discussion

This work showed that reverse signaling mediated by B7h modulated the DC response to LPS by influencing cytokine secretion and the ability to drive T cell activation and differentiation. Moreover, it modulated DC adhesiveness and migration.

Analysis of surface marker expression, endocytic activity, and cytokine secretion showed that B7h triggering per se was unable to induce DC maturation, whereas, as expected, this was induced by LPS with downregulation of endocytic activity and upregulation of B7.1, B7.2, CD83, and MHC expression, allostimulatory activity, and secretion of TNF-α, IL-6, IL-12, IL-10, and IL-23. Treatment with either ICOS-Fc or CTLA-4–Fc modulated this LPS-induced cytokine secretion with increase of IL-10 and decrease of TNF-α and IL-6, whereas IL-23 secretion was increased by ICOS-Fc only.

IL-1β and IL-6 are involved in differentiation of proinflammatory Th17 cells characterized by secretion of IL-17, IL-21, IL-22, and IL-26, whereas IL-23 is involved in their expansion (30, 47, 48). Therefore, B7h triggering in DCs may promote expansion of differentiated Th17 cells through the increased IL-23 secretion, which was supported by the finding that mDCs stimulated higher secretion of IL-17A and IL-17F than did plain mDCs in MLC assays. By contrast, they did not influence secretion of IL-21 and IL-22, secreted by subsets of Th17 cells (49, 50). Therefore, B7h triggering seemed to modulate DCs maturation by increasing their ability to expand Th17 cells, which was intriguing in light of data showing that the triggering of ICOS on T cells supports differentiation of Th17 cells in the presence of appropriate cytokine milieu (32). Therefore, the ICOS–B7h interaction between DCs and T cells may trigger bidirectional signals that bi-directionally cooperate to induce and expand Th17 cells. In the T cell activation step, ICOS triggering promotes differentiation of Th17 cells; in the DC maturation step, B7h triggering promotes secretion of IL-23, supporting Th17 cell expansion.

A further effect mediated by B7h triggering was potentiation of mDCs ability to activate the T cell response to endocytosed Ags in the context of MHC-I involving CD8+ T cells, but not in the context of MHC-II involving CD4+ T cells. This seemed to be due to increased cross-presentation of the Ag and not to increased expression of either MHC-I or costimulatory molecules involved in activation of CD8+ T cells. The effect of B7h triggering, in fact, required Ag processing because it was detectable when KLH was loaded on maturing DCs, but not when the Flu-MA58–66 peptide was loaded on already matured DCs. Specificity was shown by the ineffectiveness of F119SICOS-Fc and by the different effect exerted by CTLA-4–Fc, which inhibited mDCs ability to activate both the MHC-I- and MHC-II–restricted responses to KLH; this is in line with reports showing that CTLA-4–Fc induces DCs to produce the suppressive molecule IDO (6). Incomplete understanding of the molecular machinery of cross-presentation makes difficult to conjecture how it may be supported by B7h triggering. However,
it is noteworthy that the Th17-switch induced by B7h-stimulated DCs might further support the cross-presentation induced by B7h stimulation because IL-17 has been shown to promote cross-presentation in mice (51).

These results are quite different from those reported in regard to mouse bone marrow–derived DCs by Tang et al. (35) showing that, in the absence of other stimuli, B7h triggering induced partial maturation of iDCs. This is mediated by a p38 MAPK-dependent signal with selective augmentation of IL-6 secretion; a similar effect was detected by Orabona et al. (5) by using CD28-Fc to trigger B7.1 and B7.2. Moreover, in these murine DCs, B7h triggering increased expression of CD80, CD83, CD86, and MHC-II, phagocytosis, and Ag presentation to CD4+ Th cells, and supported their polarization to Th1 cells (35). However, the differences between these data on mice and our results on human DCs are not surprising in light of the several different functions that ICOS displays in the two species (25–31). Finally, B7h triggering substantially inhibited adhesion of both iDCs and mDCs to vascular and lymphoid ECs, and their spontaneous and chemokine-driven migration. Again, the effect was specific because it was not induced by F119SICOS-Fc and CTLA-4–Fc. Apparently, the effect was not due to modulation of the expression of adhesion molecules or chemokine receptors, as detected by surface immunofluorescence of CCR1, CCR7, ICAM-1, ICAM-2, VCAM-1, mucosal addressin cell adhesion molecule, E-selectin (CD62E), CD62P, CD15s (Sialyl Lewis X), Sialyl Lewis A, CD31, CD34, CD40, and CD44v (data not shown).

By contrast, ICOS-Fc downregulated expression of β-Pix, a Rac-1 activator recruited by activated integrins and required for rapid nascent adhesion turnover. Downregulation of β-Pix expression prevents cell spreading, lamellipodial formation, and increase of Rac1 activity (44, 52, 53). By contrast, in DCs, we did not detect any effect in ERK phosphorylation that we have previously shown to be downmodulated by ICOS-Fc in ECs (34), and similar negative results were obtained on JNK, p38, AKT, STAT1, and STAT3 (data not shown).

DCs are the most potent APC of the immune system involved in the initiation of the acquired immune response, and they are the
only type of APC that can activate naive T cells and initiate the immune response (36, 43, 54). Key features of this activity are the abilities of iDCs to migrate to the sites of inflammation in response to inflammatory chemokines, to endotype Ags, and to mature to mDCs moving to the T cell areas of secondary lymphoid organs, where they present the endocytosed Ags to both MHC-I- and MHC-II-restricted naive T cells, and initiate the immune response. Most iDCs reside within the tissues and do not recirculate, but some of them constitutively traffic to lymph nodes via the afferent lymph and play a role in the induction of peripheral tolerance (36, 54). Infection and inflammation dramatically increase DC migration as the result of maturation events that increase DC motility and responsiveness to lymph tropic chemottractants (36). In this process, mDCs downregulate chemokine receptors, such as CCR1, CCR2, CCR5, and CXCRI, required for proinflammatory chemotaxis in the tissues, and upregulate those, such as CCR7, required for migration to the lymph nodes. Their entry in the afferent lymphatics involves ICAM-1 and VCAM-1, and several other adhesion molecules only partly shared with those involved in the leukocyte interaction with vascular ECs (36).

In this scenario, our data suggest that B7θ triggering may play a key role in modulating DC function. Its ability to block iDC migrations would be crucial for arresting these cells in the inflamed tissues where they can load the Ags in both MHC-II and MHC-I, favored by the effect of B7θ triggering on cross-presentation. Then its ability to block mDC migration would be crucial to arrest these cells in the secondary lymphoid tissues where they can activate both CD4+ and CD8+ T cells and, possibly, support differentiation of Th17 cells. In this context, the inhibitory effect exerted by B7θ signaling on DC adhesion to ECs might be important to inhibit the transeendothelial migration of these cells and favor their permanence in the tissues.

ICOS-Fc has been used to treat several pathological conditions in mice with the aim of blocking the ICOS–B7θ interaction. However, this and previous reports suggest that it may also act as an agonist of B7θ signaling, and that these multifaceted effects may account for the heterogeneous results obtained in different disease models and using different types of ICOS antagonists. Therefore, before proposing therapeutic uses of ICOS-Fc, more work is needed to depict its global effect both in vitro and in vivo.

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Disclosures

The authors have no financial conflicts of interest.

References


**Supplementary Table I: Cytokine secretion induced in different lymphocyte population by allogeneic DCs treated with different stimuli.**

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*Data are expressed as pg/ml and are medians [IQR]. All differences were not significant.*