

properly stimulate CD8⁺ T cells (10), it could not rule out the possibilities that specific miRNAs are involved in LC-mediated cross-presentation, as overall deleted miRNAs in Dicer KO mice could include miRNAs that both positively and negatively regulate LC cross-presentation. To investigate whether miR-150 is involved in LC cross-presentation process, epidermal LCs pulsed with ovalbumin were co-cultured with antigen-specific CD8⁺ or CD4⁺ T cells from OT-I/OT-II mice for 3 days. As shown in Fig. 2, LCs from miR-150KO mice showed significantly decreased capacity of stimulating antigen-specific CD8⁺ T-cell proliferation (Fig. 2a) and IFN- γ production (Fig. 2b) compared to LCs from WT mice. Interestingly, their capacity of stimulating antigen-specific CD4⁺ T cells was not significantly changed (Fig S2). Thus, miR-150 is required for normal LC cross-presentation to CD8⁺ T cells.

Conclusions

MiRNA miR-150 is expressed in epidermal LCs, and its expression is significantly down-regulated during *in vitro* LC maturation. Lack of miR-150 interrupts the capacity of LCs to cross-present a soluble antigen to antigen-specific CD8⁺ T cells, but does not disturb the development, maturation, migration and phagocytic

capacity of LCs. To the best of our knowledge, this is the first identified miRNA that positively regulates LC cross-presentation. The exact antigen-processing mechanisms that lead to cross-presentation in DCs/LCs are still incompletely understood. Further identification of miR-150 downstream targets involved in LC cross-presentation may illuminate new underlying molecular mechanisms, which will help to develop new intervention strategies for LCs-related therapies.

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Conflict of interests

The authors have declared no conflicting interests.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. The percentage of epidermal LCs after the culture *in vitro*.

Figure S2. miRNA miR-150 is not required for LCs-mediated CD4 T cell proliferation.

Data S1. Materials and methods.

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Letter to the Editor

Testosterone exerts selective anti-inflammatory effects on human skin mast cells in a cell subset dependent manner

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Abstract: Androgens are known to exert anti-inflammatory effects but their impact on mast cells (MCs) remains to be determined. Here, we show that MCs isolated from human foreskin samples (male) and those from breast skin (female) express the androgen receptor, albeit with a 10-fold difference between the subsets.

While fundamental MC properties (Fc ϵ RI, c-Kit, tryptase; histamine release upon Fc ϵ RI cross-linking) were unaffected or slightly reduced (chymase) by testosterone, the hormone had a more profound impact on the production of cytokines, with IL-6 being a target (reduction by 53%). Interestingly, this effect was

limited to breast skin MCs (15 of 16 donors displayed this phenomenon), but was not reproduced by foreskin MCs. Collectively, effector functions of human skin MCs are modulated by androgens in a gene-selective and MC subset-specific fashion. Possibly, MCs from women are more susceptible to testosterone.

Background

Sex steroids shape immune responses and contribute to sex-specific prevalences of allergic and autoimmune diseases (1,2). Mast cells (MCs) are versatile cells of innate immunity. They participate in host defenses, immunoregulation and tissue remodelling, but are most commonly known for their involvement in type I allergy (3–7). Their role in innate immunity is further reinforced by the recognition that MCs are activated by Toll-like receptors (8) and can dictate responses to bacterial toxins in the skin (9).

Befitting their localization close to blood vessels, MCs are exposed to high concentrations of serum factors, including hormones. The impact of the hormonal network on MCs is incompletely understood, and little is known about the interaction of MCs with androgens. Interestingly, a recent study reported that human foreskin-derived MCs expressed high levels of the androgen receptor (AR) (10). Despite this, no functional consequences resulting from AR ligation were detected, so that the impact of androgens on MCs remains undefined. In the first place, MCs influence immune responses by the range of mediators they release upon stimulation (7,11). We and others reported that human skin-derived MCs (skMCs) produce a mainly proinflammatory cytokine profile, which comprises IL-6, IL-8 and TNF- α (12–14).

Questions addressed

We asked in this study whether human skin MCs (skMCs) are targeted by androgens and instructed to alter their phenotype or effector functions. We used foreskin (fs)-derived (male) fsMCs and breast skin (bs)-derived (female) bsMCs in direct comparison to determine potential differences between the subsets.

Experimental design

MC purification was achieved by magnetic sorting of c-Kit positive cells as described (13–15). AR expression was studied in bsMCs and fsMCs *ex vivo*. MCs were pretreated with dihydrotestosterone (DHT) and tested for survival, MC marker/mediator expression and stimulation by Fc ϵ RI cross-linking. IL-6, IL-8 and TNF- α were quantified by ELISA. The methods are described in detail in Data S1.

Results

MCs showed prominent expression of the AR. fsMCs had clearly higher levels than bsMCs both by conventional (Fig. 1a) and quantitative RT-PCR (10.8-fold, Fig. 1b). The phenomenon that female cells can express lower AR levels has been observed previously in other cells (16). On direct comparison with other myelocytes, skMCs (even bsMCs) expressed considerable levels of the receptor (data not shown). We studied whether DHT would affect the phenotype or function of skMCs. The hormone had no impact on the expression of Fc ϵ RI and c-Kit (the best characterized MC cell surface receptors), or on MC tryptase (an abundant granule-associated mediator), but slightly reduced the level of MC chymase in bsMCs (supplementary Table 1). Likewise, Fc ϵ RI-mediated

We also demonstrate that MC IL-6 production is highly variable among individuals.

Key words: androgen receptor – cytokines – interleukin-6 – mast cells – testosterone

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activation and MC survival were comparable in the hormone-treated and untreated group. The lacking effect on stimulability is in accordance with a recent report (10). We conclude that fundamental MC properties are not under decisive control of sex-specific hormonal environments.

We then assessed whether androgens would influence MC cytokine production. To this end, MCs isolated from large groups of individuals were pretreated with DHT and stimulated by Fc ϵ RI cross-linking for 24 h, after which time cytokines were quantified by ELISA. IL-8 production was largely homogenous among subjects (low SEM), did not differ between bsMCs and fsMCs and

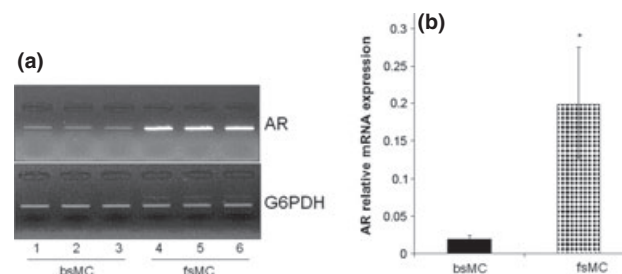


Figure 1. Comparative androgen receptor expression in fsMCs and bsMCs. (a) MCs isolated from three separate skin specimens (bsMCs) or pooled skin specimens (fsMCs) were investigated for AR expression by conventional RT-PCR. Note that a faint band corresponding to the AR product was detectable in all bsMC preparations in the original gel. (b) Quantification of AR mRNA expression by real-time quantitative PCR. MCs from eight breast skin and eight foreskin samples were included. Note that the apparently weak expression in bsMCs was substantially higher than in other immune cells tested (monocytes, neutrophils and eosinophils).

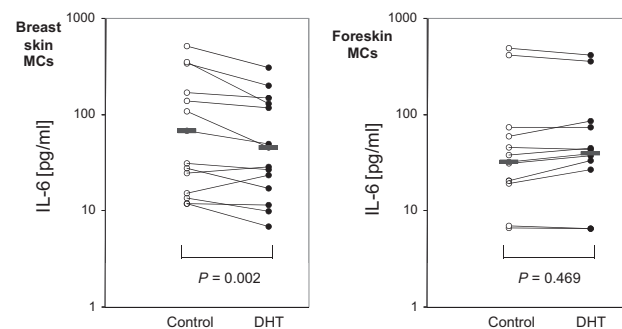


Figure 2. MC IL-6 production is highly variable among human subjects and selectively suppressed by DHT in (female) bsMCs but not in (male) fsMCs. Skin MCs from the two compartments were pretreated with DHT, sensitized with IgE and stimulated by anti-IgE for 24 h, as detailed in Methods. IL-6 levels were assessed by ELISA in cell-free supernatants. Each dot shows MCs from one human subject, the bar corresponds to the median. Note that MC IL-6 production is highly variable among individuals and that this applies to bsMCs (left) and fsMCs (right) alike (logarithmic scale for y-axis). IL-6 is reduced by DHT in 15 of 16 bsMC preparations (left), whereas no such trend is found for fsMCs (right).

was not affected by the hormone in either MC subset (supplementary Fig. 1A). TNF- α was likewise produced in a rather consistent fashion, and levels were not altered by testosterone (supplementary Fig. 1B). In contrast, IL-6 production was strikingly variable in MCs from different donors (Fig. 2, note that a logarithmic scale is used to account for the interindividual variance) with levels ranging from 12 to 514 pg/ml in bsMCs (median, 68; mean, 138 pg/ml) and 7 to 488 pg/ml (median, 37.8; mean, 106 pg/ml) in fsMCs. The findings for IL-6 substantiate a pronounced MC variability in the human population, as uncovered recently (Babina *et al.*, in preparation). The wide range in MC IL-6 production also indicated that this cytokine may be particularly susceptible to extracellular cues, *for example*, hormones. In fact, IL-6 turned out a target of androgen action. Its production was strongly inhibited by DHT in bsMCs, and the reducing effect was found in 15 of 16 bsMC preparations (Fig. 2, left). Interestingly, no such tendency was found for fsMCs, where both reducing and enhancing effects of the androgen were noted; the median even increased slightly to 115% of control (Fig. 2, right). This implies that the two MC subsets display fundamental differences in their responsiveness to androgens.

Taken together, human skMCs not only express substantial amounts of AR, but its ligation also modulates MC function in a selective fashion. We have uncovered MC IL-6 as a target of androgens, but interestingly only in bsMCs and not in fsMCs.

Conclusions

Human skin MCs are functionally altered by androgens by which they are instructed to lower their IL-6 production. The susceptibility of IL-6 to androgens has been described in other contexts and seems partially associated with NF κ B interference (17,18 and references therein). Interestingly, only bsMCs display IL-6 reduction, whereas fsMCs are largely resistant, even though the latter express higher AR levels. Although factors like age and location may

contribute to this pattern, the gender of the donor is probably involved in this variable responsiveness, as women are known to respond differently to androgens in various settings and can be more susceptible to testosterone than men (19,20). In this study, we also uncover that MC IL-6 expression differs strongly in the human population. Of particular note, MC-derived IL-6 has important functions in pathophysiological contexts, imparting protection against sepsis and tumor growth but contributing to obesity (21–23). The variability in MC IL-6 production, as uncovered here, may play a part in the donor-dependent contribution of MCs to such conditions. Interestingly, IL-6 can be induced (rather than reduced) by DHT in particular settings and this has been associated with male pattern baldness (24). Conversely, DHT does not promote, but even seems to improve, female hair loss (25). If MC IL-6 participates in hair loss in both genders, its reduction by androgens in women may contribute to this improvement. Together, androgens influence functional MC programs in a selective fashion.

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Author contributions

S.G. and M.B. designed the study, S.G.; M.A. and M.B. performed the experiments; S.G., M.B., M.A. and T.Z. analysed and interpreted the data; M.B. wrote the manuscript; S.G., M.A. and T.Z. revised the manuscript.

Conflict of interests

The authors declare no competing financial interests.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Methods.

Figure S1. IL-8 and TNF- α production are unaffected by androgens in both (female) bsMCs and (male) fsMCs.

Table S1. Fundamental MC characteristics are not profoundly altered by androgens.

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LETTER TO THE EDITORS

Supplementary Material

Testosterone exerts selective anti-inflammatory effects on human skin mast cells in a cell subset dependent manner

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Supplementary material

Methods

Skin MC purification

MC purification from human foreskin and breast specimens was performed as described (13-15). The skin was obtained either from circumcisions or cosmetic breast reduction surgeries, with informed consent of the patients. All experiments were conducted according to the Declaration of Helsinki Principles and approved by the Ethics Committee of the Charité Universitätsmedizin Berlin.

Skin was cut into strips and treated with dispase (BD Biosciences, Heidelberg, Germany) at 3.5 U/ml and 4° C overnight. After removal of the epidermis, the dermis was chopped into small pieces and digested with collagenase type 4 at 10 mg/ml (Worthington, Lakewood, NJ), hyaluronidase 1S at 5 mg/ml (Sigma, Deisenhofen, Germany), DNase I at 10 µg/ml (Roche, Basel, Switzerland), and 5 mM MgSO₄ for 1 h at 37° C. In the case of foreskin, hyaluronidase was omitted.

The dispersates obtained were separated from remaining tissue by three steps of filtration. In case of breast skin, the tissue was subjected to a second digestion step of 1 h at 37° C after the first filtration. MC purification was achieved by positive selection with anti-human c-Kit microbeads and an Auto-MACS separation device (both from Miltenyi Biotec, Bergisch

Gladbach, Germany). MC purity always exceeded 98%, as assessed by acidic toluidine-blue staining (0.1% in 0.5 N HCl).

RT-PCR

Total cellular RNA was isolated using the RNeasy Total RNA Kit, digested with RNase free DNase (Qiagen, Hilden, Germany), and quantitated using the RiboGreen RNA Quantitation Kit (Molecular Probes, Leiden, The Netherlands). Total RNA was reverse-transcribed with a first strand synthesis kit (Roche, Mannheim, Germany), and aliquots of the reaction product mixed with specific primers, and a PCR Master mix (Roche) containing all components required for PCR amplifications. Optimum conditions were determined in preliminary tests using cDNA from various cell types as controls. AR primers used for conventional PCR were as follows: AR sense 5'-AGATGGGCTTGACTTTCCCAGAAAG and AR antisense 5'-ATGGCTGTCATTTCAGTACTCCTGGA (annealing temperature 63° C, 38 cycles). Real-time quantitative PCR was performed, as described (14,15). Briefly, PCR was carried out with the LC Fast Start DNA Master SYBR Green kit (Roche Applied Science). The primer pairs were as follows: β -actin sense 5'-CTGGAACGGTGAAGGTGACA and antisense 5'-AAGGGACTTCCTGTAACAATGCA; AR sense 5'-CGGAAGCTGAAGAACTTGG and AR antisense 5'-ATGGCTTCCAGGACATTCAG. PCR was performed for 40 cycles at 95° C for 20 s, 60° C, for 5 s and 72° C for 8 s. Quantification of cDNA samples was performed automatically with reference to a standard curve. Values were normalized to β -actin. The LightCycler device Type 1.5 was used and data analysis was carried out with the LightCycler software 3.5.3 (Roche Molecular Biochemicals).

Hormone treatment and MC stimulation

Skin MC were kept at 5×10^5 /ml in basal Iscove's Medium supplemented with 10% FCS, 100 U/ml Penicillin, 100 μ g/ml Streptomycin, 2 mM L-Glutamin, 0.002% alpha-monothioglycerol (226.4 μ M). To mimic the long-term consequences of a distinct chronic micromilieu, the treatment with DHT (400 nM, Sigma) was performed for a total of 4 d with renewal after 2 d

(semi-depletion). For stimulation experiments, human IgE (Calbiochem-Novabiochem, Bad Soden, Germany; 5 µg/ml) was added to the cultures on day 3 to maximally saturate FCεRI. On day 4, MCs were washed extensively, counted in triplicate by the use of the Casy II cell counter and analyzer (Schärfe System, Reutlingen, Germany), and plated into fresh medium at 1×10^6 /ml. Cells were stimulated with mouse-antihuman IgE (Calbiochem-Novabiochem, dilution 1:20,000) for 24 h at 37° C. Remaining cells and debris were pelleted by centrifugation, and aliquotted supernatants kept at -80° C until assaying for cytokine concentration.

Flow-cytometry

MCs were washed twice in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free PBS, blocked for 15 min at 4° C with human AB-serum (Biotest, Dreieich, Germany) and incubated with either anti-CD117-PE (clone 95C3, Beckmann-Coulter-Immunotech, Krefeld, Germany), or anti-FCεRIα-FITC (clone 29C6, kindly provided by Dr. Hakimi, Nutley, NJ, labeled with FITC). Isotype matched non-binding control mAbs were from R&D Systems and used at the same concentrations. For intracellular staining of tryptase and chymase cells were fixed with 4% paraformaldehyde for 30 min at RT and permeabilized with PBS/0,1% saponine, as described (15). After blocking with 20% human AB serum, cells were incubated with specific antibodies and matching isotype controls for 30 minutes on ice. The following antibodies were used: for tryptase mouse IgG1 anti human MC tryptase (clone AA1; #MCA-1438; Serotech), for chymase mouse IgG1 anti human chymase (clone B7; #MAB1254; Chemicon). The binding of primary antibodies was visualized with a secondary, FITC conjugated goat (Fab) anti mouse IgG (H+L) antibody.

Histamine release

Histamine release assays were performed as described elsewhere (13-15). MCs preloaded with human IgE myeloma as above were washed twice with PAG-CM in order to remove unbound IgE (PAG-CM = Piperazine-N,N-bis[2-ethanesulfonic acid]-Albumin-Glucose buffer containing 3 mM CaCl_2 and 1.5 mM MgCl_2 , pH 7.4). Cell suspensions were resuspended in

PAG-CM, divided into aliquots and challenged for 30 min at 37° C with anti-IgE (1:20,000) or kept in PAG-CM for spontaneous release. Supernatants were stored at -20° C until measurement. Total histamine content was assessed upon cell lysis with 1% perchloric acid and the percentage of released histamine calculated accordingly. Histamine determination was performed by an automated fluorescence method, using an autoanalyzer (Borgwald Technik, Germany, Hamburg). All histamine determinations were performed in triplicate.

ELISA

Concentrations of IL-6 and TNF- α were determined by high-sensitivity enzyme-linked immunosorbent assays, while standard kits were utilized to detect IL-8 (all from R&D Systems, Wiesbaden, Germany), as described previously (13).

Statistics

The statistical significance of differences between values (DHT pretreated versus control cells) was assessed by paired Student's t-test or (when data were not normally distributed) by non-parametric Mann-Whitney test, using the program GraphPad Prism. Statistical significance was assumed at $p < 0.05$.

Suppl. Table 1

	BsMCs		FsMCs	
	Control	DHT	Control	DHT
c-Kit	100	100.0±14.2	100	98.0±1.5
FcεRI~	100	100.8±4.0	100	104.5±2.2
Tryptase	100	112.4±16.4	100	104.0±6.0
Chymase	100	81.3±1.2*	100	105.7±18.2
Histamine release	16.2±14.0	17.5±16.8	13.2±10.9	13.4±12.2
Survival (%)	71.1±19.1	78.0±6.4	78.0±3.0	76.6±1.7

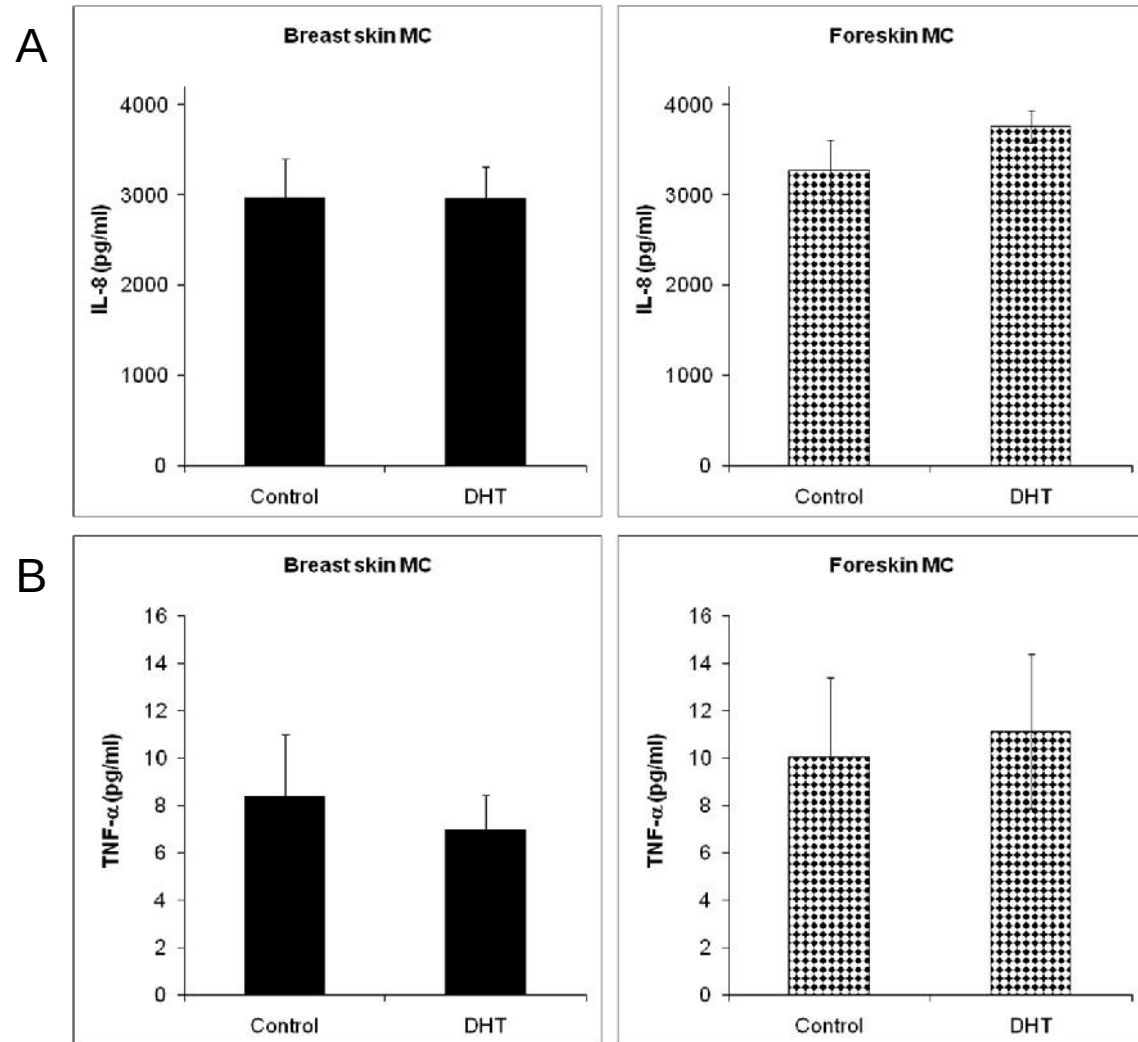
Fundamental MC characteristics are not profoundly altered by androgens

Skin MCs from breast skin and foreskin were kept in medium (control) or treated with DHT as described in Materials and Methods. c-Kit and FcεRI surface and intracellular tryptase and chymase expression were determined by flow-cytometry and mean density is expressed in % ± SD of control. Histamine release was determined in hormone pretreated and control MCs by an autoanalyzer without and following FcεRIα crosslinking. The net histamine release is given (release following FcεRIα crosslinking – spontaneous release)/total histamine content (in %). MC numbers were measured by an automatic cell counter and by manual counting in a Neubauer chamber (excluding dead cells stained by trypan blue) and are given in % of the cell number plated. The data are from 5 separate skin preparations each except for tryptase and chymase in breast skin (n=3). *p < 0.05. No other statistically significant effects from DHT were noted.

Suppl. Figure 1

IL-8 and TNF- α production are unaffected by androgens in both (female) bsMCs and (male) fsMCs

Supernatants were tested for IL-8 (A) and TNF- α (B) as in Fig. 2. Results are shown as mean $\pm$ SEM of 5 separate experiments for both subsets in the case of IL-8 (A) and 12 (bsMCs) and 8 (fsMCs) independent experiments for TNF- α .



Supplementary Fig 1