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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 If5 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.

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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). 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
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 (43–46), 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 (ERα) 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 subject studies 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.

Mice

Mice selectively lacking ERα 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 (ERαfl/+) with B6 mice expressing the Cre recombinase under the control of the Piezopromoter-enhancer (Piez2-EREαfl/+) or the CD11c promoter (CD11c-EREαfl/+) 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 reseeded in R-10 at a concentration of 1.5 million cells/ml, and 1 ml PBMCs was stimulated in FACS tubes with 1 µg/ml CL097, a synthetic TLR7 ligand (imidazoquinoline; Invivogen, San Diego, CA). A total of 5 µg/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 µg/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 CD11c–CD19–CD56–HLA-DR–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 (eBioscience, San Diego, CA), anti–IFN-γ-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 permeabilized by being permeabilized by washing twice in PBS containing 1% 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 µl 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 µl R-10 on poly-L-lysine-coated plates and simultaneously stimulated with 1 µg/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, mounted with 2% paraformaldehyde 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 wash. 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×/1.3NA objective in combination with a PCO (Kelheim, Germany) monochrome 12-bit CCD camera. This slide scanning system uses a z-axis overshoot technique 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 IFR5 fluorescence was then separately determined using a cytoplasm mask for IFR5, a nuclear mask for IFR7, and a whole-cell mask for IFR5. For each sample, a minimum of 150 pDCs was imaged to determine the mean intensity of IFR5.

**IFR5 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 and 10,000 cells/μl were mixed with Cascade Blue-labeled 3-kDa dextran molecules, for control of delivery, and with either 0.03–0.06 μg/μl IFR5 recombinant protein (Abcam or Origene, Rockville, MD) or 0.05–0.1 μg/μl 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 (SQX 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 IFR5–producing capacity of pDCs subjected to mechanical deformation. Delivery of TUBA1A protein, which is not involved in the IFR5 production pathway, was used as control for determining any nonspecific effect on IFR5 production. IFR5 levels were subsequently measured by flow cytometry as described earlier. Cells were then stimulated for 2 h with 1 μg/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. IFR5 secretion was measured using Miltenyi’s IFR5 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-dioleoyl-3-trimethylammonium-propane (DOTAP) [8 μl of a cationic liposome preparation (DOTAP; Roche) mixed with 1 μg PolyU in 150 μl RPMI, in a polystyrene tube]. BM cells were stimulated with PolyU-DOTAP preparation for 4 h, and 5 μg/ml brefeldin A (eBioscience) was added for the last 20 min of culture. Specific staining was performed with 5 μg/ml anti-CD16/CD32 (2.4G2; American Type Culture Collection). BM cell suspensions were then stained with PE-Cy7–labeled anti-CD11c (all from eBioscience). To control for nonspecific probe interaction, we replaced type4-IFR5 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 IFR5, ESR1, and B2M probes were determined by subsequent analysis using FlowJo software. Values were excluded if 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 IFR5 MFIs between IFR-α–secerting pDCs and nonsecerting pDCs was calculated using the paired Wilcoxon rank tests. Linear regression was calculated using Spearman rank-based correlation. For IFR5 protein delivery experiments, we used Wilcoxon signed rank for comparison of the increase in the percentage of IFR-α secretion relative to the control therefore normalized to 1. Comparison between WT mice and ERαKO mice was calculated using the unpaired t tests.

**Results**

**Sex differences in the IFR5-α/TLR7 pathway in pDCs**

We and others have previously reported that pDCs isolated from females produce markedly more IFR5-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 IFR5-α–producing pDCs in a first group of 31 healthy individuals (17 females, 14 males) (Supplemental Table I). A significantly higher percentage of IFR5-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 IFR5-α production by pDCs (p = 0.1, r = 0.3, Spearman rank-based correlation; p = 1.0, Fisher Exact test). The mean frequency of IFR5–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 IFR5-α production remain to be elucidated. IFR7 and IFR5 are two crucial transcription factors activated upon TLR7 stimulation that modulate IFR5-α production (33). Ex vivo levels of IFR5 and IFR7 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 IFR7 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 IFR5 expression (females: n = 29, males: n = 19; p = 0.02, two-tailed Wilcoxon Rank test; Fig. 1D). IFR5 expression among pDCs appeared heterogeneous with some pDCs expressing no or very low levels of IFR5 as determined by the use of an isotype control (Fig. 1C). Notably, no difference in IFR5 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 IFR5 protein were also examined in CD3+ BM cells using anti-CD19 beads (Miltenyi). Cells were lysed in lithium dodecyl sulfate (LDS) 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 IFR5 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 (LDS) 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 IFR5 and IFR7 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. 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 IFR5 and IFR7 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–ΔΔCt.

In situ IFR5 mRNA expression assay by flow cytometry

Five million PBMCs were pelleted and surface stained on ice for 30 min. Cells were subjected to the QuantGene FlowRNA assay (eBiocience, San Diego, CA) as per manufacturer’s instructions with typep-B2M probe, type1-ESR1 probe, and a customized ultrasensitive type4-IFR5 probe (probes are all from eBiocience). To control for nonspecific probe interaction, we replaced type4-IFR5 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 IFR5, ESR1, and B2M probes were determined by subsequent analysis using FlowJo software. Values were excluded if the duplicates exhibit >20% difference.
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 periods 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
obtained using the 10-4 × 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 × 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 cytokines 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-1α, and MIP-1β protein secretion were induced. pDCs in which IRF5 protein was overexpressed exhibited increased TNF-α, IL-8, MIP-1α, and MIP-1β protein secretion were induced. pDCs in which IRF5 protein was overexpressed exhibited increased TNF-α, IL-8, MIP-1α, and MIP-1β protein secretion were induced. pDCs in which IRF5 protein was overexpressed exhibited increased TNF-α, IL-8, MIP-1α, and MIP-1β protein secretion were induced. pDCs in which IRF5 protein was overexpressed exhibited increased TNF-α, IL-8, MIP-1α, and MIP-1β protein secretion were induced. pDCs in which IRF5 protein was overexpressed exhibited increased TNF-α, IL-8, MIP-1α, and MIP-1β protein secretion were induced. pDCs in which IRF5 protein was overexpressed exhibited increased TNF-α, IL-8, MIP-1α, and MIP-1β protein secretion.
derived from female than in age-matched male mice and from WT mice as compared with ERα knockout mice (56). We therefore investigated ERα-dependent regulation of IRF5 in female mice using tissue-specific ERα-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-ERαKO mice, lacking ERα in the hematopoietic compartment, as compared with ERαfloxflox WT mice (p = 0.04, unpaired t test; Fig. 4A, 4B) (56). We then sorted BM pDCs from ERαfloxflox WT, Tie2-ERαKO, and CD11c-ERαKO female mice, which specifically lack ERα in dendritic cells (Fig. 4C). We observed that BM pDCs derived from unmanipulated Tie2-ERαKO female mice and CD11c-ERαKO 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-ERαKO pDCs (p = 0.6, unpaired t test; Fig. 4E). We further demonstrated that reduced Irf5 expression in BM-derived pDCs from Tie2-ERαKO mice and from CD11c-ERαKO 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-ERαKO or CD11c-ERαKO 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 ERα signaling.

To address the question of ERα-dependent regulation of the IRF5 gene in humans, we quantified ESR1 (ERα 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 ERα 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
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-ERαKO (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-ERαKO as compared with WT mice. (C-E) pDCs from the BM of female WT Tie2-ERαKO and CD11c-ERαKO 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 β-actin QuantiTect Primer Assays with SYBR green PCR Mastermix (Qiagen). Gene transcripts were normalized to Hprt mRNA levels, and relative mRNA levels were calculated by the expression 2−ΔΔCt. Comparison between mice groups was calculated using the unpaired t test. (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-ERαKO (n = 14) and CD11c-ERαKO (n = 9) than in pDCs from WT mice (n = 12). (E) RNA was isolated from five individual WT mice and nine individual CD11c-ERαKO (n = 9) mice. Irf7 mRNA levels in pDCs did not differ between WT and CD11c-ERαKO mice. (F and G) BM cells from WT, Tie2-ERαKO, and CD11c-ERαKO 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+PDCA1+). (G) ERα deletion in hematopoietic compartment (Tie2-ERαKO) or in the DC lineage (CD11c-ERαKO) was associated with diminished frequency of IFN-α/β–producing pDCs from mice upon TLR7 stimulation. Comparison between mice groups was calculated using the unpaired t test. Error bars indicate the mean and SEM.

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). Human pDCs express four distinct 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). 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
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 ERα signaling (26, 27). ERs are expressed on all PBMCs including pDCs (26, 55, 62–64). The interaction of ERα 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-κB, 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 ERα knockout mice as compared with WT mice (56). pDCs from Tie2-ERαKO and CD11c-ERαKO mice are also altered in their ability to produce IFN-α after ex vivo TLR7 stimulation, and that this was associated with decreased Ifr5 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 ERα 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 (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 ERα 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 ERα protein in the same cells, previous reports suggested that ESR1 mRNA and ERα protein expression correlate (70–72). Importantly, the ERαKO 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 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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References

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