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TLR7 Ligands Induce Higher IFN-α Production in Females[1]

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IFN-α exercises multiple immune modulatory and antiviral activities and has been suggested to play a critical role in the pathogenesis of systemic lupus erythematosus (SLE). Plasmacytoid dendritic cells (pDCs) release IFN-α upon TLR7 and TLR9 ligation. With respect to the nine times higher incidence of SLE in women and the clinical use of synthetic TLR ligands as novel immune adjuvants, we analyzed IFN-α and TNF-α production in healthy human individuals. Blood samples were incubated with synthetic TLR7 and TLR9 ligands. In three independent groups ($n_1 = 120$, $n_2 = 101$, and $n_3 = 123$), analysis revealed a capacity of female PBLs to produce significantly higher IFN-α levels after TLR7 stimulation ($p_1 < 0.0000001$, $p_2 < 0.0000001$, and $p_3 < 0.0001$) compared with male PBLs. In contrast, no sex differences were evident after TLR9 stimulation. TNF-α production after TLR7 stimulation and also total pDC numbers were not different between females and males. X-inactivation escape of the *TLR7* gene was investigated in monoclonal B cell lines and, independently, in pDCs after cell sorting and single-cell picking, indicating regular silencing of one *TLR7* allele in females. Additionally, exogenous 17β-estrogen and estrogen receptor antagonism did not indicate a significant role on TLR7-induced IFN-α production. Our data reveal for the first time a profound sex-dependent pathway of TLR7-induced IFN-α with higher production in females. These findings may explain the higher prevalence of SLE in females and the reported decreased therapeutic efficacy of synthetic TLR7 ligands in male individuals. *The Journal of Immunology,* **2006, 177: 2088–2096.**

Interferon α is a critical player in the immune system linking innate and adaptive immunity (1). It is the most widely used cytokine for clinical therapy of chronic viral hepatitis and different malignancies (2). IFN-α enhances the first defense against viral infections and has several direct effects in modulating immune cells: IFN-α primes maturation and activation of dendritic cells (DCs)[3] to generate effective APCs with increased capacity to stimulate allogenic $CD4^+$ and $CD8^+$ T cells (3–6). Thereby, it mediates IFN-γ release and promotes the killing activity of $CD8^+$ T cells and NK cells (7). Consequently, Th1 differentiation is supported by IFN-α, whereas suppression of IL-4/5 blocks Th2 skewing (8, 9). Furthermore, IFN-α enhances mature lymphocyte survival (10) and promotes B cell class switching (11, 12). Recently, plasmacytoid DCs (pDCs) have been identified to represent the principal source of this cytokine in the immune system (13, 14). Subsequent reports demonstrated that the IFN-α production of pDCs is mediated through stimulation of TLR7 and TLR9 (15–18).

Besides its clinical importance as the major antiviral and antiproliferative cytokine, IFN-α has been identified as the central cytokine in the pathogenesis of the autoimmune disease systemic lupus erythematosus (SLE) (19). SLE is characterized by autoantibodies against dsDNA that form immune complexes. These complexes induce systemic injury by inflammation of multiple organs (skin, joints, CNS, and various blood elements). SLE patients show elevated IFN-α levels in circulation (20–24), and the sera of SLE patients trigger IFN-α production by pDCs (25–29). Interestingly, pDC numbers in blood of SLE patients are decreased (6) because pDCs are actively recruited to inflamed tissues, lupus skin lesions, and lymphoid organs (30, 31). Furthermore, IFN-α-regulated genes are overexpressed in peripheral blood cells of SLE patients (25, 32–36), and rIFN-α used for the treatment of viral hepatitis or certain malignancies was found to induce SLE-associated autoantibodies and occasionally clinical SLE in patients (37–39).

With respect to the pivotal role of IFN-α in SLE pathogenesis, it is remarkable that SLE belongs to the autoimmune diseases with the strongest deviation in both sexes: it has a nine times higher incidence in women than in men (40).

In our present study, we identify for the first time a sex-dependent pathway for IFN-α induction in healthy human subjects. Analyses of three independent cohorts ($n_1 = 120$, $n_2 = 101$, and $n_3 = 123$) of healthy blood donors stimulated with different TLR ligands revealed that TLR7 stimulation promotes significantly higher IFN-α production in female subjects than in male subjects. Further analyses excluded estrogen signaling and revealed no evidence of putative X-inactivation escape of the *TLR7* gene as major causes for sex-dependent IFN-α production. The identification of increased IFN-α production in female subjects through TLR7 stimulation may explain the higher prevalence of SLE in females and has direct clinical relevance with respect to the use of TLR7 ligands as novel immune adjuvants.

Materials and Methods

Subjects

A total of 344 healthy blood donors ($n = 175$ female; $n = 169$ male subjects) entered the study after they gave their informed consent. All blood donors included in the study are Caucasians and have been randomized for sex and age (18–65 years). The mean age for females and males

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[3] Abbreviations used in this paper: DC, dendritic cell; BDCA, blood DC Ag; pDC, plasmacytoid DC; SLE, systemic lupus erythematosus; SNP, single nucleotide polymorphism.

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in the three study cohorts was almost identical: n1 (120 subjects), 33.5 vs 35.7 years; n2 (101 subjects), 31.4 vs 33.6 years; and n3 (123 subjects), 31.2 vs 32.5 years. The study has been reviewed and approved by an appropriate institutional review committee.

Preparation of PBLs and purification of pDCs: cultivation and stimulation

Human PBLs were isolated from heparin-anticoagulated blood samples (in case of experiments with 17β-estrogen and ICI 182,789: EDTA-anticoagulated) by ammonium chloride lysis (Puregene RBC Lysis solution; Gentra Systems). A total of 2×10^6 cells was cultured in 96-well round-bottom plates in 200 μl of medium, comprising RPMI 1640 with L-glutamine, penicillin/streptomycin, 10% heat-inactivated FCS Gold (PAA Laboratories), nonessential amino acids (Sigma-Aldrich), sodium pyruvate, and HEPES (Invitrogen Life Technologies). The stimuli were used at comparable stimulatory activity. Optimal dose response to R848 was found at a concentration of 1 μg/ml (Fig. 1*A*). Optimal time point for measurement of IFN-α in male and female subjects in TLR7- and TLR9-stimulated cultures was estimated after 20 h of stimulation (Fig. 1*B*). Stimulation of the cells was performed with the following different stimuli: 1 μg/ml resiquimod (R848; InvivoGen), 1 μg/ml imiquimod (R837; 3M Pharmaceuticals), and 3 μg/ml CpG-ODN 2216 (InvivoGen), unless otherwise noted.

In several experiments, 17β-estradiol (500, 50, 5, and 0.5 nM; Sigma-Aldrich Laborchemikalien) or estrogen receptor antagonist ICI 182,789 (1000, 100, and 10 nM; Biotrend Chemikalien), diluted in DMSO, was supplemented to the cell culture at $t = 0$ min. Controls consisted of DMSO only. Culture supernatants were collected after 20 h, unless otherwise noted.

pDCs were purified from PBMCs, isolated by Ficoll-Paque density gradient centrifugation, using the blood DC Ag (BDCA)-4 DC isolation kit (Miltenyi Biotec). pDCs were labeled with anti-BDCA-4 Ab coupled to colloidal paramagnetic MicroBeads and passed twice through a magnetic separation column using the magnetic cell sorter autoMACS (Miltenyi Biotec). Purity of the cells was at average 92.9%. Cultivation of isolated pDCs was performed in the medium mentioned above. For quantification of pDCs and purity determination, cells were stained with anti-BDCA-2-PE (Miltenyi Biotec) and analyzed by flow cytometry using BD Biosciences FACSCalibur flow cytometer (BD Biosciences).

Detection of cytokines

Human IFN-α and TNF-α were measured by ELISA (Human IFN-α ELISA kit, PBL Biomedical Laboratories; BD OptEIA ELISA Sets, BD Biosciences).

Polyclonal EBV-immortalized human B cell cultures

Human PBMCs, isolated by Ficoll-Paque density gradient separation, were washed twice with RPMI 1640, resuspended in 0.3 ml of virus-enriched supernatant of the EBV-transformed B95-8 marmoset cell line, and incubated with 500 μl of RPMI 1640 containing 20% FCS, 0.1% gentamicin (Invitrogen Life Technologies), and 0.5% cyclosporine (Sigma-Aldrich) in sterile 4.5-ml tubes (Greiner Bio-One) at 37°C and 5% CO_2. Every 2 days, 0.7 ml of medium (RPMI 1640 containing 20% FCS, 0.1% gentamicin, and 0.25% cyclosporine) was added, and after 3 wk, cells were transferred to 40-ml flasks (Nunc) in 15 ml of IMDM with Glutamax I plus 4% MEM-α medium with ribonucleosides and deoxyribonucleosides (Invitrogen Life Technologies), 15% heat-inactivated FCS Gold (PAA Laboratories), 0.5% penicillin/streptomycin (Invitrogen Life Technologies), 7.7 mM insulin (Sigma-Aldrich), and 1.15 mM oxaloacetate (Sigma-Aldrich). Half of the cell suspension was removed and substituted by new medium every 2–3 days.

Establishment of monoclonal EBV-transfected B cell lines

EBV-immortalized B cell cultures were diluted to 10, 1, and 0.3 cells/100 μl (IMDM plus supplements mentioned above completed with 2.5 μg/ml CpG-ODN 2006) and cultivated in 96-well round-bottom plates (200 μl/well) in the presence of irradiated mononuclear feeder cells (1×10^5 cells in 100 μl, 30 Gy). Growing cells were transferred to 24-well plates and later to 40-ml flasks.

Clonality of cell lines was tested with the Ag Receptor Gene Rearrangement Assay (InVivoScribe Technologies). DNA was amplified by PCR using specific primers for conserved framework and J regions. Readout was performed according to the manufacturer's description by using the ABI Prism 3100 Genetic Analyzer (PerkinElmer). In case of successful rearrangement and monoclonality, 1 amplicon of expected size was displayed.

DNA and RNA isolation

Genomic DNA was extracted from EDTA-anticoagulated blood samples using the Puregene DNA isolation kit (Gentra Systems).

DNA from EBV-immortalized human B cell cultures was isolated using the QIAamp Blood Mini kit (Qiagen). The RNA of EBV-immortalized B-cells, PBMCs, and pDCs was isolated by using the Qiagen RNeasy Mini kit (Qiagen). Quantity and quality measurements were performed by using UV spectrometry (NanoDrop ND-1000; NanoDrop Technologies).

Single nucleotide polymorphism (SNP) genotyping

The *TLR7* gene is located on chromosome Xp22.2. The *TLR7* exon polymorphism we analyzed was SNP *rs179008* (*A*>*T*), bp 17,961 relative to start codon ATG on the *TLR7* gene on exon 3. SNP genotyping of the *TLR7* exon polymorphism was performed by TaqMan allelic discrimination using the Assay-by-Design SNP Genotyping Assays (Applied Biosystems). Both alleles were scored in one well by using Primers and TaqMan minor groove binder probes labeled with VIC and FAM dye (forward primer, 5′-CTT TCA GGT GTT TCC AAT GTG GAC-3′, and reverse primer, 5′-CCC CAA GGA GTT TGG AAA TTA GGA T-3′; probes, 5′-TGA

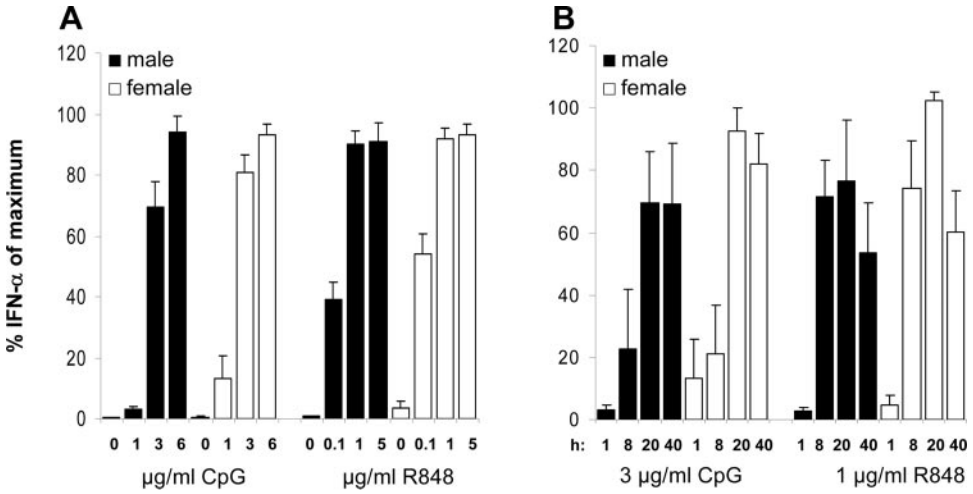

FIGURE 1. Similar time kinetic and dose response of female and male subjects with respect to each individuals maximum IFN-α production after TLR7 and TLR9 stimulation. IFN-α was quantified by ELISA. The data are plotted as percentage of maximal IFN-α production (mean ± SE) and stratified for sex to compare relative stimulatory concentrations for TLR7 and TLR9 ligands. Dose response (*A*) of PBLs ($n = 13$ males; $n = 12$ females) stimulated with the indicated concentrations of TLR7 ligand R848 and TLR9 ligand CpG-ODN 2216 (*A*). TLR9 stimulation through CpG-ODN 2216 resulted in higher absolute IFN-α production when compared with TLR7 stimulation (mean absolute value of maximal IFN-α: 584 pg/ml, R848-stimulated; 1782 pg/ml, CpG-ODN 2216-stimulated culture; 5×10^5 PBLs). Time course (*B*) of IFN-α production of PBL ($n = 5$ males; $n = 5$ females) stimulated with CpG-ODN 2216 or R848 (mean absolute value of maximal IFN-α: 90.1 pg/ml, R848-stimulated; 662 pg/ml, CpG-ODN 2216; 5×10^5 PBLs).

AGA GAC **A**AA TTC-3′, and 5′-ACT GAA GAG AC**T** AAT TC-3′; bold characters indicate the polymorphism). PCR was conducted according to the manufacturer's protocols on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems) using the following program: 50°C, 2 min; 95°C, 10 min; and 40 cycles at 95°C, 15 s and 60°C, 1 min. The accuracy of genotyping was confirmed by known sequenced DNA samples of each genotype and water samples running as controls for each run.

cDNA synthesis

For cDNA synthesis, 1 μg of RNA diluted in H_2O was heated to 70°C for 10 min and then cooled on ice for 5 min. Afterward, 7.5 μl of MasterMix containing 2 μl of 10× buffer II, 2 μl of $MgCl_2$ (25 mM), 1 μl of dNTPs (10 mM), 1 μl of random hexamers (50 μM), 1 μl of reverse transcriptase Moloney murine leukemia virus (50 U/μl), and 0.5 μl of RNase inhibitor (20 U/μl) were added to a final volume of 20 μl (Applied Biosystems; dNTPs, Eurobio). The reaction was performed as follows: 10 min at 20°C, 75 min at 42°C and stopped by incubating at 95°C for 5 min.

mRNA quantification by real-time PCR

Starting material for RT-PCR was RNA isolated from 10 ml of EDTA-anticoagulated blood samples or 6 ml of EBV-immortalized B cell suspension containing 1.8×10^7 cells and transcribed to cDNA according to the protocol above. Real-time PCR was performed on an ABI PRISM 7000 Sequence detection system (Applied Biosystems). The following validated PCR primers and TaqMan minor groove binder probes (6FAM-labeled) were used: TLR7 (assay ID: Hs00152971_m1), TLR9 (assay ID: Hs00152973_m1), and for endogenous control GAPDH (Hs99999905_m1). PCR mix was prepared according to the manufacturer's instructions. Amplification was conducted as follows: 50°C, 2 min; 95°C, 10 min; and 40 cycles at 95°C, 15 s and 60°C, 1 min.

Single-cell picking of purified pDCs

Single-cell picking using the Laser Microbeam System (P.A.L.M.) was performed by our group as described in detail previously (41, 42). One milliliter of the purified pDCs in suspension was spun down for 8 min at 1200 rpm on glass slides (SuperFrost Plus; Menzel-Glaeser) using the Cytospin 2 centrifuge (Thermo Shandon). After hemalaun staining for 45 s, the cytospins were immersed in 70, 96, and 100% ethanol until immediate use. Single pDCs were isolated by a sterile 30-gauge needle mounted on a micromanipulator and were transferred into a reaction tube containing 4.5 μl of first strand buffer (52 mM Tris-HCl (pH 8.3), 78 mM KCl, and 3.1 mM $MgCl_2$).

Single-cell PCR

cDNA synthesis was performed as described above, except in a total volume of 11 μl and elongation for 60 min. Reagents, as well as primers, were applied as described previously (41). Eleven microliters of cDNA was subjected to PCR amplification. Primers were added to a final concentration of 200 nM each (forward, 5′-TCA ACC AGA CCT CTA CAT TCC-3′, exon 2; reverse, 5′-AAA CCA TCT AGC CCC AAG GA-3′, exon 3) in a total volume of 50 μl. Cycling conditions were 95°C for 6 min, followed by 55 cycles of 95°C, 20 s; 62°C, 30 s; and 72°C, 30 s. Gel electrophoresis was performed to check correct amplicon length (130 bp). Routinely applied negative controls included samples with needles that were transferred directly to the first strand buffer (needle control) and samples containing the PCR mix (buffer control). Additionally, a positive control with respective cDNA was amplified in parallel. Furthermore, intron-spanning (intron 2, 17,930 bp) primers were designed to avoid amplification of contaminating genomic DNA.

Determination of monoallelic and biallelic expression by PCR-RFLP

cDNA obtained from single pDCs was used for additional amplification. PCR was performed consisting of 50 ng of cDNA, 10× PCR buffer with $MgCl_2$ (Applied Biosystems), 0.2 mM each of the dNTPs (Pfizer), 0.5 μM of each primer (sequence see above), and 0.5 U of AmpliTaq Gold (Applied Biosystems). The PCR conditions were as follows: 95°C, 10 min (pre-PCR activation step); 40 cycles at 95°C, 30 s; 62°C, 30 s; 72°C, 30 s; and 10°C, 15 min. PCR product was diluted 1/10 and subjected to another round of PCR amplification. PCR product was digested by 10 U of *Apo*I at

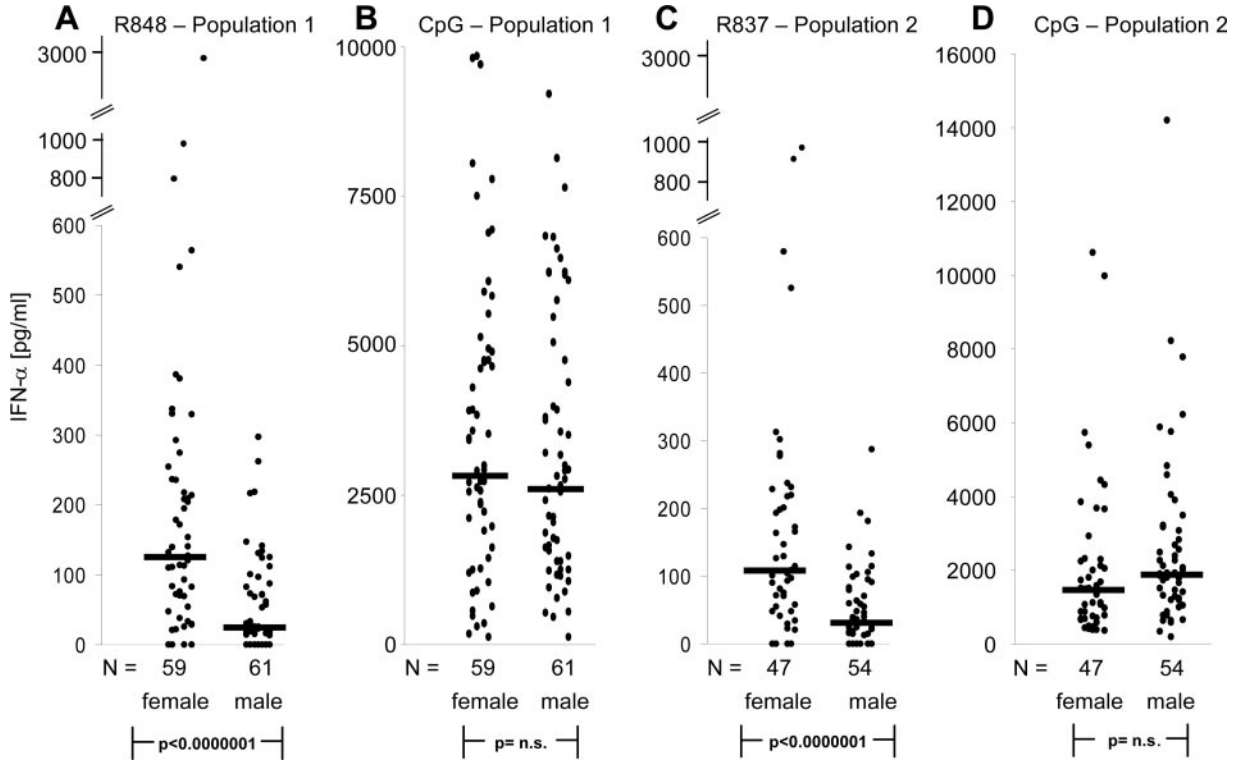

FIGURE 2. Sex-dependent IFN-α production through TLR7 stimulation. PBLs of two independent cohorts of healthy individuals were stimulated with TLR7 agonists R848 (*A*) and R837 (*C*), and IFN-α was quantified by ELISA. In control experiments, PBLs of the same individuals were stimulated with TLR9 agonist CpG-ODN 2216 (*B* and *D*). In two independent groups, the TLR7 agonists induced significantly higher IFN-α in female than in male individuals ($p_1 < 0.0000001$; $p_2 < 0.0000001$). Results show individual data points in a dot plot diagram, with the median indicated as ■, of $n = 120$ (*A* and *B*) and $n = 101$ (*C* and *D*) individuals. Values of *p* refer to two-tailed Mann-Whitney *U* test. IFN-α levels of unstimulated PBL cultures were below the detection limit (12.5 pg/ml).

50°C for 2 h in NEBuffer 3 supplemented with 100 μg/ml BSA. Control preparation was digested by 10 U of Tsp509 I at 65°C for 2 h in NEBuffer 1 (New England Biolabs). Fragments were visualized after gel electrophoresis (2.5% agarose gel) and ethidium bromide staining under UV lamp.

Results

Sex-specific IFN-α induction through TLR7 stimulation

pDCs represent the principal source of IFN-α in human blood and express TLR7 and TLR9 that mediate high IFN-α production. The imidazoquinolines resiquimod (R848) and imiquimod (R837) represent synthetic TLR7 agonists that induce IFN-α production in pDCs in a TLR7-specific manner (43, 44). The TLR9 agonist CpG-ODN 2216 has been shown recently to specifically induce IFN-α in pDCs (14).

Analysis of 120 healthy individuals revealed a highly significant difference of female and male PBLs to produce IFN-α after TLR7 stimulation with R848 (Fig. 2*A*; $p < 0.0000001$, two-tailed Mann-Whitney *U* test). In contrast, IFN-α induction in the same females and males after TLR9 stimulation with CpG-ODN 2216 did not differ significantly (Fig. 2*B*), indicating that preferential IFN-α induction in female subjects is TLR7 dependent.

To confirm this finding, we repeated the experiments with a second independent cohort of 101 healthy individuals and a second TLR7 agonist, R837. The results again indicated a highly significant preference of TLR7 but not TLR9 agonists to promote IFN-α production in female individuals (Fig. 2, *C* and *D*; $p < 0.0000001$, two-tailed Mann-Whitney *U* test). To control for possible differences in the total number of IFN-α-producing cells between female and male subjects, we quantified the pDC numbers of the first cohort by flow cytometry based on BDCA-2 expression. There were no differences in the total pDC numbers between female and male subjects ($n = 109$), excluding the possibility that preferential IFN-α induction in females after TLR7 stimulation is related to different pDC numbers (Fig. 3).

Since pDCs are the major IFN-α producer, we investigated in a small cohort if the sex-dependent IFN-α production could also be shown in purified pDCs (sorted to >92% purity). In confirmation with the results found in the PBL experiments with large cohorts of blood donors, we found elevated absolute IFN-α in female vs male subjects after TLR7 stimulation of purified pDCs (median IFN-α production of 50,000 pDCs: 8,258 pg/ml in females vs 4,804 pg/ml in males).

Sex-specific cytokine induction through imidazoquinolines is restricted to IFN-α

Since imidazoquinolines have been reported to represent no exclusive IFN-α inducer and to exert additional stimulatory activity through TLR8, we analyzed a second pDC-independent cytokine, TNF-α. TNF-α is produced in significant amounts by many different types of leukocytes. Stimulation of 123 individuals with R848 revealed no significant TNF-α level differences between female and male individuals, whereas IFN-α levels again differed significantly ($p = 0.0001$) between the two groups (Fig. 4, *A* and *B*). These results suggest that sex-dependent cytokine induction through R848 is restricted to IFN-α.

TLR7-induced IFN-α levels are not influenced by exogenous 17β-estradiol or estrogen receptor antagonism in vitro

Significantly increased expression of IFN-α by female PBLs after TLR7 stimulation led us to analyze the impact of estrogen signaling on IFN-α production. Therefore, the PBLs of 12 individuals were stimulated with 1 μg/ml R848, in the presence of different 17β-estradiol concentrations (0.5, 5, 50, and 500 nM) to analyze a putative influence of 17β-estradiol on IFN-α production in females and males.

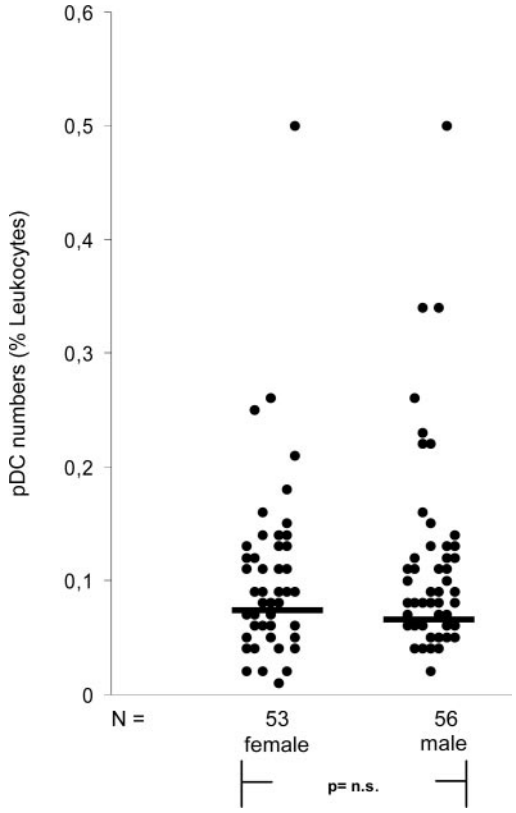

FIGURE 3. pDC numbers in male PBLs correspond to females. pDC numbers in males and females were quantified by flow cytometry according to BDCA-2 surface expression in $n = 109$ individuals. Individual data points are shown in a dot plot diagram. The black bar indicates the median.

We found that IFN-α levels in R848-stimulated cultures without 17β-estradiol corresponded to 17β-estradiol supplemented cultures in the entire group, as well as in male and female subgroups. 17β-Estradiol had no significant impact on IFN-α production (two-sided paired *t* test, Wilcoxon signed-rank test). It should be noted that, in agreement with the above described experiments, the median and mean IFN-α values in all female PBL cultures were consistently higher than in corresponding male PBL cultures (Table I).

To independently address the question if estrogen receptor signaling interferes with IFN-α expression in male or female individuals after TLR7 stimulation, we investigated the impact of estrogen receptor antagonist ICI 182,780 on TLR7-induced IFN-α production. In contrast to the partial estrogen receptor antagonist tamoxifen, ICI 182,780 is an exclusive antiestrogen due to its lack of estrogen-like activity (45–48). Furthermore, ICI 182,780 causes disassociation of receptor-associated proteins, an impaired receptor dimerization and increased receptor degradation (49, 50).

We investigated IFN-α production in PBL cultures of 12 individuals after TLR7 stimulation with R848 in the presence of different ICI 182,780 concentrations (10 nM, 100 nM, and 1 μM). Again, IFN-α levels in R848-stimulated cultures were not significantly affected by the presence of different ICI 182,789 concentrations (two-sided paired *t* test, Wilcoxon signed-rank test). Similar to the results above, male PBL cultures showed lower IFN-α values compared with identically stimulated female cultures (Table II).

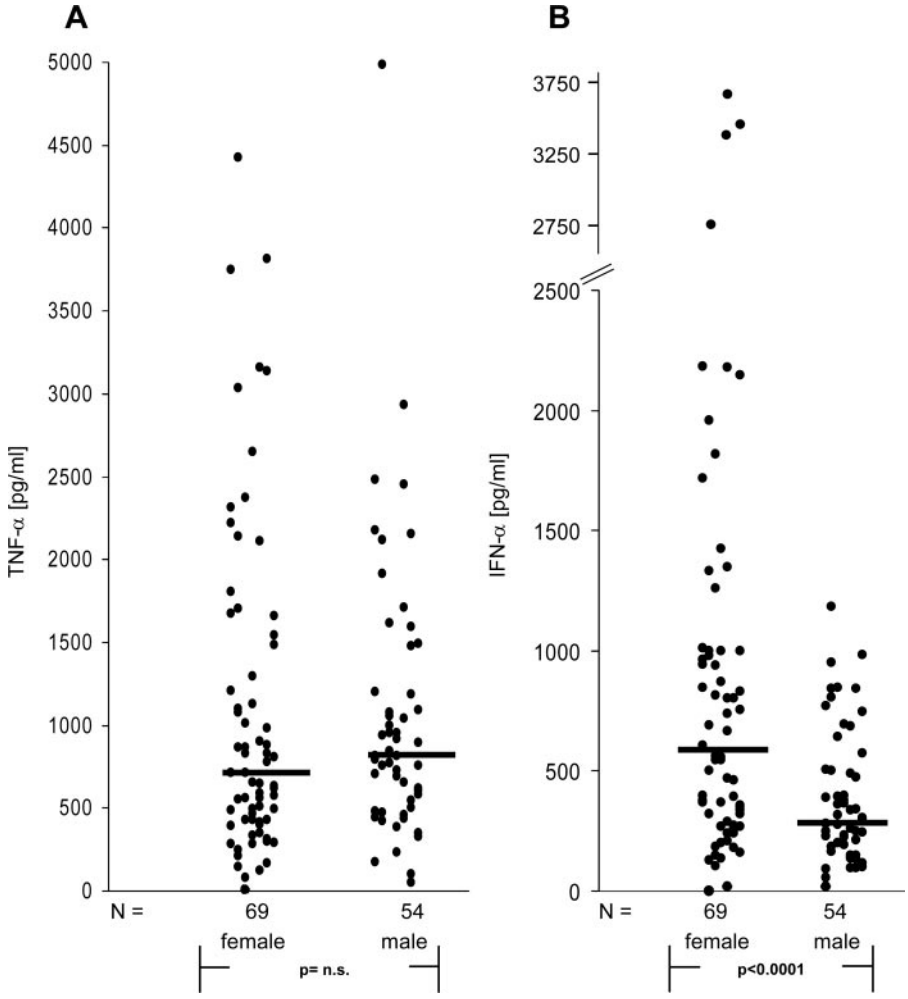

FIGURE 4. Sex-dependent cytokine induction of TLR7 agonists is restricted to IFN-α. PBLs of healthy individuals were stimulated with TLR7 agonist R848. IFN-α and TNF-α were quantified by ELISA. TLR7 stimulation induced significantly higher IFN-α in female than in male individuals (*B*; $p = 0.0001$), whereas TNF-α production in the same individuals was not affected (*A*). Results show individual data points of $n = 123$ individuals in a dot plot diagram with the median (■). Values of p refer to two-tailed Mann-Whitney U test. IFN-α levels of unstimulated PBL cultures were below the detection limit (12.5 pg/ml).

TLR7 does not to escape from X-inactivation in human EBV-immortalized monoclonal B cell lines

Next, we examined through analysis of EBV-immortalized monoclonal B cell lines whether increased IFN-α levels after TLR7 stimulation of female subjects were due to *TLR7* gene X-inactivation escape causing the gene dosage imbalance between females and males.

We first confirmed the expression of TLR7 in EBV-immortalized polyclonal B cell lines by TaqMan real-time PCR. Subsequently, we generated monoclonal EBV-immortalized B cell lines from female donors who were heterozygous at *TLR7* coding SNP *rs179008* (*A>T*), confirmed by TaqMan SNP genotyping on DNA level. The monoclonality was proven by molecular analysis of B cell AgR gene rearrangement with PCR primers for conserved

Table I. *Effect of 17β-estradiol addition on IFN-α levels in PBL cultures (n = 12)*[a]

	Gender	Mean	SEM Value	Median	SD
1 μg/ml R848	Male	184.00	24.76	194.10	60.66
	Female	337.50	83.24	343.50	203.89
	Total	260.75	47.42	221.50	164.30
+ 0.5 nM estradiol	Male	171.35	29.41	158.40	72.03
	Female	387.85	121.13	345.40	296.70
	Total	279.6	67.79	165.2	234.85
+ 5 nM estradiol	Male	176.12	19.80	187.75	48.49
	Female	373.87	123.93	274.50	303.55
	Total	274.99	66.84	189.90	231.56
+ 50 nM estradiol	Male	173.38	26.65	171.75	65.28
	Female	357.65	107.70	294.80	263.82
	Total	265.52	59.75	187.25	206.96
+ 500 nM estradiol	Male	175.18	22.80	180.00	55.86
	Female	357.65	107.70	294.80	263.82
	Total	244.95	49.33	209.30	170.87

[a] Six female and six male subjects.

Table II. *Effect of ICI 182,789 on IFN-α levels in PBL-cultures (n = 12)*[a]

	Gender	Mean Value	SEM Value	Median	SD
1 μg/ml R848	Male	200.20	59.27	164.20	145.17
	Female	364.17	55.89	338.70	136.89
	Total	282.18	46.03	300.95	159.47
+ 10 nM ICI 182,789	Male	217.35	64.45	184.55	157.87
	Female	355.03	58.32	336.30	142.86
	Total	286.20	46.35	299.30	160.55
+ 100 nM ICI 182,789	Male	231.58	65.74	217.80	161.03
	Female	351.25	65.05	324.40	159.34
	Total	291.42	47.64	294.00	165.02
+ 1 μM ICI 182,789	Male	178.77	41.73	173.50	102.21
	Female	343.90	55.07	329.80	134.89
	Total	261.33	41.29	272.90	143.03

[a] Six female and six male subjects.

framework and J regions (data not shown). One amplicon of expected size suggested successful rearrangement of one single B cell clone, whereas polyclonal B cell cultures displayed several amplicons of different sizes. Monoallelic expression (X-inactivation occurred) or biallelic expression (X-inactivation escape) of TLR7 RNA was then analyzed by RT-PCR and subsequent RFLP analysis of *TLR7* exon SNP *rs179008*. The RFLP of digested cDNA fragments of the monoclonal B cell lines revealed that no X-inactivation escape of *TLR7* had occurred (Fig. 5). Either the *A*-allele or the *T*-allele was expressed by monoclonal B cell cultures of female subjects being heterozygous at SNP *rs179008*. Polyclonal cultures showed expression of both alleles. An additional control included the parallel digestion with Tsp509 I (Fig. 5*A*). PCR-RFLP of PBMC RNA from subjects with known heterozygous genotype confirmed digestibility of amplified cDNA and specificity of the method (Fig. 5*B*).

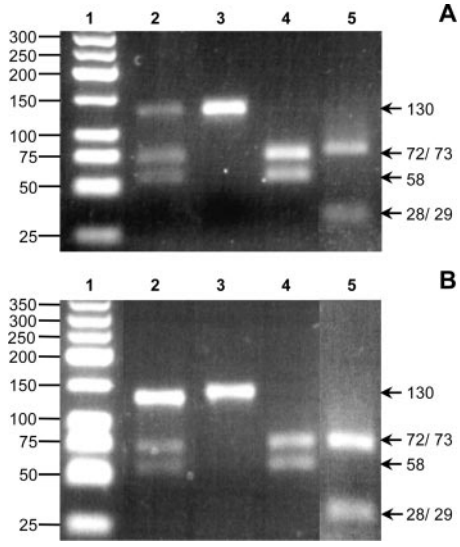

FIGURE 5. Human *TLR7* gene undergoes X-inactivation in monoclonal EBV-immortalized B cell lines of heterozygous females. RNA of polyclonal and monoclonal EBV-immortalized B cell lines (*A*) and PBMCs (*B*) was subjected to reverse transcription and subsequent PCR-RFLP with *Apo*I (*lanes 2–4*) or Tsp509 I *(lanes 5*) at TLR7 SNP *rs179008*. *Apo*I digests at SNP *rs179008* in case of wild-type A-allele, but mutant T-allele is not digested. Tsp509 I digests the PCR product at *A*- and *T*-allele and at a second digestion site of the PCR product. The figure shows agarose gel electrophoresis of digested fragments. Sizes are indicated in base pairs. *A*, *lane 1*, DNA ladder for length standard; *lane 2*, polyclonal heterozygous B cell culture; *lane 3*, monoclonal heterozygous culture that transcribed the mutant *T*-allele; *lane 4*, monoclonal heterozygous culture that transcribed the wild-type *A*-allele; and *lane 5*, monoclonal *T*-allele expressing culture that is digested for control with Tsp509 I. *B*, Control experiment to validate the PCR-RFLP method. *Lane 1*, DNA ladder for length standard; *lane 2*, heterozygous PBMCs; *lane 3*, homozygous mutant *T*-allele expressing PBMCs; *lane 4*, homozygous wild-type *A*-allele expressing PBMCs; and *lane 5*, homozygous *T*-allele expressing PBMCs digested for control with Tsp509 I.

Single-cell RT-PCR indicates X-inactivation of human TLR7 in female pDCs

Based on our data in immortalized B cell lines, we decided to validate the results through analysis of X-inactivation status in single native pDCs. Therefore, we used, according to their high TLR7 expression level, magnetic bead-sorted single pDCs of a heterozygous female donor after single-cell picking to discriminate monoallelic and biallelic expression. RNA isolated from single cells was analyzed by RT-PCR and RFLP analysis ($n = 7$). Three of seven heterozygous cells expressed the wild type *A*-allele and four expressed the mutant *T*-allele. Thus, in all cases, one *TLR7* allele of the heterozygous cells was inactivated, indicating X-inactivation of human TLR7 in female pDCs (Fig. 6).

To further confirm the data, we have performed real-time quantitative RT-PCR for TLR7 and TLR9 mRNA in sorted human native pDCs ($n = 16$) and human monoclonal B cell lines from blood donors ($n = 8$) and did not find significant differences of TLR7 or TLR9 mRNA levels between female and male samples (Fig. 7).

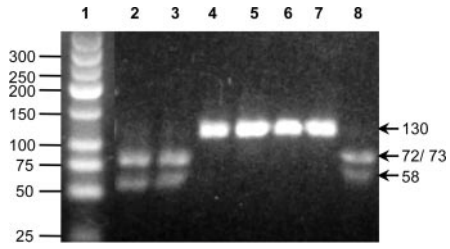

FIGURE 6. X-inactivation status of the human *TLR7* gene in single native pDCs. Forty single pDCs of one SNP *rs179008* heterozygous female were analyzed after cell picking, and RNA was individually subjected to RT-PCR. The seven successfully amplified PCR products were digested with *Apo*I, cutting at TLR7 SNP *rs179008* in case of *A*-allele expression. The figure shows agarose gel electrophoresis of digested fragments in comparison to DNA ladder (*lane 1*). *Lanes 2*, *3*, and *8*, These single heterozygous pDCs transcribed the wild-type *A*-allele and the *T*-allele was inactivated. *Lanes 4–7*, These single heterozygous pDCs transcribed the mutant *T*-allele and the *A*-allele was inactivated.

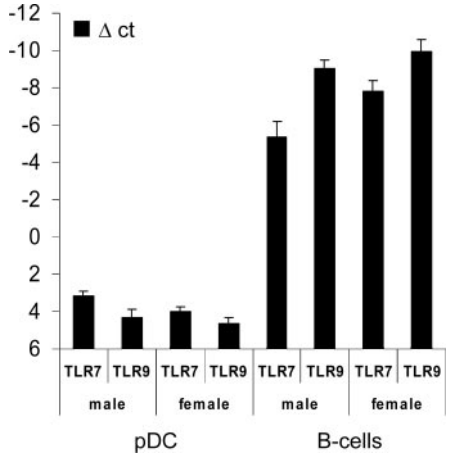

FIGURE 7. Similar TLR7 mRNA expression in female and male pDCs and B cells. mRNA expression of sorted pDCs ($n = 8$ females; $n = 8$ males) and immortalized B cell lines ($n = 4$ females; $n = 4$ males) was measured by quantitative TaqMan RT-PCR. Threshold cycle (ct) is normalized on a housekeeper gene (Δct = ct of housekeeper minus ct of target gene; higher ct value means lower expression level). Bars show the mean values with SE indicated. Please note scaling of the ordinate.

Discussion

In our present study, we demonstrate for the first time a sex-dependent difference of IFN-α production by healthy human subjects. We show in three independent cohorts that female PBLs produce significantly higher levels of IFN-α in response to TLR7 stimulation compared with male PBLs. These findings may have direct implications with respect to the pathogenesis of SLE and the clinical use of synthetic TLR7 ligands as immune adjuvants. It was noted that women are affected primarily by autoimmune diseases. SLE shows the most striking preference for female subjects (90%) (40, 51), and elevated IFN-α levels have been demonstrated by many independent groups to play a key role in the development of clinical and experimental SLE (20, 37–39, 52). Our presented data, which show a sex-dependent higher capacity of females to produce IFN-α after TLR7 stimulation, add to the understanding of the higher prevalence of SLE in females.

DNA, ssRNA, and dsRNA triggering cell activation via TLR7 and TLR9 provide good candidates for transducers of the signals that induce high IFN-α in SLE (53–55). Experimental models have recently highlighted the importance of TLR7 signaling for the development of SLE. Pawar et al. (56) demonstrated that the TLR7 ligand imiquimod, which is structurally related to guanosine nucleosides, aggravated murine lupus nephritis. Barrat et al. (57) revealed that mammalian DNA and RNA immune complexes are potent self-Ags for TLR7 and TLR9 and excite IFN-α production by pDCs, indicating that these TLRs have a critical role in the promotion of lupus. Furthermore, Lau et al. (58) reported that RNA and RNA-associated autoantigens can activate autoreactive B cells through sequential engagement of TLR7 and the BCR. These responses were markedly enhanced by IFN-α, indicating further evidence for TLRs playing also a role in autoantibody response in SLE.

IFN-α is produced primarily by pDCs in response to TLR7 and TLR9 stimulation. The TLR7- and TLR9-triggered pathway downstream the receptor was previously suggested to deal with the same components. However, our data reveal that TLR9 (located at autosome 3p21.3) activation in contrast to TLR7 activation does not lead to sex-dependent IFN-α induction, but both sexes produce at average the same levels. Remarkably, we found the sex-dependent cytokine induction restricted to IFN-α when peripheral blood leukocytes were stimulated via TLR7/8 with imidazoquinolines and absent with respect to TNF-α (primarily secreted by macrophages and activated T cells). Total pDC numbers were shown by us to be not affected by the sex of the subject.

To identify the molecular mechanism for sex-dependent IFN-α production through TLR7 stimulation, we have tested two hypotheses: 1) estrogen-dependent modulation of TLR7 signaling; and 2) X-inactivation escape of the human *TLR7* gene being located on Xp22.2.

Based on our data with estrogen and estrogen receptor antagonist, we found that estrogen signaling is unlikely to be responsible for the sex-dependent effects of synthetic TLR7 ligands on IFN-α levels in contrast to other cytokines of immune cells and T cell lines that are modulated by sex hormones (59–61).

Second, we hypothesized X-inactivation escape of the human *TLR7* gene playing a critical role in the ability of increased TLR7 signaling and resulting higher IFN-α production in females. Our experiments with monoclonal B cell lines and, independently, analysis of single-picked pDCs (the principal TLR7 producer) also did not support evidence for significant X-inactivation escape of the human *TLR7* gene. However, it is important to note that ~10% of genes that show X-inactivation escape display variable patterns of inactivation and are expressed in different extents from some inactive X chromosomes in females (62). However, we did not find significant differences of TLR7 mRNA expression in purified female and male pDCs. This observation does not necessarily exclude the possibility that sex-dependent TLR7 mRNA differences do exist since we observed major interindividual differences. Additionally, sex-dependent epigenetic mechanisms may contribute to modulate IFN-α synthesis, such as RNA-editing mechanisms interfering with mRNA stability (63, 64) of TLR7 or sex differences in the IFN-α-inducing pathway downstream the receptor. Early response genes encoding for transcriptional activators or repressors that may modulate the levels of upstream targets are also likely to be involved in sex-dependent IFN-α gene expression. Currently, the TLR7 and TLR9 pathways for IFN-α induction are believed to deal with the same signaling components. Based on our results, we have initiated experiments to identify different signaling molecules in the TLR7- and TLR9-induced IFN-α induction in purified human pDCs and to analyze their sex-dependent modulation.

Interestingly, the same TLR7 ligands we have investigated in this study are being used in clinical therapy of genital warts, which are caused by human papillomavirus. In independent studies, these TLR7 ligands have been demonstrated to exhibit better efficiency in female than male patients, and there have been several speculations about the underlying mechanism, such as, for example, sex-dependent skin resorption of the drug in female patients (65–67). Our results link the improved clinical efficacy directly with a sex-dependent IFN-α induction and consequently higher antiviral activity in female patients. Hence, our findings provide novel insight into the clinical action of these agents, and sex-dependent differences in clinical activity may lead to different treatment protocols for male and female patients.

Taken together, our studies identify for the first time a sex-dependent pathway of IFN-α induction in healthy female subjects. Sex-dependent IFN-α induction is TLR7 dependent, and estrogen signaling or X-inactivation escape is unlikely to account for the observed effects. Our results provide the basis for further studies of the epigenetic sex-dependent regulation of TLR7 signaling that might lead to novel insights with respect to the molecular pathogenesis and the therapeutic treatment of SLE in females.

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Disclosures

The authors have no financial conflict of interest.

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