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Sex Differences in Plasmacytoid Dendritic Cell Levels of IRF5 Drive Higher IFN- α Production in Women

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Increased IFN- α production contributes to the pathogenesis of infectious and autoimmune diseases. Plasmacytoid dendritic cells (pDCs) from females produce more IFN- α upon TLR7 stimulation than pDCs from males, yet the mechanisms underlying this difference remain unclear. In this article, we show that basal levels of IFN regulatory factor (IRF) 5 in pDCs were significantly higher in females compared with males and positively correlated with the percentage of IFN- α -secreting pDCs. Delivery of recombinant IRF5 protein into human primary pDCs increased TLR7-mediated IFN- α secretion. In mice, genetic ablation of the estrogen receptor 1 (*Esr1*) gene in the hematopoietic compartment or DC lineage reduced *Irf5* mRNA expression in pDCs and IFN- α production. *IRF5* mRNA levels furthermore correlated with *ESR1* mRNA levels in human pDCs, consistent with *IRF5* regulation at the transcriptional level by *ESR1*. Taken together, these data demonstrate a critical mechanism by which sex differences in basal pDC IRF5 expression lead to higher IFN- α production upon TLR7 stimulation in females and provide novel targets for the modulation of immune responses and inflammation. *The Journal of Immunology*, 2015, 195: 5327–5336.

Differences in immune responses between females and males, including responsiveness to vaccination (1), have been reported (2–5) but often remain overlooked in immunological studies and particularly in human viral infections (6), because most studies have been carried out in rodents (7–10). As a general rule, females exhibit more robust humoral and cell-mediated immune responses to antigenic challenges compared with males (1, 11–13). Furthermore, females are also often more prone to immune-related pathology and autoimmunity (14). The heightened inflammatory immune responses observed in females have been suggested to contribute to sex differences in the clinical manifestations, immune responses and outcome of viral diseases,

including influenza A virus (15), hantavirus (16), hepatitis C virus (17, 18), and HIV-1 (19, 20).

The pathways underlying these sex differences in the manifestations of viral and autoimmune diseases are not well understood, but increasing data suggest a critical role of the TLR7 pathway and resulting type I IFN production in the outcome of these diseases (21–23). Our group and others have previously shown that plasmacytoid dendritic cells (pDCs) derived from females produced significantly more IFN- α in response to TLR7 ligands than pDCs derived from males, resulting in stronger immune activation (24, 25), and that sex hormones can regulate the IFN- α response to TLR7 stimulation (26, 27). However, the

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Abbreviations used in this article: BM, bone marrow; cDC, conventional dendritic cell; DOTAP, 1,2-dioleoyloxy-3-trimethylammonium-propane; ER α , estrogen receptor α ; *Esr1*, estrogen receptor 1; IRF, IFN regulatory factor; MFI, mean fluorescence intensity; pDC, plasmacytoid dendritic cell; PFA, paraformaldehyde; WT, wild-type.

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mechanisms underlying this sex difference in TLR7-induced IFN- α production by pDCs remain unknown.

IFN- α induction is regulated primarily at the transcriptional level by the IFN regulatory factors (IRF) family (28–30). In response to stimulation, these transcription factors are phosphorylated on serine residues, a modification that stimulates protein dimerization, nuclear translocation, and interaction with transcriptional coactivators (31, 32). pDCs constitutively express high levels of IRF5 and IRF7 (33–35). TLR7 activation of pDCs leads to the activation and phosphorylation of both IRF5 and IRF7 (31, 36, 37). IRF7 is widely recognized as the “master regulator” of type I IFN production (32), whereas IRF5 has been shown to be a central mediator of TLR7 signaling (33, 38). In addition, IRF5 polymorphisms have been associated with multiple autoimmune diseases, and in particular systemic lupus erythematosus and rheumatoid arthritis (39–42), two autoimmune diseases characterized by overproduction of type I IFN and by significant sex differences in prevalence. Autoimmune-risk haplotypes exhibit higher IRF5 levels (43) and are associated with increased levels of IFN- α (44–46), suggesting that expression of IRF5 contributes to the development of autoimmune diseases (47).

In this study, we investigated the role of IRF5 and IRF7 for the difference in IFN- α production observed between females and males. Our results demonstrate that IRF5 levels are regulated by the estrogen receptor α (ER α) in mice, and that sex difference in IRF5 expression in human pDCs can lead to higher IFN- α production in females compared with males after TLR7 stimulation, providing potential novel targets for the modulation of inflammation and immune responses in both chronic viral and autoimmune diseases.

Materials and Methods

Study subjects and samples

Human samples were collected from individuals recruited and enrolled at Massachusetts General Hospital, and all subjects gave written, informed consent for participation in these studies. The study was approved by the Partners Human Research Committee. Characteristics of the patient cohort are available in Supplemental Table I. No significant differences in age ($p = 0.18$, two-tailed Mann–Whitney U test) or ethnicity were noticed ($p = 0.1$, Fischer Exact test) between the 53 females and 37 males included in this study. When available ($n = 26$), information on the use of oral contraceptives containing sex hormones was collected. The female study subjects included 18.9% ($n = 10$) of postmenopausal or surgically sterile females; 50% ($n = 8$) and 18% ($n = 3$) of premenopausal females reported using oral contraceptives and using an intrauterine device, respectively. Subgroups were used in the different analyses performed, with some donors being tested across multiple assays. Blood was collected in lithium heparin tubes, and PBMCs were separated from whole blood by Ficoll-Histopaque density centrifugation (Sigma-Aldrich, St. Louis, MO). Cells were resuspended in R-10 [RPMI 1640 (Sigma-Aldrich) containing 10% heat-inactivated FBS (Sigma-Aldrich), 2500 U/ml penicillin, 2500 μ g/ml streptomycin, 100 mM L-glutamine (Corning, Lowell, MA)] and counted. Blood was processed within 5 h after venipuncture to prevent the loss of pDC responsiveness to TLR ligands (48).

Mice

Mice selectively lacking ER α in the hematopoietic compartment or in the DC lineage were generated by crossing B6 mice carrying an estrogen receptor 1 (*Esr1*) gene in which exon 2 was flanked by loxP sites (ER $\alpha^{n/n}$) with B6 mice expressing the Cre recombinase under the control of the Tie2 promoter-enhancer (Tie2-ER α^{KO}) or the CD11c promoter (CD11c-ER α^{KO}) as described elsewhere (49). Littermate wild-type (WT) mice were used as controls. Mice were bred and maintained in a specific pathogen-free animal facility. Eight- to 12-wk-old female mice were used in all experiments. The INSERM U1043 Institutional Review Board for animal experimentation approved protocols.

Measurement of single-cell cytokine production by flow cytometry

Intracellular cytokine staining assays were carried out as previously described (25). In brief, freshly isolated PBMCs were resuspended in R-10 at a con-

centration of 1.5 million cells/ml, and 1 ml PBMCs was stimulated in FACS tubes with 1 μ g/ml CL097, a synthetic TLR7 ligand (imidazoquinoline; Invivogen, San Diego, CA). A total of 5 μ g/ml brefeldin A (Sigma-Aldrich) was added to each tube immediately after addition of the stimulant to inhibit cellular cytokine release. Unstimulated cells with 5 μ g/ml brefeldin A added served as a negative control. Intracellular cytokine content of pDCs was determined after 20 h of stimulation as previously described (50). PBMCs were stained for surface markers using anti-CD3 Alexa Fluor 700, anti-CD19 Alexa Fluor 700, anti-CD56 Alexa Fluor 700, anti-CD11c PE, anti-CD14 allophycocyanin-Cy7, anti-HLA-DR Pacific blue, and anti-CD123 PE-Cy5 (all from BD Biosciences, San Jose, CA). pDCs were defined as CD3^{neg}CD19^{neg}CD56^{neg}HLA-DR^{pos}CD14^{neg}CD11c^{neg}CD123^{bright} cells. Cells were fixed and permeabilized using Fix&Perm Medium A and B (Invitrogen, Carlsbad, CA) and stained intracellularly with anti-IFN- α FITC (PBL IFN Source, Piscataway, NJ), anti-IL-12 allophycocyanin, and anti-TNF- α PE-Cy7 (BD Biosciences). Flow cytometry data were acquired within 2 h of staining on a BD Biosciences LSRII equipped with four lasers. Spectral overlap was corrected by appropriate compensation, and rainbow beads were used to maintain the consistency of the fluorescence intensity between experiments. The frequency of cytokine-producing pDCs was determined by subsequent analysis using FlowJo software (version 8.5.2; Tree Star, Ashland, OR). Unstimulated cells were used to define background cytokine production level and subtracted from the frequency in stimulated samples.

Measurement of ex vivo protein levels of transcription factors by flow cytometry

Freshly isolated PBMCs were fixed by adding cold 4% paraformaldehyde (PFA) directly into the culture medium to obtain a final concentration of 2% PFA. Cells were incubated in fixative for 30 min at 37°C, then washed and pelleted. Cells were permeabilized by vortexing to resuspend in 500 μ l ice-cold methanol and incubated for 10 min at –20°C, and subsequently washed twice. For IRF5 staining, fixed and permeabilized cells were incubated for 10 min at room temperature and in the dark with the unconjugated rabbit monoclonal IRF5 Ab (Abcam, Cambridge, MA), washed, and then stained with a secondary goat anti-rabbit Alexa Fluor 700 Ab (Invitrogen). Unconjugated rabbit IgG (Cell Signaling Technology, Danvers, MA) was used as isotype control. Cells were stained for surface markers for 30 min at room temperature as described earlier. For IRF7 staining, anti-IRF7 Alexa Fluor 488 (BD Biosciences) was also added to the surface stain mix. Finally, the cells were washed, pelleted, and resuspended in 100 μ l PBS containing 2% heat-inactivated FBS. All washes were performed with PBS containing 2% heat-inactivated FBS at 4°C. The IFN- α secretion assay (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) was used in combination with this protocol as per manufacturer's instructions. Samples were acquired on the BD Biosciences LSRII within 2 h of staining. The mean fluorescence intensities (MFIs) of IRF5 and IRF7 in pDCs, CD3⁺ T cells, and monocytes/conventional dendritic cells (cDCs) and the frequencies of IFN- α -secreting pDCs were determined by subsequent analysis using FlowJo software.

Subcellular quantification of IRF5 protein levels in pDCs using the TissueFAXS slide scanning system

Two million fresh PBMCs were seeded in 50 μ l R-10 on poly-D-lysine-coated plates and simultaneously stimulated with 1 μ g/ml CL097 for 2 h. Cells were then fixed with 4% PFA for 20 min at room temperature and permeabilized with ice-cold methanol (10 min at –20°C). Unconjugated anti-IRF5 (Abcam) or rabbit IgG (Cell Signaling Technology) was added and the plates were incubated at 4°C overnight. The slides were subsequently washed in PBS supplemented with 2% normal goat serum and incubated in goat anti-rabbit IgG Alexa Fluor 546 (Invitrogen) for 30 min at room temperature. Cells were successively stained at room temperature with CD123 allophycocyanin (BD Biosciences) for 1 h, goat anti-mouse IgG2a Alexa Fluor 647 (Invitrogen) for 30 min, HLA-DR Alexa Fluor 488 for 1 h (Exbio, Vestec, Czech Republic), goat anti-mouse IgG1 Alexa Fluor 488 (Invitrogen) for 30 min, with three washes in PBS supplemented with 2% normal goat serum between each stain. All slides were mounted in Prolong Gold Antifade reagent with DAPI (Invitrogen). The sample slides were scanned using the TissueFAXS (TissueGnostics GmbH, Vienna, Austria) slide scanning system based on a Zeiss Axio Imager Z2 upright epifluorescence microscope. Images were captured using a Zeiss EC Plan-Neofluar 100 \times 1.3NA objective in combination with a PCO (Kelheim, Germany) monochrome 12-bit CCD camera. This slide scanning system uses a Maerzhaeuser motorized scanning stage with eight-slide holder to permit scanning and stitching together of many fields of view into one image. In this way, all of the plated cells could be scanned on each coverslip and then the fluorescence intensity of the different markers evaluated on a per-cell basis

using TissueQuest imaging analysis software (TissueGnostics GmbH). pDCs were identified as HLA-DR⁺CD123⁺ cells. Each fluorescence channel was thresholded to visually segment the cells based on average per-cell fluorescence intensity. Cytoplasm and nuclear IRF5 fluorescence was then separately determined using a cytoplasm mask for IRF5, a nuclear mask for IRF5, and a whole-cell mask for IRF5. For each sample, a minimum of 150 pDCs was imaged to determine the mean intensity of IRF5.

IRF5 recombinant protein delivery using a vector-free microfluidic platform

pDCs were enriched from PBMCs using the pDC Enrichment Kit (Stemcell, Vancouver, Canada) following manufacturer's instructions. Cells were resuspended in R-10 at 1000 cells/ μ l and were mixed with Cascade Blue-labeled 3-kDa dextran molecules, for control of delivery, and with either 0.03–0.06 μ g/ μ l IRF5 recombinant protein (Abcam or Origene, Rockville, MD) or 0.05–0.1 μ g/ μ l of the control TUBA1A recombinant protein (Abcam), and subsequently placed in the device's inlet reservoir. Delivery was performed using a vector-free microfluidic platform as previously described (51, 52) and illustrated in Supplemental Fig. 1A. In brief, cells were mechanically deformed while passing through the microfluidic device (SQZ Biotechnologies, USA) at a pressure of 80 or 120 psi, resulting in the transient formation of holes in the cell membrane allowing content from the surrounding buffer to diffuse into the cytosol. Cells were incubated at room temperature in the delivery solution for 5 min after treatment to ensure closure of membrane holes before being subjected to any further treatment, as previously described (52). Delivery efficiency was assessed using FITC-labeled, 70-kDa dextran probes and/or Cascade blue-labeled, 3-kDa dextran molecules mimicking protein and small interfering RNA deliveries, respectively. Appropriate controls were included to correct for reduced IFN- α -producing capacity of pDCs subjected to mechanical deformation. Delivery of TUBA1A protein, which is not involved in the IFN- α production pathway, was used as control for determining any nonspecific effect on IFN- α production. IRF5 levels were subsequently measured by flow cytometry as described earlier. Cells were then stimulated for 2 h with 1 μ g/ml CL097. Supernatants were collected and run on the Milliplex Human 29 cytokine/chemokine magnetic bead panel kit (Millipore, Billerica, MA). Viability was assessed by using the Aqua LIVE/DEAD staining (Invitrogen) as per manufacturer's instructions. IFN- α secretion was measured using Miltenyi's IFN- α secretion assay following manufacturer's instructions.

Intracellular cytokine staining of murine pDCs

Murine bone marrow (BM) cell suspensions were activated with a preparation of oligonucleotides PolyU (Sigma-Aldrich) with 1,2-dioleoyloxy-3-trimethylammonium-propane (DOTAP) [8 μ l of a cationic liposome preparation (DOTAP; Roche) mixed with 1 μ g PolyU in 150 μ l RPMI, in a polystyrene tube]. BM cells were stimulated with PolyU-DOTAP preparation for 4 h, and 5 μ g/ml brefeldin A (eBioscience) was added for the last 2 h of culture. Nonspecific staining was blocked with 5 μ g/ml anti-CD16/CD32 (2.4G2; American Type Culture Collection). BM cell suspensions were then stained with PE-Cy7-labeled anti-CD11c (N418) and allophycocyanin-labeled mouse plasmacytoid dendritic cell Ag-1 (all from eBioscience). Intracellular cytokine staining was performed with mixed FITC-labeled IFN- α / β -specific Abs (RMMA-1/RMMB-1; PBL). Unstimulated cells and isotypes were used as control staining. Data were acquired on a Fortessa (BD Biosciences).

Quantification of IRF5 protein expression in mouse splenic B cells

B cells were individually purified from mouse spleens by positive selection using anti-CD19 beads (Miltenyi). Cells were lysed in lithium dodecyl sulfate sample buffer (Invitrogen) and analyzed by immunoblotting. Membranes were probed with anti-IRF5 (polyclonal rabbit IgG; Cell Signaling 4950) or anti- β -actin (monoclonal mouse IgG1; Sigma A1978) Abs, followed by incubation with appropriate HRP-conjugated secondary Abs. Densitometric analysis was performed using Image Lab software v5.0 (Bio-Rad).

Quantification of IRF5 and IRF7 mRNA expression in mouse pDCs

BM cell suspensions were stained with allophycocyanin-labeled mouse plasmacytoid dendritic cell Ag-1 and PE-Cy7-labeled anti-CD11c (all from eBioscience) for 30 min at 4°C, and double-positive cells were sorted using a FACSAria (BD Biosciences). RNA from purified pDCs was extracted using the NucleoSpin RNA XS and treated with DNase I following manufacturer's instructions (Macherey-Nagel). RNA samples were retrotranscribed into

cDNA using oligo-dT, random primers, and the SuperScript III Reverse Transcriptase (Life Technologies). Quantitative PCRs were performed using *Ir5* and *Ir7* QuantiTect Primer Assays with SYBR green PCR Mastermix (QIAGEN). Gene transcripts were normalized to *Hprt* gene abundance, and relative mRNA levels were calculated by the expression $2^{-\Delta\Delta C_t}$.

In situ IRF5 mRNA expression assay by flow cytometry

Five million PBMCs were pelleted and surface stained on ice for 30 min. Cells were subjected to the QuantiGene FlowRNA assay (eBioscience, San Diego, CA) as per manufacturer's instructions with type6-B2M probe, type1-*ESR1* probe, and a customized ultrasensitive type4-*IRF5* probe (probes are all from eBioscience). To control for nonspecific probe interaction, we replaced type4-*IRF5* probe and type1-*Esr1* probe by type4-*TLR7* probe and type1-*TLR9* probe. The bacterial *DapB* probes were used as a control. To gain sensitivity, we increased target incubation time from 2 to 3 h. Similarly, preamplification and amplification incubation times were increased from 1.5 to 2 h. Samples were run in duplicates and acquired on the BD Biosciences Fortessa within 2 h of staining. The MFIs of *IRF5*, *ESR1*, and *B2M* probes were determined by subsequent analysis using FlowJo software. Values were excluded if the duplicates exhibit >20% difference.

Statistical analysis

Comparison between females and males was calculated using Wilcoxon rank tests (Mann–Whitney) or unpaired *t* tests. Comparison of IRF5 MFI between IFN- α -secreting pDCs and nonsecreting pDCs was calculated using the paired Wilcoxon rank tests. Linear regression was calculated using Spearman rank-based correlation. For IRF5 protein delivery experiments, we used Wilcoxon signed rank for comparison of the increase in the percentage of IFN- α secretion relative to the control therefore normalized to 1. Comparison between WT mice and *ER α* ^{KO} mice was calculated using the unpaired *t* tests.

Results

Sex differences in the IFN- α /TLR7 pathway in pDCs

We and others have previously reported that pDCs isolated from females produce markedly more IFN- α in response to TLR7 ligands than pDCs derived from males (24–26). These results were confirmed in this study by measuring the frequency of IFN- α -producing pDCs in a first group of 31 healthy individuals (17 females, 14 males) (Supplemental Table I). A significantly higher percentage of IFN- α -producing pDCs after 20 h of stimulation with the synthetic TLR7/8 ligand CL097 was observed in females than in males ($p = 0.04$, two-tailed Mann–Whitney *U* test; Fig. 1A). Neither age nor ethnicity influenced IFN- α production by pDCs ($p = 0.1$, $r = 0.3$, Spearman rank-based correlation; $p = 1.0$, Fisher Exact test). The mean frequency of IFN- α -producing pDCs was 50.15% in females and 39.53% in males, in line with previous reports (25). In contrast, no sex difference was noticed in the percentage of TNF- α -producing pDCs ($p = 0.54$, two-tailed Mann–Whitney *U* test; Fig. 1A).

Mechanisms underlying sex difference in IFN- α production remain to be elucidated. IRF7 and IRF5 are two crucial transcription factors activated upon TLR7 stimulation that modulate IFN- α production (33). Ex vivo levels of IRF5 and IRF7 in pDCs were measured subsequently by flow cytometry in a second group of healthy donors (Supplemental Table I). No sex difference was observed in the ex vivo levels of IRF7 in pDCs ($p = 0.64$, two-tailed Mann–Whitney *U* test; Fig. 1B). In contrast, pDCs derived from females contained 1.6 times more IRF5 than pDCs derived from age- and ethnicity-matched males, as measured by the MFI level of ex vivo IRF5 expression (females: $n = 29$, males: $n = 19$; $p = 0.02$, two-tailed Wilcoxon Rank test; Fig. 1D). IRF5 expression among pDCs appeared heterogeneous with some pDCs expressing no or very low levels of IRF5 as determined by the use of an isotype control (Fig. 1C). Notably, no difference in IRF5 protein levels was noticed between premenopausal females under hormonal birth control ($n = 8$) and those without hormonal birth control ($n = 11$). Ex vivo expression levels of IRF5 protein were also examined in CD3⁺

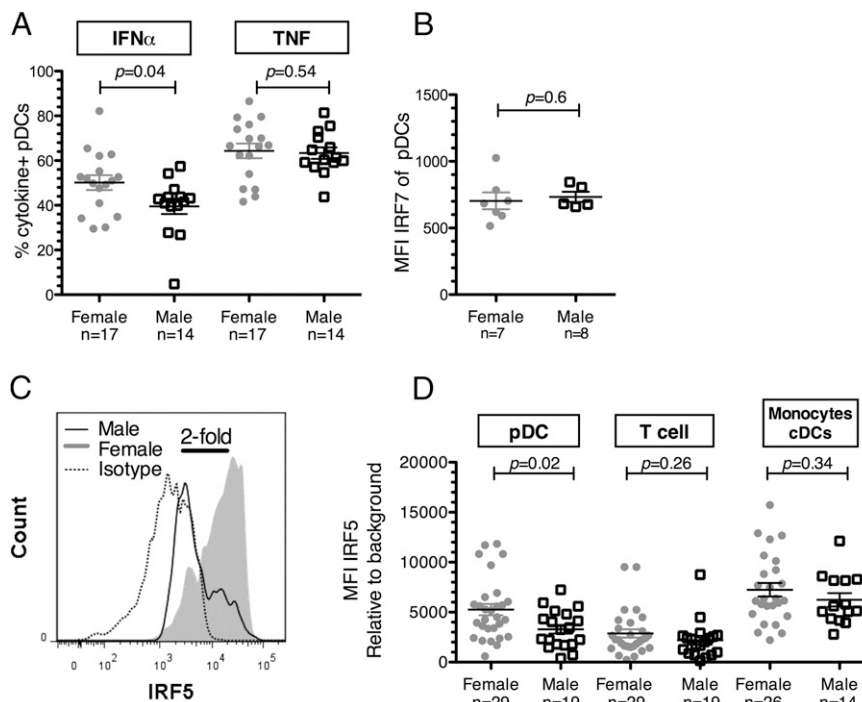


FIGURE 1. Sex differences in TLR7 signaling in pDCs. **(A)** The percentage of IFN- α -secreting pDCs was significantly higher in females ($n = 17$) than in males ($n = 14$), whereas the percentage of TNF- α -secreting pDCs did not differ between females and males after 20 h of stimulation with 1 μ g/ml CL097. Brefeldin A was added at 5 μ g/ml at time of stimulation to block cytokine secretion. **(B)** MFI of IRF7 was determined ex vivo in pDCs from females ($n = 7$) or age-matched male donors ($n = 5$) using FlowJo software. No difference between sex in IRF7 expression in pDCs was observed. **(C)** Flow cytometry histogram overlays show the MFI of the IRF5 in pDCs from a male representative of the male average (empty curve) and in pDCs from a female representative of the female average (filled gray curve). There was a 2-fold difference in the MFI from those two samples. Isotype control is represented with dotted curve. **(D)** MFI of IRF5 was determined ex vivo in pDCs, T cells, and monocytes/cDCs from females ($n = 29$ for pDCs and T cells, and $n = 26$ from monocytes/cDCs) or age-matched male donors ($n = 19$ for pDCs and T cells, and $n = 14$ from monocytes/cDCs) using FlowJo software. pDCs derived from females exhibited significantly higher IRF5 levels than pDCs derived from males. IRF5 levels in T cells did not differ between females and males. Comparison between sexes was calculated using the Wilcoxon rank tests. Error bars indicate the mean and SEM.

T cells and in monocytes/cDCs, in which the MFI of IRF5 was significantly lower and higher than in pDCs ($p < 0.0001$, Wilcoxon matched-pairs signed rank test), respectively. Ex vivo expression levels of IRF5 in CD3 $^{+}$ T cells and monocytes/cDCs did not significantly differ between females and males ($p = 0.26$ and $p = 0.34$, respectively, two-tailed Mann-Whitney U test; Fig. 1D), suggesting that the sex difference in ex vivo levels of IRF5 is cell type dependent. These results suggest a potential role of IRF5, but not IRF7, levels in the observed sex differences in IFN- α production.

Sex difference in basal IRF5 protein levels can influence pDC responses to TLR7 stimulation

To gain a better understanding of the biological consequences of the sex difference in basal protein levels of IRF5 in pDCs, we measured IRF5 subcellular localization after TLR7 stimulation using the TissueFAXS slide scanning system. PBMCs were stimulated for 2 h with CL097 before staining for IRF5. pDCs were identified among all of the plated cells as HLA-DR $^{+}$ CD123 $^{+}$ (Fig. 2A), and the levels of IRF5 in both the cytoplasm and the nucleus from all pDCs on the slide were subsequently quantified. The ratio of nuclear IRF5 to cytoplasmic IRF5 was calculated to determine the changes in IRF5 translocation to the nucleus. Higher ratios of IRF5 in the nucleus after stimulation were observed in females than in males ($p = 0.038$; unpaired t test; Fig. 2B). The total amount of IRF5 among pDCs did not vary between baseline and after 2 h of stimulation with CL097 ($p = 0.19$, paired Mann-Whitney U test). This suggests that the sex difference in IRF5 levels observed before stimulation might translate to higher nuclear IRF5 content after TLR7 stimulation.

The consequences of the sex difference in basal levels of IRF5 in pDCs on IFN- α production by pDCs were subsequently examined by measuring ex vivo IRF5 levels and the percentage of IFN- α -secreting pDCs by flow cytometry. Ex vivo IRF5 levels before stimulation positively correlated with the percentage of IFN- α -secreting pDCs after 2 h of stimulation with CL097 ($r = 0.8$, $p = 0.02$, Spearman rank-based correlation; Fig. 2C). Nevertheless, a subset of IFN- α -secreting pDCs expressed no or low IRF5 levels, suggesting that IRF5 may not be the sole factor involved in IFN- α production. Altogether, these data demonstrate a link between the basal quantity of IRF5 in pDCs and the production of IFN- α .

Delivery of exogenous IRF5 protein increases IFN- α secretion in response to TLR7 stimulation in pDCs

We further examined the direct impact of IRF5 protein levels on IFN- α production. Given that pDCs left in culture for >6 h have significantly reduced capabilities to produce IFN- α in response to TLR7 stimulation (48), techniques involving long incubation period such as small interfering RNA or transfection of vectors containing IRF5 cannot be applied. Thus, we used a technique recently described by Sharei and colleagues (51) for the direct delivery of IRF5 recombinant protein into human primary pDCs. A microfluidic device injects cells in narrow lanes inducing cell constriction, creating transient holes in plasma membranes, and enabling passive entry of molecules. Different microfluidic devices (described in Supplemental Table II) were tested as the size of the constriction and the number of constrictions were previously shown to influence delivery efficiency (51). The best delivery efficiency without significant loss of cell viability was

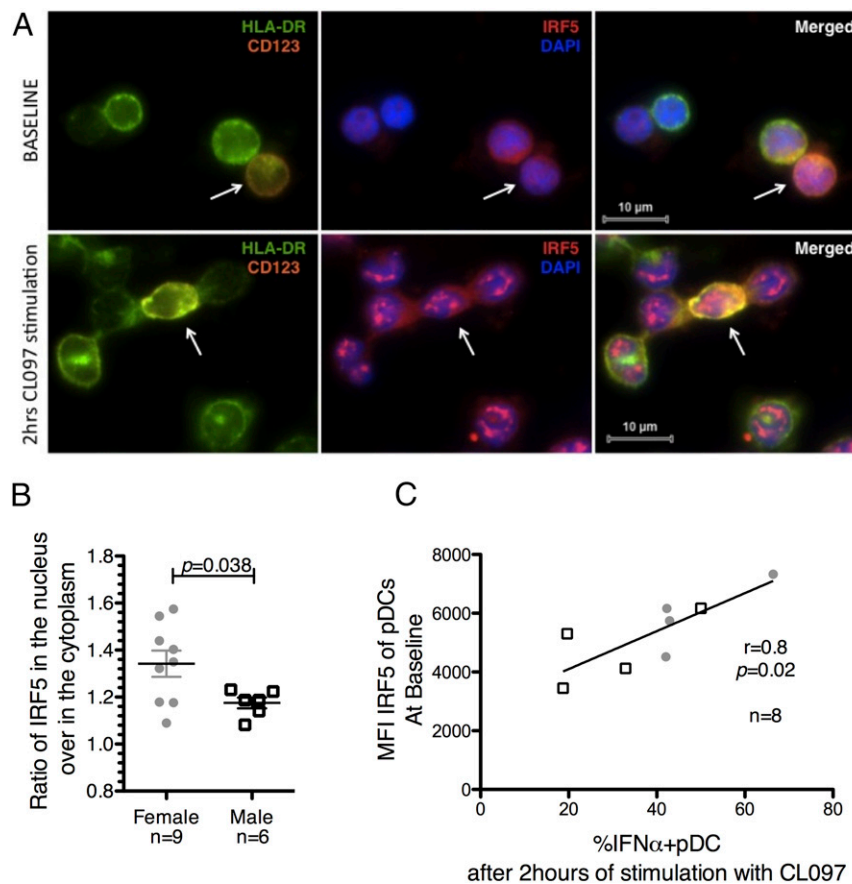


FIGURE 2. IRF5 in pDCs is activated upon TLR7 stimulation and associated with IFN- α secretion. (**A** and **B**) PBMCs were stimulated with CL097 at 1 μ g/ml for 2 h and then stained for IRF5–Alexa Fluor 546, HLA-DR–Alexa Fluor 488, and CD123–Alexa Fluor 647. The sample slides were scanned in Prolong Gold using the slide scanning system based on a Zeiss Axio Imager Z2 upright epifluorescence microscope. Images were captured using a Zeiss EC Plan-Neofluar 100 \times 1.3NA objective in combination with a PCO monochrome 12-bit CCD camera. pDCs were identified among all of the plated cells as HLA-DR⁺CD123⁺ cells. Quantification was performed using the TissueQuest analysis software. (**A**) Representative images at baseline and after 2 h of stimulation are shown with CD123 in orange, HLA-DR in green, DAPI staining of the nucleus in blue, and IRF5 in red. (**B**) The dot plot represents the ratio of nuclear IRF5 on cytoplasmic IRF5 in pDCs after 2 h of stimulation with CL097. Higher IRF5 ratio in the nucleus in females ($n = 9$) than in males ($n = 6$). Error bars indicate the mean and SEM. Comparison between sexes was calculated using unpaired t tests. (**C**) IRF5 and IFN- α expression were measured in pDCs at baseline and after 2 h of stimulation with CL097, respectively. A significant correlation between the basal levels of IRF5 in pDCs and the percentage of IFN- α -secreting pDCs after 2 h of stimulation with CL097 was observed. Linear regression was calculated in eight healthy donors (females: $n = 4$, close round shapes; males: $n = 4$, open squares) with Spearman rank-based correlation.

obtained using the 10-4 \times 5iS chip where 10 is the length of constriction in micrometers, 4 is the width of the constriction in micrometers, and 5 is the number of times the constriction is repeated through each channel (Supplemental Fig. 1C, 1D). pDCs subjected to this optimized delivery method exhibited decreased IFN- α secretion in response to TLR7 ligand compared with untreated pDCs, whereas no unspecific IFN- α production was induced in the absence of TLR7 ligand. The 10-4 \times 5iS device enabled efficient delivery of IRF5 protein into live primary pDCs (Fig. 3A, Supplemental Fig. 1B), allowing us to assess the impact of IRF5 protein delivery into primary pDCs on IFN- α production. Fig. 3B shows representative plots of IFN- α -secreting pDCs with or without IRF5 protein delivery. IRF5 delivery resulted in a significant increase (3-fold) in the percentage of IFN- α -secreting pDCs ($n = 7$, $p = 0.02$, Wilcoxon signed rank test; Fig. 3C). In contrast, delivery of TUBA1A protein, a protein irrelevant for the pDC TLR7/IFN- α pathway, did not lead to changes in IFN- α production (Fig. 3C), validating that the increase of IFN- α secretion was not due to unspecific stimulation by the protein delivery method.

Apart from its role in IFN- α production, IRF5 has been described to also promote transcription of proinflammatory cyto-

kines such as TNF- α upon TLR stimulation (38). Supernatants were therefore collected after 2 h of CL097 stimulation from control pDCs and pDCs in which IRF5 protein was overexpressed, and cytokines were measured using the Milliplex Human 29 cytokine/chemokine magnetic bead panel kit. Consistent with previous work (53), 2 h of CL097 stimulation did not induce the production of IL-2, IL-4, IL-5, IL-7, IL-10, IL-13, IL-15, IL-17, IFN- γ , MIG, and eotaxin, whereas TNF- α , IL-8, MIP-1 α , and MIP-1 β protein secretion were induced. pDCs in which IRF5 protein was overexpressed exhibited increased TNF- α , IL-8, MIP-1 α , and MIP-1 β production compared with control pDCs (Fig. 3D). Overall, we showed increased production of IFN- α and other inflammatory cytokines after delivery of exogenous IRF5 protein into primary human pDCs using a novel method of protein delivery, confirming the role of IRF5 in mediating TLR7 signaling and cytokine production in human pDCs (54).

Sex difference in IRF5 levels in pDCs is associated with ER α signaling

Previous reports have shown that estrogens can modulate IFN- α production by pDCs both in mice (26) and in humans (55). Studies have also shown higher *Irf5* mRNA levels in splenic B cells

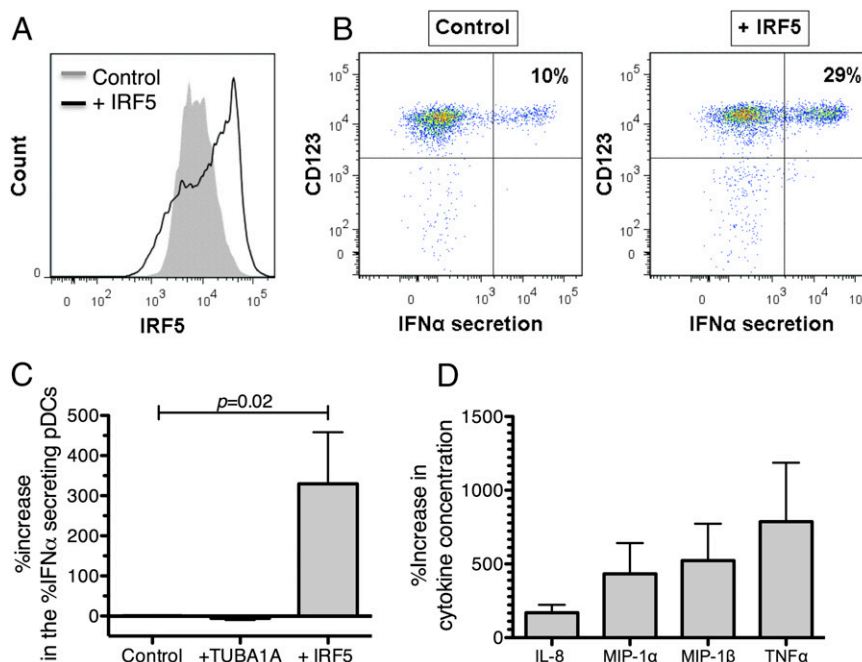


FIGURE 3. Exogenous IRF5 enhance IFN- α production after TLR7 stimulation. Enriched pDC population was subjected to the microfluidic device with or without (control) recombinant IRF5 protein in the surrounding milieu. IRF5 levels were measured at baseline. pDCs were then stimulated with CL097 for 2 h and IFN- α secretion was measured. **(A)** Flow cytometry histogram overlays show the mean intensities of the IRF5 in pDCs after IRF5 delivery (empty curve) and in the control (filled curve) after treatment with the microfluidic device. A 2-fold increase in the MFI of IRF5 in pDCs is observed upon IRF5 delivery. **(B)** Primary flow data of IFN- α secretion in pDCs after 2 h of TLR7 stimulation in the control sample and after IRF5 delivery. **(C)** The percentage of IFN- α -secreting pDCs increased specifically upon delivery of IRF5 protein. The percentage of IFN- α -secreting pDCs upon delivery of IRF5 was normalized to the control. In contrast, no significant changes occurred in the percentage of IFN- α -secreting pDCs upon delivery of TUBA1A, a protein irrelevant to the IFN- α pathway. Data for IRF5 delivery are representative of seven independent experiments. Wilcoxon signed rank for comparison of the percentage increase in the percentage of IFN- α secretion relative to the control therefore normalized to 1. **(D)** Supernatants were collected after 2 h of stimulation and run on the Milliplex Human 29 cytokine/chemokine magnetic bead panel kit (Millipore). Increase in the concentration of TNF- α , MIP-1 α , MIP-1 β , and IL-8 is observed upon delivery of IRF5 protein. Error bars indicate the mean and SEM.

derived from female than in age-matched male mice and from WT mice as compared with ER α knockout mice (56). We therefore investigated ER α -dependent regulation of IRF5 in female mice using tissue-specific ER α -deficient mice (26, 49). Before assessing levels of *Irf5* mRNA in pDCs, and in agreement with previous works (56), we observed a lower expression of IRF5 protein in splenic B cells from Tie2-ER α ^{KO} mice, lacking ER α in the hematopoietic compartment, as compared with ER α ^{flx/flx} WT mice ($p = 0.04$, unpaired t test; Fig. 4A, 4B) (56). We then sorted BM pDCs from ER α ^{flx/flx} WT, Tie2-ER α ^{KO}, and CD11c-ER α ^{KO} female mice, which specifically lack ER α in dendritic cells (Fig. 4C). We observed that BM pDCs derived from unmanipulated Tie2-ER α ^{KO} female mice and CD11c-ER α ^{KO} female mice exhibited significantly less *Irf5* mRNA expression than in pDCs from WT mice ($p = 0.003$ and $p = 0.007$, respectively, unpaired t test; Fig. 4D). By contrast, *Irf7* mRNA levels were similar between WT and CD11c-ER α ^{KO} pDCs ($p = 0.6$, unpaired t test; Fig. 4E). We further demonstrated that reduced *Irf5* expression in BM-derived pDCs from Tie2-ER α ^{KO} mice and from CD11c-ER α ^{KO} mice was associated with an impaired capacity of pDCs to produce IFN- α in response to TLR7 engagement. Indeed, significantly less IFN- α was produced in response to TLR7 stimulation by pDCs from Tie2-ER α ^{KO} or CD11c-ER α ^{KO} mice than from WT littermate controls ($p = 0.02$ and $p = 0.01$, respectively, unpaired t test; Fig. 4G). These results demonstrate that *Irf5* expression correlates with the level of TLR7-mediated IFN- α production in pDCs and is, at least partially, regulated by ER α signaling.

To address the question of ER α -dependent regulation of the *IRF5* gene in humans, we quantified *ESR1* (ER α gene) and *IRF5*

mRNA transcripts in the same pDCs purified from humans using a novel flow cytometry-based in situ hybridization assay, the QuantiGene FlowRNA Assay. This assay based on the use of specific oligonucleotide probes coupled with branched DNA signal amplification offers the advantage over standard quantitative RT-PCR techniques to control for the potential heterogeneity in the expression among a defined cell type. Fig. 5A shows histograms of the detection of *IRF5* mRNA and *ESR1* mRNA. A significant correlation between the *ESR1* and *IRF5* transcripts was observed in pDCs from females, but not in pDCs from males (females: $n = 10$, $r = 0.81$, $p = 0.04$; males: $n = 7$, $r = 0.45$, $p = 0.31$, Spearman correlation; Fig. 5B). In addition, *IRF5* mRNA and *ESR1* mRNA in the pDC population correlated at the single-cell levels in both male and female healthy donors (Fig. 5C). Potential nonspecific interactions between the type1-*Esr1* and type4-*IRF5* probes were controlled for by simultaneously testing two different probes (type4-*TLR7* and type1-*TLR9*) coupled to the same fluorophores as used for *ESR1* and *IRF5* probes. Overall, these data demonstrate that estrogen-dependent regulation of IRF5 transcription via ER α can result in sex differences in IRF5 levels of pDCs and downstream IFN- α production.

Discussion

Important differences exist between males and females in the outcome of infectious diseases and occurrence of autoimmune diseases for which the pDC IFN- α response has been implicated in the pathology. Sex differences in IFN- α production by pDCs upon TLR7 stimulation have been previously described by our group and others (24–26). In this study, we investigated the potential role

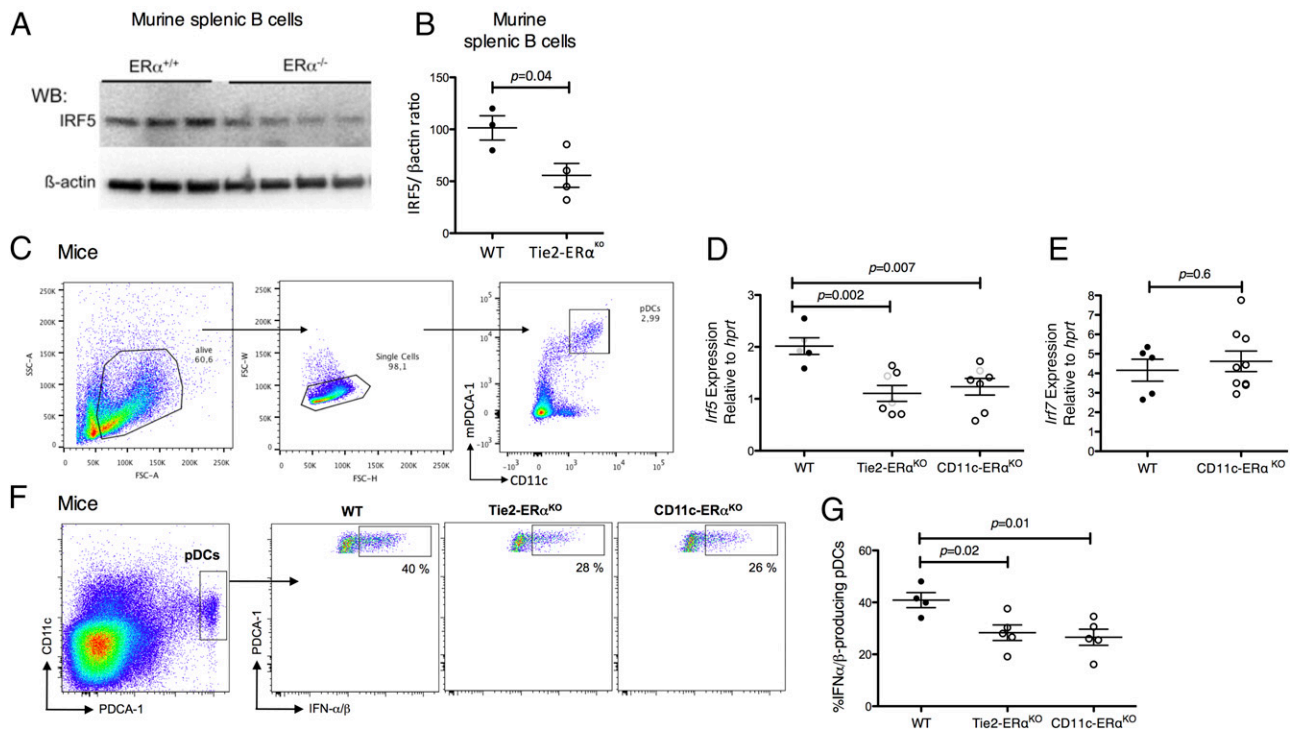


FIGURE 4. Defective estrogen signaling in mice is associated with reduced IRF5 levels and diminished TLR7-mediated production of IFN- α by pDCs. (**A** and **B**) B cells were individually purified from WT ($n = 3$) and Tie2- $ER\alpha^{KO}$ ($n = 4$) mice spleens by positive selection using anti-CD19 beads (Miltenyi). Protein lysates of B cells were analyzed by immunoblotting for IRF5 and β -actin protein expression. (**A**) Representative Western blot of the results is shown. (**B**) Densitometry results normalized to β -actin show decreased IRF5 protein levels in splenic B cells from Tie2- $ER\alpha^{KO}$ as compared with WT mice. (**C–E**) pDCs from the BM of female WT Tie2- $ER\alpha^{KO}$ and CD11c- $ER\alpha^{KO}$ mice were sorted based on the expression of CD11c and PDCA-1 from BM cells. RNA samples were retrotranscribed into cDNA, and quantitative PCRs were performed using *Irf5* and *Irf7* QuantiTect Primer Assays with SYBR green PCR Mastermix (Qiagen). Gene transcripts were normalized to *Hprt* gene abundance, and relative mRNA levels were calculated by the expression $2^{-\Delta Ct}$. Comparison between mice groups was calculated using the unpaired t tests. (**C**) Gating strategy for pDC sorting is shown. (**D**) RNA was isolated for each group from one to two pools of four to five mice (in gray) and from three to six individual mice (in black). *Irf5* mRNA levels were significantly lower in pDCs from Tie2- $ER\alpha^{KO}$ ($n = 14$) and CD11c- $ER\alpha^{KO}$ ($n = 10$) than in pDCs from WT mice ($n = 12$). (**E**) RNA was isolated from five individual WT mice and nine individual CD11c- $ER\alpha^{KO}$ ($n = 9$) mice. *Irf7* mRNA levels in pDCs did not differ between WT and CD11c- $ER\alpha^{KO}$ mice. (**F** and **G**) BM cells from WT, Tie2- $ER\alpha^{KO}$, and CD11c- $ER\alpha^{KO}$ mice were stimulated with the TLR7 ligand PolyU for 4 h, in the presence of brefeldin A for the last 2 h. (**F**) Representative flow cytometry plots showing IFN- α production by pDCs (CD11c⁺PDCA-1⁺). (**G**) $ER\alpha$ deletion in hematopoietic compartment (Tie2- $ER\alpha^{KO}$) or in the DC lineage (CD11c- $ER\alpha^{KO}$) was associated with diminished frequency of IFN- α -producing pDCs from mice upon TLR7 stimulation. Comparison between mice groups was calculated using the unpaired t tests. Error bars indicate the mean and SEM.

of two key regulators of IFN- α production, IRF7 and IRF5, in sex differences in IFN- α production by pDCs. Although no sex-based difference in IRF7 expression was observed, significantly higher protein levels of IRF5 were detected in pDCs purified from females than in pDCs derived from males. Basal protein levels of IRF5 were positively associated with the level of IFN- α secretion by TLR7-stimulated pDCs, and experimental increase in IRF5 protein levels resulted in increased IFN- α secretion by human pDCs. BM-derived pDCs from female mice with conditional $ER\alpha$ knockout exhibited impaired capacity to produce IFN- α in response to TLR7 engagement and significantly less *Irf5* mRNA expression than pDCs from WT mice. Altogether, these results identify sex hormone-mediated regulation of IRF5 levels in pDCs as an underlying mechanism for the higher percentage of IFN- α -secreting pDCs in females as compared with males after TLR7 stimulation.

Previous studies have reported conflicting results regarding the role of IRF5 in IFN- α transcription (33, 38, 57, 58). These studies differed in cell type and stimulations used, suggesting that the impact of IRF5 on type I IFNs transcription may be cell type and stimulus dependent. In our study, the basal levels of IRF5 were found to be significantly higher in pDCs from females than in those from males. IRF5 was furthermore observed to be functional upon TLR7 stimulation, with IRF5 levels before stimulation being

correlated with the percentage of IFN- α -secreting pDCs after 2 h of TLR7 stimulation. However, it should be noted that a subset of IFN- α -secreting pDCs with undetectable IRF5 levels was also present. This observation suggests that IRF5 alone does not account for all the IFN- α produced by pDCs and may reflect the well-known role of IRF7 in IFN- α production. Altogether, these data demonstrate that sex difference in basal levels of IRF5 can drive sex differences in IFN- α production upon TLR7 stimulation.

Although sex-based differences were observed in pDCs, no sex difference in IRF5 expression was observed in T cells, confirming a cell-specific regulation of IRF5 expression. A potential explanation for the cell-specific differences in IRF5 might be the presence of multiple alternatively spliced isoforms of IRF5. These different IRF5 isoforms have been shown to have distinct cell type-specific expression, regulation, cellular localization, and function (59). Human pDCs express four distinct alternatively spliced isoforms (V1, V2, V3, and V4), with V3 and V4 being the most predominant transcripts expressed in unstimulated pDCs. In contrast, the V4 (and also V1) isoforms are not detected in unstimulated T cells (59). In addition, cell type-specific posttranscriptional regulation, for example, by the expression of cell-specific microRNAs, may be involved in differences in IRF5 expression (60, 61). Basal IRF5 expression among pDCs is heterogeneous, with some pDCs expressing no or low levels of IRF5. Although the Ab against

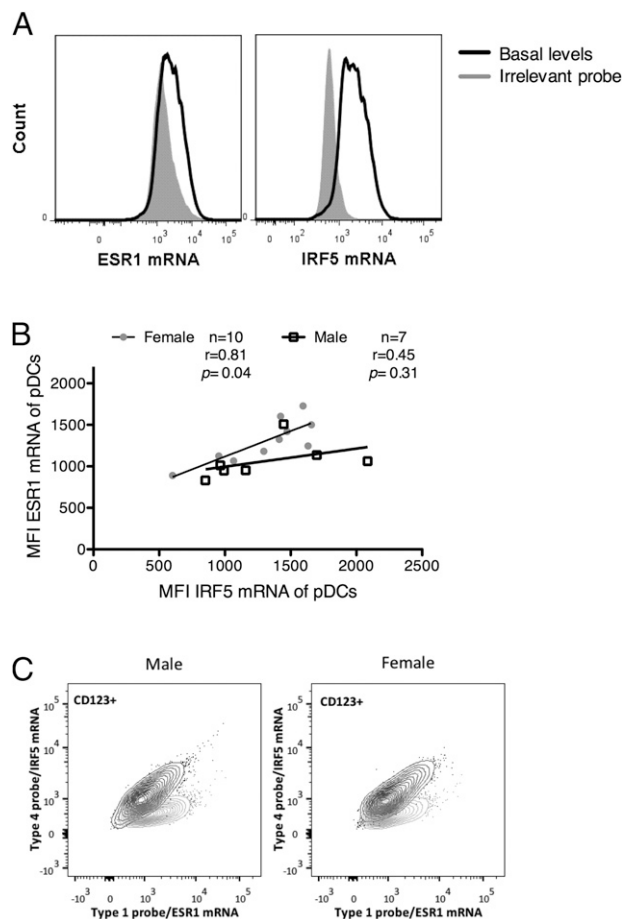


FIGURE 5. Sex difference in IRF5 levels in human pDCs is associated with estrogen signaling. mRNA levels of *IRF5* and *ESR1* were measured in human pDCs using the QuantiGene FlowRNA assay. **(A)** Flow cytometry histogram overlays show the mean intensities of *IRF5* or *Esr1* mRNA (empty curve) compared with control with the irrelevant *dapB* probes (filled gray curve) in pDCs. **(B)** A significant correlation between *IRF5* and *ESR1* mRNA levels is observed in human pDCs derived from females (closed round shapes, $n = 10$, $p = 0.04$, $r = 0.84$), but not in pDCs derived from males (open squares, $n = 7$, $p = 0.31$, $r = 0.45$). Samples were run in duplicates. Linear regression was calculated with Spearman rank-based correlation. Error bars indicate the mean and SEM. **(C)** Flow cytometry contour plots show correlated *ESR1* mRNA and *IRF5* mRNA expressions in the pDC population at the single-cell level, respectively, in representative male (left panel) and female (right panel) healthy donors. Control with irrelevant type 1 and 6 probes is shown in gray in both plots.

IRF5 used in this study is recognizing all IRF5 isoforms, it is possible that differences in the affinity for the various isoforms may account for the low IRF5 levels reported in some pDCs. Overall, our data show that the role of IRF5 in IFN- α transcription is cell type specific and results in differential sex-based expression of IRF5 as well as IFN- α expression in pDCs.

To clarify the impact of IRF5 on IFN- α transcription in primary human pDCs, we used a novel technique previously described to deliver exogenous recombinant protein into human primary pDCs (51). The delivery of recombinant IRF5 protein led to an increase in the percentage of IFN- α -secreting pDCs, confirming the ex vivo association observed between IRF5 levels and TLR7-induced IFN- α production. In addition to IFN- α production, IRF5 is also involved in the transcription of proinflammatory cytokines such as IL-6 and TNF- α (38). In agreement with this, we observed an increase in the production of TNF- α , IL-8, MIP-1 α , and MIP-1 β upon IRF5 delivery in cells and after 2 h of TLR7 stimulation.

Previous studies by our group and others did not detect sex differences in TNF- α production after overnight stimulation with TLR7 ligand (24, 25). In contrast, Seillet and colleagues (26) recently reported an increased frequency in TNF- α -producing pDCs in women as compared with men upon 5 h of stimulation with a TLR7 ligand. Such discrepancy could be explained by the shorter stimulation times used in Seillet et al.'s study (26), which may have unmasked the sex differences by limiting cytokine-specific feedback regulatory mechanisms. Therefore, although increased IRF5 may lead to increased TNF- α production by pDCs upon short stimulation time, feedback regulatory mechanisms may later be dampening this production so that no difference is observed with longer stimulation time.

Biological sex differences in the human immune responses to infections or autoimmune diseases can be caused by genetic factors linked to sex chromosomes and/or the modulation of immunity by sex hormones. The precise functional mechanisms by which sex hormones might regulate the IFN- α response of pDCs are unknown but are thought to involve ER α signaling (26, 27). ERs are expressed on all PBMCs including pDCs (26, 55, 62–64). The interaction of ER α with target gene promoters can occur either directly, through specific estrogen response elements, or indirectly through contacts with other DNA-bound transcription factors such as the specificity protein 1, but also as the AP-1 or the NF- κ B, both involved in the transcription of type I IFNs and proinflammatory cytokines (65–68). A previous study in mice showed higher *Irf5* mRNA levels in splenic B cells from female than from age-matched male mice and lower levels of *Irf5* mRNA in ER α knockout mice as compared with WT mice (56). pDCs from Tie2-ER α ^{KO} and CD11c-ER α ^{KO} intact female mice lack ER α in the hematopoietic compartment or in CD11c⁺ DC lineage, respectively, and have been shown to be impaired in their capacity to produce IFN- α in response to TLR9 engagement (26). We further show in this article that pDCs from Tie2-ER α ^{KO} and CD11c-ER α ^{KO} mice are also altered in their ability to produce IFN- α after ex vivo TLR7 stimulation, and that this was associated with decreased *Irf5* mRNA expression levels as compared with their littermate controls.

Species-specific differences, in particular in splice patterns of *IRF5*, may bias the translation of our results obtained from mice to humans. Indeed, spliced variants of *IRF5* were identified only in human cells, whereas in inbred strains of mice *Irf5* encodes for a dominant unspliced transcript (59). Interestingly, an ER α binding site has been identified using the University of California Santa Cruz Genome Browser (<http://genome.ucsc.edu>) at position 128561334–128561609 on human chromosome 7, which is 16,385 bp upstream the *IRF5* gene (*IRF5* chr7:128577994–128590088), suggesting that IRF5 may also be regulated by sex hormones in humans. Furthermore, one of the described *IRF5* polymorphism in humans, the CGGGG indel, is associated with increased expression of IRF5 itself because of the presence of an additional SP1 binding site, an ER α cofactor (47, 69). In this study, we found a significant correlation between *IRF5* and *ESR1* mRNA levels in pDCs from females but not in pDCs from males in humans, highlighting the dependency of *IRF5* mRNA on estrogen signaling in females. Although we were not able to simultaneously measure *IRF5* mRNA and ER α protein in the same cells, previous reports suggested that *ESR1* mRNA and ER α protein expression correlate (70–72). Importantly, the ER α ^{KO} mice models used in this study do not exclude that nonhormonal pathways and particularly X-chromosome-linked factors participate in sex-specific regulation of IRF5 in pDCs. Recently, it was shown that X chromosome dosage contributed independently from sex hormones to the sex bias in the pDC TLR7-mediated IFN- α response (55). Accord-

ingly, we observe a trend toward higher IRF5 levels persisting in postmenopausal females as compared with age-matched males ($p = 0.06$). This might also explain why we did not observe significant difference in IRF5 levels between premenopausal females under hormonal birth control and premenopausal females having regular menstrual cycle.

In conclusion, this study demonstrates that pDCs from healthy females exhibit higher basal levels of IRF5 than pDCs from healthy males. Furthermore, higher levels of IRF5 in pDCs are directly associated with higher IFN- α responses to TLR7 stimulation. This sex-based difference appeared to be partially due to ER α signaling-mediated modulation, because *Irf5* mRNA expression was significantly reduced in female mice with a conditional knockout for ER α and correlated, in humans, to *ESR1* mRNA expression in pDCs from females. These data provide new insights into the mechanisms underlying the higher inflammation observed in females in infectious and autoimmune diseases, and identify IRF5 as an attractive target for specific modulation of the IFN- α pathway.

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Disclosures

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