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RESEARCH HIGHLIGHT

Estrogen receptor-dependent modulation of dendritic cell biology of mice and women

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Autoimmune and infectious diseases differentially affect women from men. Women tend to develop stronger immune responses and thus in general men are more susceptible to infectious diseases whereas women are more likely to develop autoimmune diseases. These differences could be in part attributable to the pro-inflammatory role of the female sex hormone estrogen on immunity and particularly on dendritic cells (DCs), a key subset of innate immune cells. For several years now, we have undertaken studies to understand how estrogens influence the biology of murine and human DCs. We and others have demonstrated that estradiol (E2) was required for the optimal *in vitro* differentiation of murine DCs and acquisition of their effector functions. These effects on DC biology were dependent on the activation of the estrogen receptor α (ERα). More recently, we focused our interest on plasmacytoid dendritic cells (pDCs). Indeed, this subset, that produces large amount of IFN-α/β in response to viral or endogenous nucleic acids through activation of their TLR-7 and TLR-9, shows gender differences with enhanced IFN-α production by pDCs from women, compared to men. We could establish, in Human and in mice, that *in vivo* treatment with E2 enhanced the TLR-dependent production of IFNα by pDCs. In mice, we demonstrated that the amplifying effect of endogenous and exogenous estrogens was dependent on the activation of ERα by E2 in a cell-intrinsic manner. We also provided evidence for ER expression in human pDCs, and we showed that blockade of ER-signaling in developing human pDCs *in vitro* blunted their TLR7-dependent responses. Finally, in a humanized mouse model, we showed that beside the female sex hormone estrogens, X chromosome complement also contributed to the enhanced TLR-7-mediated response of pDCs from women. Altogether, our work demonstrates that estrogen-mediated activation of ER signaling is a key regulator of DC biology both in Human and in mouse, which may account for the sex-based differences in autoimmune and infectious diseases.

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Cumulative evidence indicate that estrogens can positively regulate innate immune responses *in vivo*, which may account for the immunological advantages of females, but also for their higher propensity to develop autoimmune diseases [1,2]. Estrogen effects are mediated through estrogen receptors (ER) ERα and ERβ, which are encoded by the ESR1 and ESR2 genes, respectively [3]. ERs are members of the nuclear receptor super family and mainly function as ligand-inducible transcription factors, and a growing body of evidence supports the notion that immunocompetent cells of the innate and adaptive immune systems functionally express estrogen receptors (ERs), particularly ERα in mouse [4-10].

Alternatively, estrogens, through their ERs, may act on precursor cells to regulate the differentiation and functions of lymphoid and myeloid cells [11-17].

Dendritic cells (DCs) are professional antigen-presenting cells bridging innate and adaptive immunity. They are essential for activation of naive T cells specific for self or non-self protein antigens and for their subsequent differentiation into effector T cells through the secretion of specific cytokines [18]. Multiple DC subsets have been identified, which are divided into four main cell types: conventional/classical DCs (cDC), plasmacytoid DCs (pDCs), Langerhans cells and monocytes-derived $CD11b^+$ inflammatory/migratory DCs [18,19]. While cDCs are specialized for antigen processing and presentation at the steady state, under inflammatory conditions, circulating blood monocytes can be rapidly mobilized and differentiate into cells with many features of cDC [18]. By contrast, pDCs are a distinct lineage, characterized by their capability to rapidly produce great amount of type I interferons in response to viral infections [20]. Due to the importance of DCs in linking innate and adaptive immunity, this cell lineage is likely to represent a key target of E2.

Several studies have shown a role for ERα-signaling in the differentiation and functions of various DC subsets not only *in vitro* [12,15-17], but also *in vivo* [10]. DCs can be generated from bone marrow (BM) precursors cultured in the presence of GM-CSF or Flt3 ligand (Flt3L). GM-CSF induces mainly myeloid DCs (GM-DC) thought to be equivalent to monocyte-derived $CD11b^+$ DCs, while Flt3L allows for the development of DCs (FL-DCs), similar in phenotype and function to splenic resident conventional (cDC) and plasmacytoid DCs (pDCs) [18,19,21]. Agonists or antagonists of ERα can differentially regulate the development of these distinct DC populations *in vitro* [12,15,16,22,23]. For instance, E2 is crucial for the GM-CSF-dependent differentiation of DCs [12,16], through ERα but not ERβ [16]. Interestingly, Lin^- $c\text{-}kit^+$ $Flt3^+$ myeloid progenitors (MP) express high levels of ERα but not ERβ [15], and respond to E2 by up regulating interferon regulatory factor (IRF)-4 in the presence of GM-CSF, thereby promoting DC differentiation [17]. Besides differentiation, E2/ERα activation was subsequently shown to augment the innate function and T cell-stimulatory activity of GM-DCs [16].

Since selective estrogen receptor modulators (SERM) like tamoxifen and raloxifen are in current clinical use, it is important to decipher the mechanisms by which ERα-signaling regulates inflammatory and homeostatic DC development and functions. Full-length ERα (66 kDa) consists of six domains (A–F) and two separate transactivation functions (AF), AF1 and AF2, which reside in the N-terminal A/B domain and the C-terminal E domain, respectively [24,25]. AF-2, in the C-term ligand-binding domain, exerts a ligand-dependent transcriptional activity and has been shown to be required for all the genomic actions of ERα [26]. Although, interactions between AF domains are essential for full ligand-dependent transcriptional activity of ERα, AF-1 and AF-2 can activate transcription independently in a promoter-specific and cell-specific manner [25,27,28]. It has been proposed that the relative contribution of AF1 in ERα transcriptional activity may depend upon the differentiation stage of the cells [29] or the tissue examined [30]. For instance, ERα AF1 has been shown to be dispensable for E2-induced vascular protection [31] and osteoporosis prevention [32]. By contrast, AF-1 was necessary for the proliferation of breast cancer cells [33] and for the normal E2-induced increase in uterine weight *in vivo* [31]. These results indicate that the requirement for AF-1 is largely tissue dependent. We therefore recently investigated whether AF1 domain of ERα could differentially regulate the development and functional properties of the DC subsets that develop in the presence of GM-CSF or Flt3L [34].

In agreement with previous work [17], we showed that E2-mediated *Irf-4* expression in MPs was critically dependent on ERα, as it was lost in $ER\alpha^{-/-}$ cells despite similar basal expression at steady state. Moreover, we demonstrated that both AF-1 and AF-2 domains of ERα were required for sustained *Irf-4* expression in GM-CSF-stimulated MPs at early stage of differentiation in the presence of E2. By contrast, at later time points, we observed a significant increase in IRF-4 expression in fully differentiated $ER\alpha AF\text{-}1^0$ DCs, but not in $ER\alpha AF\text{-}2^0$ or $ER\alpha^{-/-}$ DCs. Thus, in the absence of AF-1, E2/ERα signaling in GM-CSF-stimulated DC precursors could still lead to an enhanced IRF-4 expression to levels compatible with the development of the more functional $Ly6C^-$ DC subsets. By contrast, in Flt3L-driven DC differentiation, activation of AF1 domain was required to promote the development of more functionally competent cDCs and pDCs [34]. Notably, we showed that E2/ERα activation in developing cDC and pDC was associated with an enhanced production of pro-inflammatory cytokines, such as IL-16 and IL-12, upon stimulation of their endosomal TLRs, TLR-9 or TLR-7, respectively. In addition, lack of ERα AF-1 blunted the TLR-7 mediated IFN-α response of female pDCs *in vivo*. In conclusion, this work provided the first evidence that discrete AF domains of ERα could differentially regulate the development and functional properties of the DC subsets that develop in the presence of GM-CSF or Flt3L [34]. Moreover, we showed that E2/ERα activation during DC development exerted proinflammatory effects on both GM-CSF-derived and Flt3L-dependent conventional DC subsets, probably by promoting the differentiation of DCs that exhibit superior

innate functions.

Although our work [34] suggests that the impact of E2 on cytokine-driven DC differentiation mainly results from AF1-dependent or -independent genomic effects, the contribution of non-genomic mechanisms could also be at play. Indeed, the PI3K/PKB signaling pathway can be activated by acute exposure to E2 *in vitro* in different cell types including endothelial cells and cortical neurons through membrane-initiated steroid signaling (MISS) effects [35-37]. MISS is mediated by a fraction of ERα that is localized at the cytosolic face of the plasma membrane through palmitoylation of Cys 451 in mouse. MISS effects are rapid and include mobilization of intracellular calcium, and the stimulation of several kinases such as PI3K/Akt, MAPK, or PKC [30]. Whether both pathways operate in a coordinate manner in DC to regulate one unique effect (e.g. differentiation or TLR responsiveness) is presently not known. In the GM-DC model, we demonstrated that AF-2 mutant mice, which selectively lack nuclear ER actions [26], exhibited the same phenotype as full-ERα-deficient mice. In these mice, GM-CSF-driven DC differentiation was strongly impaired demonstrating that MISS cannot substitute for the lack of genomic action of ERα [34]. Alternatively, MISS and nuclear action of ERα could represent parallel pathways with distinct or antagonistic effects. For instance, in a similar model, it has been recently shown that short-term acute exposure of GM-DC to E2, in a dose-dependent manner, resulted in the down-regulation of Nf-κB activation upon TLR-stimulation [38]. This anti-inflammatory effect of E2 required higher doses of hormone as compared to the doses required to promote GM-DC development, and was due to the transcriptional repression of NEMO (*Ikbkg*) the regulatory subunit of the inhibitor of the IKK complex [38]. Although, it remains to be demonstrated that this inhibitory action of E2 on DC function is mediated through ERα activation, we may speculate that it could involve some MISS actions of ERα. The use of recently described mouse models, such as mice lacking C451 palmitoylation site which lack known MISS effects [26], may help to answer this question and will be useful to provide a detailed understanding of the respective contribution of membrane versus nuclear actions of a steroid hormone receptor in DC biology.

An important issue concerns the relevance of the *in vitro* effects of E2 on DC biology in mouse models to the *in vivo* situation, and its translation to human DC biology. We decided to focus on a particular DC subset, the pDCs, which functions appear to be highly regulated by sex-dependent factors [39,40]. pDCs are specialized type I IFN (IFN-α/β)-producing cells that sense viral nucleic acid in the context of infections through intracellular Toll-like receptor (TLR)-7 and TLR-9. Aberrant type I interferon (IFN) signaling has been shown to be implicated in the enhanced susceptibility of females to some viral infections or autoimmune diseases. Sex-dependent differences in TLR-mediated type I IFN production by pDCs from healthy human subjects have been recently reported [39,40]. It was shown that pDCs from women exhibited an enhanced capacity to produce type I IFNs in response to TLR-7-stimulation [39,40]. We have recently investigated the molecular mechanisms leading to this sexual dimorphism in the functional properties of pDCs [10,34,41].

We first tested the hypothesis that abundance of the female sex hormone estrogens may regulate TLR responsiveness of human pDCs. We first showed that pDCs from post-menopausal women exhibited a reduced TLR-mediated response as compared to pre-menopausal women. To directly demonstrate that estrogen deprivation could be responsible for this reduced TLR-mediated response of pDCs in post-menopausal women, we evaluated the effect of E2 supplementation on pDC functions using doses and routes of E2 administration that are currently used in the clinic for hormonal replacement therapy. Our data show that E2-treatment substantially enhanced TLR-mediated IFN-α and TNF-α production by blood pDCs in response to TLR-9 and TLR-7 stimulation. Enhanced cytokine production by pDC was not only observed in response to synthetic ligands for TLR-7 or TLR-9, but also in response to natural ligands such as self-nucleic acid-containing immune complexes present in SLE/lupic patient sera [10]. Using ERα-deficient mice, we provided direct *in vivo* evidence for endogenous and exogenous estrogen-dependent modulation of the TLR responses in mouse pDCs through hematopoietic expression of ERα. Lastly, using mice lacking ERα in the CD11c lineage, we demonstrated that the ligand inducible up-regulation of IFN-α production by TLR-7 and TLR-9-activated pDCs required ERα expression within the DC compartment, including pDCs. Altogether, our results point to a cell-intrinsic role for ERα-signaling in the enhanced TLR-mediated activation of female pDCs [10].

To gain further insight into how sex-linked factors may regulate human pDC biology, we designed a humanized mouse model (HuMouse) to directly examine the respective contribution of female sex hormones and X-linked factors to the enhanced TLR-7-mediated responses of human pDCs *in vivo* [41]. Male or female NOD-SCID-ß2m$^{-/-}$ mice were transplanted with human CD34^{+} progenitor cells (HPCs) purified from either male or female donors. Human pDCs that developed in the bone marrow of HuMice were subsequently assessed for their capacity to ex vivo produce cytokines in response to TLR7/8 ligands, including influenza virus and HIV-derived ligands. We showed that, in response to TLR-7 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. We also examined whether antagonizing ER-signaling using the pure ER antagonist $ICI_{182,780}$ in developing human pDCs could regulate their TLR-7-dependent responses. We used an *in vitro* model of Flt3L/IL-7-driven human pDC differentiation from $CD34^+$ HPCs. Interestingly, the percentage of pDCs producing IFN-α was reduced by almost two-fold, when CD34-derived pDCs were generated in the presence of $ICI_{182,780}$, independently of the sex of the donor HPCs. 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* was not affected by the sex of the mice [16,34]. Indeed, we show here that ER-blockade during human pDC differentiation *in vitro* strongly diminished the frequency of cytokine-producing cells in response to TLR-7-stimulation, and to a lesser extent their capacity to up-regulate maturation markers, such as MHC class II and costimulatory molecules, such as CD86. However, unlike their mouse counterpart, we showed that human pDCs express both *ESR-1* and *ESR-2* genes [41]. As the role of ERα and ERβ within the same cell type is complex and often antagonistic [42], it will be critical to determine the respective contribution of each ER in the regulation of the TLR-7-dependent response of human pDCs, and whether this involves liganded or unliganded ERs. Works are in progress to address these issues in this important cell population critically influenced by sex-dependent factors [1].

ER activity may regulate the function of innate immune cells such as pDCs via several mechanisms, acting on precursor cells during pDC differentiation or on mature pDC to directly regulate gene expression. We believe that estrogens may regulate key signaling molecules of the TLR pathway, or components implicated in their intracellular trafficking or proteolytic cleavage [43]. In mice, it has been shown that estrogen signaling in immune cells up-regulated the expression of the trafficking TLR7 transmembrane protein, Unc93b [44], as well as IRF5 [45]. Whether such estrogen-mediated upregulation of genes also operates in human pDCs to enhance TLR-signaling warrant further investigation.

Interestingly, we also found that X chromosome dosage contributed to this sex bias as female pDCs have an enhanced TLR-7-mediated IFN-α response as compared to 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 from women [41].

Overall, our data have recently provided compelling evidence that E2/ERα-signaling is a key regulator of the development and the effector function of various DC subsets [10,16,34,41]. We established that estrogen is an important enhancer of type I IFN production by human and mouse pDCs [10]. Indeed, using estrogen receptor (ER) α deficient mice in the CD11c lineage, we provided direct *in vivo* evidence for estrogen-dependent modulation of the TLR responses in mouse pDCs through cell-intrinsic expression of ERα [10]. In human, beside estrogen, we also showed that X-linked genetic factors may also independently contribute to enhance TLR-7-mediated type I IFN production by pDC from women [10,41]. Understanding further the detailed mechanisms of ER-mediated nuclear or MISS responses that interfere with the development and effector functions of various DC subsets may help to select SERM able to differentially regulate steady-state resident or inflammatory DCs *in vivo*, in order to optimize selective ERα modulation of innate immunity in various pathophysiological contexts.

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Competing interests

The authors have declared that no competing interests exist.

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