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X-Chromosome Complement and Estrogen Receptor Signaling Independently Contribute to the Enhanced TLR7-Mediated IFN-α Production of Plasmacytoid Dendritic Cells from Women

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Human plasmacytoid dendritic cells (pDCs) play a major role in innate immunity through the production of type I IFNs after TLR engagement by pathogens. Sex-based differences in the innate function of human pDCs have been established, with pDCs from women exhibiting enhanced TLR7-mediated IFN-α production as compared with pDCs from males. In mice, we recently provided evidence for a role of estrogens as a positive regulator of pDC innate functions through cell-intrinsic estrogen receptor α signaling, but did not exclude a role for other X-linked factors, particularly in human pDCs. In this study, we investigated the respective contribution of X chromosome dosage and sex hormones using a humanized mouse model in which male or female NOD-SCID-β2m−/− were transplanted with human progenitor cells purified from either male or female cord blood cells. We showed that, in response to TLR7 ligands, the frequency of IFN-α- and TNF-α-producing pDCs from either sex was greater in female than in male host mice, suggesting a positive role for estrogens. Indeed, blockade of estrogen receptor signaling during pDC development in vitro inhibited TLR7-mediated IFN-α production by human pDCs, which expressed both ESR1 and ESR2 genes. Interestingly, we also found that X chromosome dosage contributed to this sex bias as female pDCs have an enhanced TLR7-mediated IFN-α response as compared with male ones, irrespective of the sex of the recipient mice. Together, these results indicate that female sex hormones, estrogens, and X chromosome complement independently contribute to the enhanced TLR7-mediated IFN-α response of pDCs in women. The Journal of Immunology, 2014, 193: 5444–5452.

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Abbreviations used in this article: BDCA, blood DC Ag; BM, bone marrow; DC, dendritic cell; E2, 17β-estradiol; ER, estrogen receptor; CD45, human CD45; HPC, human progenitor cell; HuMouse, humanized mouse; IRF, IFN regulatory factor; pDC, plasmacytoid DC; SLE, systemic lupus erythematosus.

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that 17β-estradiol (E2) supplementation markedly enhanced IFN-α and TNF-α production by blood pDCs in response to TLR9 and TLR7 stimulation. Estrogens mediate their effects through two estrogen receptors (ER), ERα and ERβ. Using ERα-mutant mice, we showed that exogenous and endogenous estrogens increased the TLR-mediated cytokine secretion by mouse pDCs through pDC-intrinsic ERα signaling (19).

Our results, however, do not exclude a role for other X-linked factors, particularly in human pDCs. Indeed, beside the role of sex hormone estrogens, direct effects of X-chromosome loci could participate to the sex-specific difference in TLR-mediated responses of human pDCs (21). The X chromosome carries numerous genes involved in TLR signaling including TLR7 itself (21). It has been postulated that the X chromosome could be partly responsible for the hyper responsiveness of the female immune system due to the biallelic expression of X-linked genes that escape X-inactivation (21). Indeed, despite chromosome-wide transcriptional silencing of one X chromosome in female, some genes remain expressed from both the active and inactive X alleles, resulting in a double gene dosage (22). It has been estimated that in women, ~15% X-linked genes are biallelically expressed, whereas there are only 3% in mice (22).

The present study was designed to directly examine the respective contribution of female sex hormones and X-linked factors to the enhanced TLR7-mediated responses of human pDCs in vivo by means of a humanized mouse model (HuMouse). Male or female NOD-SCID/B2m−/− mice were transplanted with CD34+ human progenitor cells (HPCs) purified from either male or female donors. Human pDCs that developed in the bone marrow (BM) of HuMice were subsequently assessed for their capacity to produce cytokines in response to TLR7/8 ligands, including influenza virus and HIV-derivied ligands ex vivo. Our results indicate that the female sex hormone estrogens and X chromosome complement both independently contribute to the enhanced TLR7-mediated responses of pDCs in women.

Materials and Methods

**Ethics statement**

All procedures were approved by the French Blood Service’s Institutional Review Board. All adult subjects provided written informed consent, and a parent of any participant child provided informed consent on their behalf (form EN08 6587/2). Animal studies were carried out under the control of the National Veterinary Services and in accordance with European regulations (EEC directive 86/609 dated November 24, 1986) and French National Charter guidelines. The protocol was approved by the Regional Ethics Committee from Région Midi-Pyrénées (approval MP/06/58/10/11) and Grenoble (approval 174-UHTA-U823-CA-05) affiliated to the “Comité National de Réflexion Ethique sur l’Experimenter-Animale.”

**Generation of humanized mice**

NOD/SCID/B2m−/− mice (NOD.Cg-Prkdcscid-lefv-1Tmc1Unc/J; Jackson ImmunoResearchLaboratories) were sublethally irradiated (110 Gy) at the age of 4 wk and injected i.v. with 1 to 2 × 10⁶ CD34+ HPCs purified from umbilical cord blood. Briefly, CD34+ HPCs were positively isolated from the mononuclear fraction using anti-CD34 magnetic microbeads and MS separation columns (Miltenyi Biotec). Purity was routinely ∼90%.

In some experiments, mice have been injected s.c. with a solution of E2 (1 μg diluted in 100 μl castor oil).

**In vitro CD34-derived pDCs**

CD34+ HPCs (2 × 10⁶ cells/well of a 24-well plate) were cocultured for 2 wk with OP9-β 1 stromal cells that were seeded the day before as described (23). Briefly, culture was performed in MEM-α complete medium (MEM-α supplemented with 20% heat-inactivated FCS, 1 mM sodium pyruvate, 1% nonessential amino acids, 2 mM L-glutamine, 50 μM 2-ME, and 50 μg/ml gentamicin) in the presence of 5 ng/ml Flt3L and 5 ng/ml IL-7 (ProproTech). Cytokine-containing medium was refreshed after 1 wk of culture. pDCs were harvested by disrupting the stromal cell layer and filtering out OP9-β1 stial cells using nylon mesh.

**Flow cytometric analysis of human pDCs and monocytes**

Cell suspensions were obtained by flushing out the BM of humanized mice. Before staining, cells were incubated at room temperature for 15 min with blocking buffer (PBS with 1% FCS, 3% normal mouse serum, 3% normal rat serum, 5 mM EDTA, and 1/50 NaN3). For surface cell staining, cells were incubated on ice for 30 min with FITC-, PE-, PE-Cy7-, PerCP-Cy5.5-, allophycocyanin-, and E450-conjugated mAbs diluted at the optimal concentration in FACS buffer (PBS 1% FCS, 5 mM EDTA, and 1/50 NaN3). Cells were stained with Abs to murine CD45, human CD45 (hCD45), CD14, blood DC Ag-4 (BDCA-4), BDCA-2, CD123, HLA-DR, or CD86. For detection of intracellular IFN regulatory factor (IRF) 7, cells were surface stained, fixed, permeabilized using the intracellular fixation/permeabilization buffer set from eBioscience, and finally stained for IRF7 using anti-IRF7 rabbit polyclonal IgG (sc-9083; Santa Cruz Biotechnology) and secondary anti-rabbit A647 Ab (Invitrogen).

For detection of cytokine in intracellular compartment, cells were stimulated as indicated with 1 μg/ml R848 (Invivogen) or formol-inactivated influenza virus H1N1 (100–200 ng/ml hemagglutinin). For stimulation with HIV-1-derived Gag RNA1166 and Gp160 RNA2093, RNA were mixed with 1,2-di-oioleoyl-3-trimethylammonium-propane (DOTAP) and 10²/µl Poly-2. Cells were stimulated and fixed for 5 h in the presence of brefeldin A (eBioscience) for the last 3 h of culture. After surface staining and fixation in PBS/2%/parafomaldehyde, cells were permeabilized with 0.5% saponin, and intracellular cytokine staining was performed with Abs specific for anti-IFN-α–PE (Miltenyi Biotech) or anti–TNF-α–Alexa 700 (BD Biosciences) Abs.

Data were acquired on a Fortessa cytometer (BD Biosciences) and further analyzed using the FlowJo software (Tree Star).

**Data normalization**

We generated several cohorts of male or female HuMice transplanted with CD34+ HPCs from umbilical cord blood of either male or female donors. For each CD34 donor HPCs, between two and four replicate mice from either sex were injected. In order to pool the results from these different cohorts, individual data were normalized within each cohort to the mean value of pDC responses in male HuMice (Fig. 2). These mean values were assigned the arbitrary unit of 100 and used to recalculate individual data that were expressed as percent of the male-associated responses.

**Measuring expression levels of ERα and -β in human leukocytes and DC subsets**

Peripheral blood myeloid DCs and pDCs were magnetically sorted with BDCA-1 and BDCA-4 cell isolation kits (Milteny Biotech), respectively (24). Macrophages, B cells, NK cells, CD4+, and CD8+ T cells were purified using appropriate cell isolation kits all purchased from Miltenyi Biotec. Total RNA was extracted using the RNasy Mini kit (Invitrogen) and Absolutely RNA Microprep kit (Stratagen) and were reverse transcribed using appropriate cell isolation kits all purchased from Miltenyi Biotec. Total RNA was extracted using the RNasy Mini kit (Qiagen) or Absolutely RNA Microprep kit (Stratagen) and were reverse transcribed using the SuperScript III reverse transcriptase (Invitrogen). ERα and ERβ mRNA transcripts were quantified by real time RTPCR analysis using SYBR Green JumpStart Taq ReadyMix for quantitative PCR (Sigma-Aldrich) in a LightCycler 480 II (Roche). ERα- and ERβ-specific primers were purchased from Qiagen (Quantitect Primer Assays Hs_ESR1_1_BG for ERα and Hs_ESR2_1_BG for ERβ). GAPDH-specific primer sequences were: GAPDH-F, 5′-ACAAGCTGCACTGACTGACG-3′ and GAPDH-R, 5′-AGCAGAGTGGGTCAGGCTG-3′. GAPDH-forward, 5′-ACAGCAGAGTGGGTCAGGCTG-3′ and GAPDH-reverse, 5′-AGCAGAGTGGGTCAGGCTG-3′. The number of ERα or ERβ transcripts were quantified using external ER standard plasmids generated by cloning the PCR products into pCR2.1-TOPO vector. Standard curves were generated by serial dilution of specific plasmids from 10⁶ to 10² copy numbers. Samples were normalized to the copy numbers of the housekeeping gene, GAPDH, measured using a similar procedure as described (25).

**Statistical analysis**

The statistical significance was estimated with the two-tailed Mann-Whitney U test, Student t test, or Wilcoxon signed-rank test as indicated using GraphPad Prism Version 4.03 (GraphPad Software). A p value ≤ 0.05 was considered significant in all cases.
Results
The female recipient environment enhances the TLR7-mediated response of engrafted human pDCs from both male and female donors

We have used a humanized mouse model (26) to study the respective contribution of sex-linked factors in the TLR7-mediated responses of human pDCs. We first evaluated the TLR-mediated response of human pDCs that had developed in either a male or a female environment. In this model, sublethally irradiated NOD/SCID β2m−/− mice of either sex were transplanted with CD34+ HPCs from umbilical cord blood of either male (XY) or female (XX) donors. HPCs from a given donor were injected into replicate mice of both sexes. Five weeks after transplantation, BM cells were analyzed for lymphoid chimerism and the presence of human pDCs (Fig. 1). In agreement with previous works (27), we observed a significant trend toward a better engraftment of human cells in the BM of female mice compared with male recipients (Fig. 1A, 1B). The frequency of CD123+BDCA-4+ pDCs among human CD45+ cells (Fig. 1C, 1D) was, however, lower in female mice. Consequently, when absolute numbers of human pDCs were calculated, no significant differences were observed between male and female recipients (Fig. 1E). Thus, despite differences in human cell engraftment, similar numbers of human pDCs developed in the BM of male and female mice.

BM cells were stimulated with TLR7/8 ligands (R-848), and the frequencies of IFN-α- and TNF-α-producing human pDCs were quantified by FACS using the gating strategy shown in Supplemental Fig. 1. Whereas the percentage of IFN-α- and TNF-α-producing pDCs was very homogenous within the replicate male or female mice transplanted with the same HPCs, we observed some variability in the pDC responses depending on the HPC donors (Fig. 2A, 2B). Therefore, the genetic makeup of the human HPC donor, rather than interindividual variability, regulates the frequency of cytokine-producing pDCs that develop into the BM of replicate mice engrafted with the same human HPCs (Fig. 2A, 2B). In agreement with our recent work (19), when we analyzed the response of human pDCs from the same donor that develop into female or male recipients, we observed a significant increase in the frequency of pDCs producing IFN-α or TNF-α in females as compared with males, after stimulation with R-848 (Fig. 2C). Similar results were observed when we analyzed in some cohorts the pDC responses after stimulation with more pathophysiological ligands that are known to activate pDCs through TLR7 such as HIV GagRNA1166 and Gp160RNA2091 sequences, as well as inactivated influenza virus (Fig. 3). Beside the analysis of the frequency of IFN-α- and TNF-α-producing pDCs (BDCA4+CD123+CD45+) upon TLR7 stimulation with R-848, we also assessed the expression level of IRF7 by intracellular staining in the same cell population, as well as the cell-surface expression of maturation markers on pDCs. We could not see any significant sex-dependent difference in the expression of IRF7 (Fig. 4), CD40, or CD86 (not shown). We noticed, however, a modest but consistent increase in HLA-DR molecule expression by TLR7-activated human pDCs in female mice as compared with male recipients (Fig. 4). This slight increase in HLA-DR expression in females was observed in 7 out of 12 experiments. Expression of the pDC markers CD123 and BDCA-4 was similar between human pDCs that developed in male and female mice.

We then assessed whether intrinsically male and female pDCs were equally sensitive to the enhancing effect of the female environment on their TLR7-dependent responses. By comparing the TLR7-mediated responses of individual mice, engrafted with either male or female HPCs, we indeed found that the enhancing effect of the female host environment was not dependent on the sex of the donor HPCs. As shown in Fig. 2D and 2E, there was a 50% increase on average in the frequency of IFN-α or TNF-α-producing...
We then tested the effect of E2 treatment on the TLR7 responsiveness of human pDCs in this model. To this purpose, male HuMice engrafted with male HPCs were treated with a single s.c. injection of E2 and their BM analyzed 5 d later. E2 treatment was associated with a slight increase in the total numbers of cells, including human CD45+ cells (Supplemental Fig. 2A). Upon R-848 stimulation, there was a trend toward an increase in the frequency and absolute number of IFN-α-producing pDCs in E2-treated mice (Supplemental Fig. 2B). At steady state, the expression of CD86, CD40, HLA-DR, or HLA-A, -B, and -C on pDCs was not affected by E2 treatment (Supplemental Fig. 2C). These results suggest that estrogen administration increases the capacity of human pDCs to produce IFN-α upon TLR7 engagement, in agreement with our previous work (19).

As human monocytes can be also activated by R-848 through TLR8 (Supplemental Fig. 3), we also analyzed the intracellular expression of TNF-α in CD14+ monocytes in the same mice. In contrast to pDCs, the frequency of TNF-α–producing monocytes was not affected by the sex of the recipient mice (Fig. 2F). Thus, the female sexual environment, likely estrogens, selectively enhances the TLR7-mediated response of pDCs independently of the sex of the donor HPCs but not the TLR8-mediated response of monocytes.

Next we investigated whether the sex of the HPC donor had an influence on the TLR7-mediated responses of pDCs that developed in male or female recipients. For this, we compared the R-848–mediated IFN-α response of male and female human pDCs from transplanted mice (Fig. 5A). We found that the frequency of IFN-α–producing pDCs from female human donors was significantly higher as compared with pDCs that developed from male HPCs. This difference was not dependent on the sex of the recipient mice (Fig. 5B). By contrast, the TNF-α responses of neither pDCs (Supplemental Fig. 4A) nor monocytes (Supplemental Fig. 4B) were affected by the

pDCs in female mice as compared with male recipients, irrespective of the sex of the HPC donor.

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pDCs from female donors exhibit an enhanced TLR7-mediated IFN-α response

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sex of the donor HPCs. Thus, the X-chromosome complement appears to selectively enhance the IFN-α response of TLR7-stimulated pDCs, but not their capacity to produce TNF-α.

**Cell-intrinsic ER signaling potentiates the TLR7 responses of CD34-derived human pDCs independently of their sex**

The female sex hormone estrogen is a good candidate for explaining the better TLR-mediated responses of human pDCs developing in female HuMice (19, 20). Because estrogens mediate their effects through binding to the nuclear ERα and ERβ, we examined the ER isotype expression profile of peripheral blood leukocytes including pDCs by quantitative PCR. Although, it has been shown that human monocyte-derived DC can express ER genes (29), the expression pattern of ERα and ERβ gene transcripts has never been examined in pDCs. To address this point, we quantified ESR1 and ESR2 gene mRNA transcripts, in pDCs and various cell types including monocytes, T, and B lymphocytes, that have been shown to differential express ESR1 and ESR2 genes (29). ESR1 expression was lower in pDCs as compared with B and T lymphocytes that expressed relatively higher levels of ESR1 mRNA (Fig. 6A). B lymphocytes expressed the highest levels of both ESR1 mRNA as compared with T cells and monocytes. Of note, B cells and pDCs had the highest levels of ESR2 mRNA compared with any other cell type examined (Fig. 6B).

We next examined whether sex could influence ER gene expression in pDCs. In Fig. 7, we quantified the expression levels of ESR1, ESR2, and TLR7 genes in purified pDCs from healthy man or woman donors. Although, we noticed a trend toward a slightly higher expression of ESR1 in pDCs from women as compared with men, this was not significant. Likewise, expression levels of ESR2 and TLR7 were similar between men and women (Fig. 7).

We next examined whether antagonizing ER signaling using the pure ER antagonist ICI 182,780 in developing human pDCs could regulate their TLR7-dependent responses. We used an in vitro model of Flt3L/IL-7-driven human pDC differentiation from CD34+ HPCs on OP9 stromal cells expressing the Notch ligand Delta1 (23). In cocultures of CD34+ HPCs and OP9-Delta1 cells, CD123+ BDCA-4+ pDCs could be readily detected after 2 wk (Fig. 8A), with an average frequency of 13.5 ± 2.8%, ranging from 4 to 21% of total human cells in the culture. Because our culture media involved regular FCS that contains estrogens at concentrations ranging from 10⁻¹⁵ to 10⁻¹⁰ M (30), we evaluated the effect of ICI182,780 in this system. Indeed, it has been previously shown that ICI182,780 at 10⁻⁷ M can potently affect the differentiation of murine DC in GM-CSF– or Flt3L-driven cultures mainly through the blockade of ERα signaling (30–32). Addition of ICI182,780 to the cocultures slightly decreased the frequency of
pDCs in some donors, although this effect was not significant (Fig. 8A, 8B). pDCs that developed in this culture exhibited an immature phenotype with low expression levels of the maturation marker HLA-DR or the costimulatory molecule CD86. Steady-state expression of these molecules was not modified by addition of ICI182,780 (Fig. 8C). By contrast, after TLR7 stimulation with R-848, upregulation of these two molecules was blunted in ICI-treated pDCs as compared with controls (Fig. 8C, 8D). We were unable to detect IFN-α+ pDC by intracellular staining in cultures stimulated with R-848 for 5 h (not shown), in agreement with previous works by others (33). By contrast, using influenza virus as a TLR7-dependent ligand, we could detect strong IFN-α production in CD34-derived pDCs by intracellular staining (Fig. 9A) and, to a lesser extent, TNF-α–producing pDCs (Fig. 9B). Interestingly, the percentage of pDCs producing IFN-α was reduced by ~2-fold, when CD34-derived pDCs were generated in the presence of ICI182,780 (Fig. 9A), independently of the sex of the donor HPCs. A similar trend was observed for TNF-α–producing pDCs (Fig. 9B). Despite a significant reduction in the frequency of cytokine-producing pDCs in ICI182,780-treated cultures, no difference was observed in the cytokine mean fluorescence intensity per cell, suggesting that ER signaling enhanced TLR7 reactivity without modifying the amount of cytokine produced at the single-cell level (not shown). Together, these data suggested that ER signaling in CD34-derived human pDCs promotes in response to TLR7 stimulation both IRF7-dependent pathway that triggers IFN-α production and NF-κB–dependent pathway that triggers pDC maturation and inflammatory cytokine production (12).

**Discussion**

In this study, we have independently evaluated in vivo the respective contribution of the sex chromosome complement and sex hormones in the female-biased responses of human pDCs. Our data support the conclusion that in addition to the female sex-steroid hormones, estrogens, X-chromosome dosage also contributes to the enhanced TLR7-mediated response of pDCs in women, particularly regarding the production of type I IFNs.

In favor of a role of sex hormones, we recently showed that estradiol-induced activation of ERα in murine pDCs enhanced their capacity to produce IFN-α and proinflammatory cytokines in response to TLR7- and TLR9-mediated stimulation (19, 20). In the present work, we extend these previous results by showing that the innate function of human pDCs is not only enhanced when they develop in female mice as compared with a male environment, but

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**FIGURE 5.** X-chromosome dosage contributes to the enhanced TLR7-mediated IFN-α response of female pDCs in vivo. (A) BM cells from mice reconstituted with CD34+ HPCs from XY (n = 5) or XX (n = 6) cord blood (CB) donors (three recipient mice of each sex per donor, with the exception of XY CD34+ HPCs transferred in only 1 male) were stimulated in vitro with R-848 as in Fig. 1. (B) The frequencies of IFN-α-producing pDCs from individual male and female recipient mice were compared according to the sex of the donor HPCs (left panel). Error bars represent SD values (SD). *p < 0.05, **p < 0.01, ***p < 0.001, one-way ANOVA with Bonferroni post hoc test.

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**FIGURE 6.** Real-time PCR analysis of ESR1 and ESR2 gene expression in pDCs and PBMC subsets from healthy donors. Expression level of ERα (A) and ERβ (B) transcripts in total PBMC (n = 11), CD4 T cells (n = 11), CD8 T cells (n = 4), B cells (LB; n = 8), monocytes (Mono; n = 9), NK cells (n = 2), pDCs (n = 7), and monocyte-derived DCs (MoDC; n = 2). All cell subsets were purified from PBMCs of healthy donors as indicated in Materials and Methods. The copy numbers of transcripts coding for ESR1 or ESR2 gene were quantified using external ER standard plasmids and normalized to the number of GAPDH transcripts quantified in the same way. Results are expressed as ER copy numbers normalized to 10^4 GAPDH copies.
also that this enhancing effect of the female environment was not dependent of the sex of the human HPC donors. Indeed, the enhanced pDC responses observed in female mice were of similar order of magnitude whether pDCs were originated from female XX or male XY HPC donors, suggesting that the sex chromosome complement does not influence estrogen responsiveness. These results are in agreement with previous work in mice showing that the modulatory effects of ER signaling on DC development and functions in vitro were not affected by the sex of the mice (20, 32).

Indeed, we show in this study that ER blockade during human pDC differentiation in vitro strongly diminished the frequency of cytokine-producing cells in response to TLR7 stimulation and, to a lesser extent, their capacity to upregulate activation markers. Again, similar results were obtained when CD34-derived pDCs from either sex were analyzed. These results suggest that ER activation in human pDCs enhances the frequency of cells able to productively respond to TLR7 ligand stimulation, rather than the amount of cytokine produced at the single-cell level. Thus, estrogens may act as a cell-extrinsic factor to positively regulate in a cell-intrinsic manner the TLR7 responses of pDCs in women, independently of the X-chromosome complement. According to our recent work in mouse (19), it is likely that ligand-dependent activation of ER is required to mediate this effect in human pDCs, too. Indeed, our preliminary results in HuMice indicate that E2 supplementation enhances the TLR-mediated responses of pDCs in vivo in agreement with our previous work in postmenopausal women (19). However, unlike their mouse counterpart, we show in the current study that human pDCs express both ESR-1 and ESR-2 genes. As the role of ERα and ERβ within the same cell type is complex and often antagonistic (34), it will be critical to determine the respective contribution of each ER in the regulation of the TLR7-dependent response of human pDCs and whether this

FIGURE 7. Sex does not influence ESR-1/2 gene expression in human pDCs. pDCs were purified from male (n = 5) or female (n = 5) human healthy donors. Relative expression of ESR-1, ESR-2, and TLR-7 genes was quantified by quantitative RT-PCR and normalized to GAPDH gene expression level.

FIGURE 8. Blocking ER signaling during CD34-derived pDC development decreases HLA-DR and CD86 expression by TLR7-stimulated pDCs. CD34+ HPCs were differentiated toward pDCs on OP9-d1 stromal cells in the presence or absence of the pure ER antagonist ICI182,780 (10-7 M). (A) After 2 wk, cells from replicate cultures were counted using the trypan blue exclusion method and analyzed for the expression of BDCA-4 and CD123 by flow cytometry. (B) Frequency and absolute numbers of pDCs per well from seven donors. Wilcoxon signed-rank test. (C) In some experiments, cells were stimulated for 24 h with 1 μg/ml R-848, and the frequency of pDCs expressing HLA-DR or CD86 molecules was further analyzed in replicate cultures (n = 4). (D) Results from four different CD34+ HPC donors. Error bars represent SD values. *p < 0.05, Student t test.
FIGURE 9. The ER antagonist ICI182,780 reduces the TLR7-mediated response of CD34-derived pDCs. CD34-derived pDCs were differentiated in the presence or not of the ER antagonist ICI182,780 as in Fig. 8. After 2 wk, cells were stimulated with influenza virus (Flu) for 5 h, in the presence of brefeldin A for the last 3 h. The frequencies of pDCs (BDCA-4+CD123+) producing IFN-α (A) or TNF-α (B) are indicated. The graphs represent results from eight different CD34+ HPCs. *p < 0.05, Wilcoxon signed-rank test. CB, cord blood.

involves liganded or unliganded ERs. Works are in progress to address these issues in this important cell population critically influenced by sex-dependent factors (35).

TLR7 triggers two signaling cascades, the IRF7 and IRF5/NF-κB pathways resulting in the production of type I IFNs and/or proinflammatory cytokines, respectively (12). Because the enhanced TLR7 responsiveness of pDCs in female recipients was observed for both IFN-α and TNF-α production, we believe that estrogens may regulate key signaling molecules of the TLR pathway or components implicated in their intracellular trafficking or proteolytic cleavage (36). In mice, it has been shown that estrogen signaling in immune cells upregulated the expression of the trafficking TLR7 transmembrane protein, Unc93b (37), as well as IRF5 (38). Whether such estrogen-mediated upregulation of genes also operates in human pDCs to enhance TLR signaling remains to be investigated.

Beside the role of estrogens, we now provide evidence for a direct effect of the X-chromosome complement as a positive regulator of type I IFN production by pDCs in response to TLR7 stimulation. Interestingly, this enhanced response of female pDCs as compared with the male cells was sex hormone independent as it was similarly observed in both male and female mouse recipients. Several X-linked factors could contribute to the enhanced TLR7-mediated production of type I IFNs by pDCs from women through nonexclusive mechanisms (21). Among them, X inactivation escape of genes directly or indirectly involved in TLR signaling can be considered. Based on expression analysis in rodent/human hybrid fibroblast cell lines that retain a human inactive X, it has been shown that up to 15% of X-linked genes display some degree of escape from X inactivation, including genes such as BTK and IRAK1 (39), which are critical components of the endosomal TLR7/9 signaling pathways (12). However, Bruton’s tyrosine kinase has been recently shown to regulate TLR9 rather than TLR7 signaling in human pDCs, suggesting that it may represent a good candidate (40). The TLR7 gene itself is also an attracting candidate, as it is located in the short arm of the human X chromosome, a region carrying several gene clusters for which X inactivation escape has been documented (39). Although previous studies (17) and our present results in Fig. 7 failed to establish sex differences in the expression of TLR7 transcripts in human pDCs at the population level (17), analysis of the allelic expression of TLR7 in human pDCs and B cells at the single-cell level is warranted. Interestingly, it has been shown in mice that Tlr7 gene duplication is sufficient to drive the development of lupus-like disease (41). Systemic lupus erythematosus (SLE) is an autoimmune disease, which preferentially affects women (2), and pDCs play a prominent role in the pathogenesis of this disease through the production of type I IFNs (42). Although a role for estrogens in SLE pathogenesis has been also clearly established, evidence exists that X-linked gene dosage may contribute to disease development in mice (43). It has been shown that XXY men with Klinefelter syndrome have a risk of developing SLE equivalent to that of women, whereas SLE in women with Turner syndrome, who have only one X chromosome, is rarely reported (44). Whether XXY males exhibit enhanced TLR7-dependent pDC responsiveness as compared with XY males is currently unknown.

In summary, our study provides direct evidence of a critical role for X-chromosome dosage on the functional competence of pDCs from women. Our data support the notion that the X-chromosome complement, together with sex hormones, may act in concert to confer elevated production of type I IFNs by pDCs in response to TLR7 ligands, thereby contributing to the enhanced TLR7-mediated response of pDCs from women. This finding might have broader implications for our understanding of the pathogenesis of RNA virus-mediated infectious diseases, such as HIV-1 infection and chronic hepatitis C, for which sex-based differences have been reported (35, 45).

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Disclosures
The authors have no financial conflicts of interest.

References


Supplemental figures

Figure S1. Flow cytometry analysis of cytokine production by pDCs. Bone marrow cells were stimulated for 5 hours with R848, in presence of brefeldin A for the last 3 hours. Cells were then harvested, surface stained, fixed, permeabilized and intracellularly stained for IFN-α and TNF-α. Gating strategies to determine the frequencies of human pDCs (hCD45⁺CD123⁺BDCA-4⁺) producing IFN-α and TNF-α in response to TLR stimulation are represented.
Figure S2: Effect of E2-treatment on pDC function and phenotype in vivo. Male mice (n = 6) were grafted with CD34⁺ HPCs from a male donor. 7 weeks after reconstitution, male mice were subcutaneously injected with a solution of 17β-estradiol (1 µg in 100 ul of castor oil). 5 days after injection, we determined (A) the absolute number of cells in the bone marrow, the frequency of human CD45⁺ cells as well as the frequency of human pDCs. Bone marrow cells were stimulated with R-848 for 5 hours, in presence of brefeldin A for the last 3 hours, and (B) the frequency and absolute number of pDC producing IFN-α were determined. (C) The Geometric mean expression of CD86, CD40, HLA-DR and HLA-A, B, C on pDCs was evaluated. This experiment has been performed twice with similar results.
Figure S3. Flow cytometric analysis of TNF-α-producing monocytes. Bone marrow cells were stimulated for 5 hours with TLR7/8 ligand R848, in presence of brefeldin A for the last 3 hours. Cells were then harvested, surface stained for hCD45 and CD14, fixed, permeabilized and intracellularly stained for TNF-α. Gating strategy to determine the frequency of human monocytes (hCD45⁺CD14⁺) producing TNF-α in response to TLR stimulation is shown.
Figure S4. Analysis of X-chromosome dosage contribution to TNF-α responses of pDCs and monocytes in vivo. Bone marrow cells from mice (3 recipient mice of each sex per donor) reconstituted with CD34+ HPCs from XY or XX CB donors were stimulated in vitro with the TLR-7/8-ligand R-848. (A) The frequencies of TNF-α-producing pDCs from individual mice (3-4 recipient mice from either sex per donor) were normalized in each cohort to the mean values of the XY HPC response independently of the sex of the recipient mice, (left panel). The same results were further represented according to the sex of the recipients (central and right panels). (B) Frequencies of TNF-α-producing monocytes from individual mice were normalized as in (A). Bars represent standard deviation values. N.S., not significant, Mann Whitney U Test.