

# Androgens have an anti-inflammatory effect on human basophils in vitro

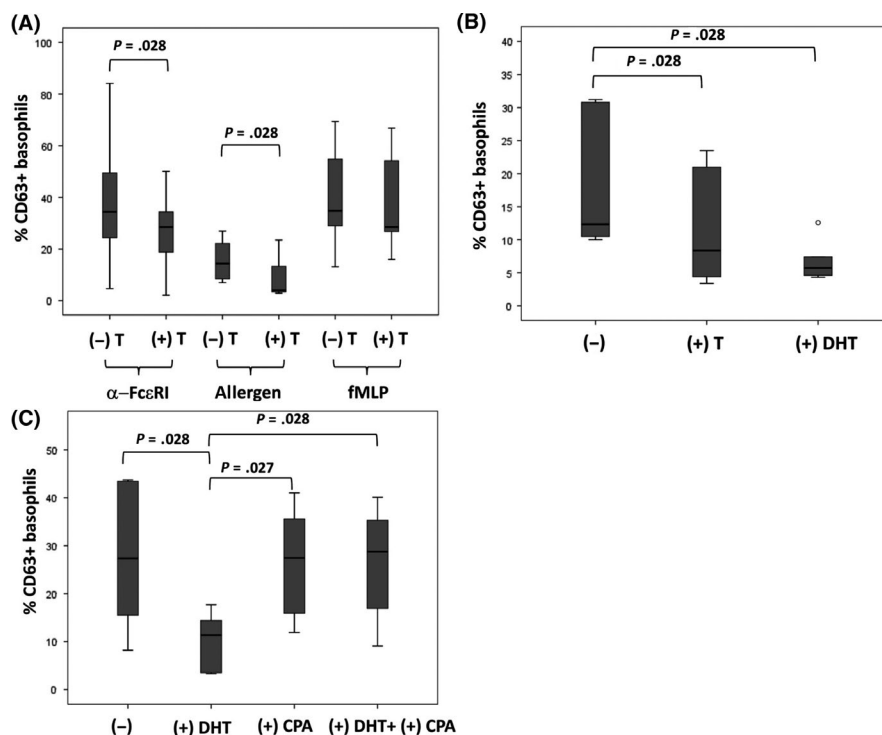
To the editor,

Hormonal influences on allergic diseases are frequently observed, but have not been studied in detail yet. Gender-specific age and disease distribution of allergic diseases exists<sup>1</sup> and it is likely that male as well as female sex hormones play a role in the development of allergic diseases.<sup>2</sup> Nevertheless, it is currently unclear how androgens and oestrogens influence certain cellular players of allergic immune responses, in particular effector cells like basophils. Basophils play a role in allergic inflammation of the airways as well as the skin.<sup>3</sup> Although their number is low in the blood, they are capable of releasing histamine in response to the challenge of the high affinity receptor for IgE (FcεRI) as well as cytokines such as IL-4. Thereby, basophils profoundly contribute to Th2 differentiation.<sup>4</sup> Basophils like mast cells are also responsible for anaphylactic reactions to physiologic allergen exposure as well as side effects of allergen exposure during allergen specific immunotherapy. Testosterone is produced by the testicles and exerts its functions via the androgen receptors. Testosterone is converted and thereby activated by 5 $\alpha$ -reductase to 5 $\alpha$ -dihydrotestosterone (DHT). Testosterone and DHT both bind to the androgen receptor (AR). Androgens have been

demonstrated to exert suppressive effects on distinct immune cells including macrophages, myeloid and plasmacytoid dendritic cells as well as T cells.<sup>5</sup> DHT has been demonstrated to suppress synthesis of pro-inflammatory leukotrienes via phosphorylation of extracellular signal-regulated kinases and inhibition of phospholipase D and is supposed to attenuate thereby inflammatory immune responses of relevance for different diseases.<sup>6</sup>

After local ethical committee approval and written informed consent were obtained, peripheral blood was taken from male and female patients with allergic diseases. The mean patient age was 40.7 years. Peripheral blood mononuclear cells (PBMC) were isolated. We performed Basophile activation tests (Flow CAST<sup>®</sup> highsense (BÜHLMANN Laboratories AG)) with PBMCs diluted in stimulation buffer, mixed 1 part to 2 parts in 10 mmol/L Hepes buffer and incubated with testosterone and ethanol (as control) in a concentration of 10<sup>-8</sup> mol/L and the different allergens according to the manufactures guidelines. We were able to show a significant downregulation after allergen challenge with insect venom in patients allergic to insect venom protein (Figure 1A). Similar effect was achieved after allergen challenge to birch and timothy grass allergen

**FIGURE 1** A, Basophil activation test with ((+T) and without ((-T) stimulation with testosterone and allergen challenge with insect venom measured with Flow CAST<sup>®</sup> highsense-system; (n = 6; mean  $\pm$  SEM; Wilcoxon test ( $\chi^2$  =  $P$  < .05)). B, Basophil activation test after stimulation with control, dihydrotestosterone (DHT), testosterone (T) and allergen challenge with aero-allergens measured with Flow CAST<sup>®</sup> highsense-system; (n = 6; mean  $\pm$  SEM; Wilcoxon test ( $\chi^2$  =  $P$  < .05)). C, Basophil activation after stimulation with anti-FcεRI, DHT, cyproterone acetate (CPA) and DHT and CPA combined measured with Flow CAST<sup>®</sup> highsense-system (n = 6; mean  $\pm$  SEM; Wilcoxon test ( $\chi^2$  =  $P$  < .05))



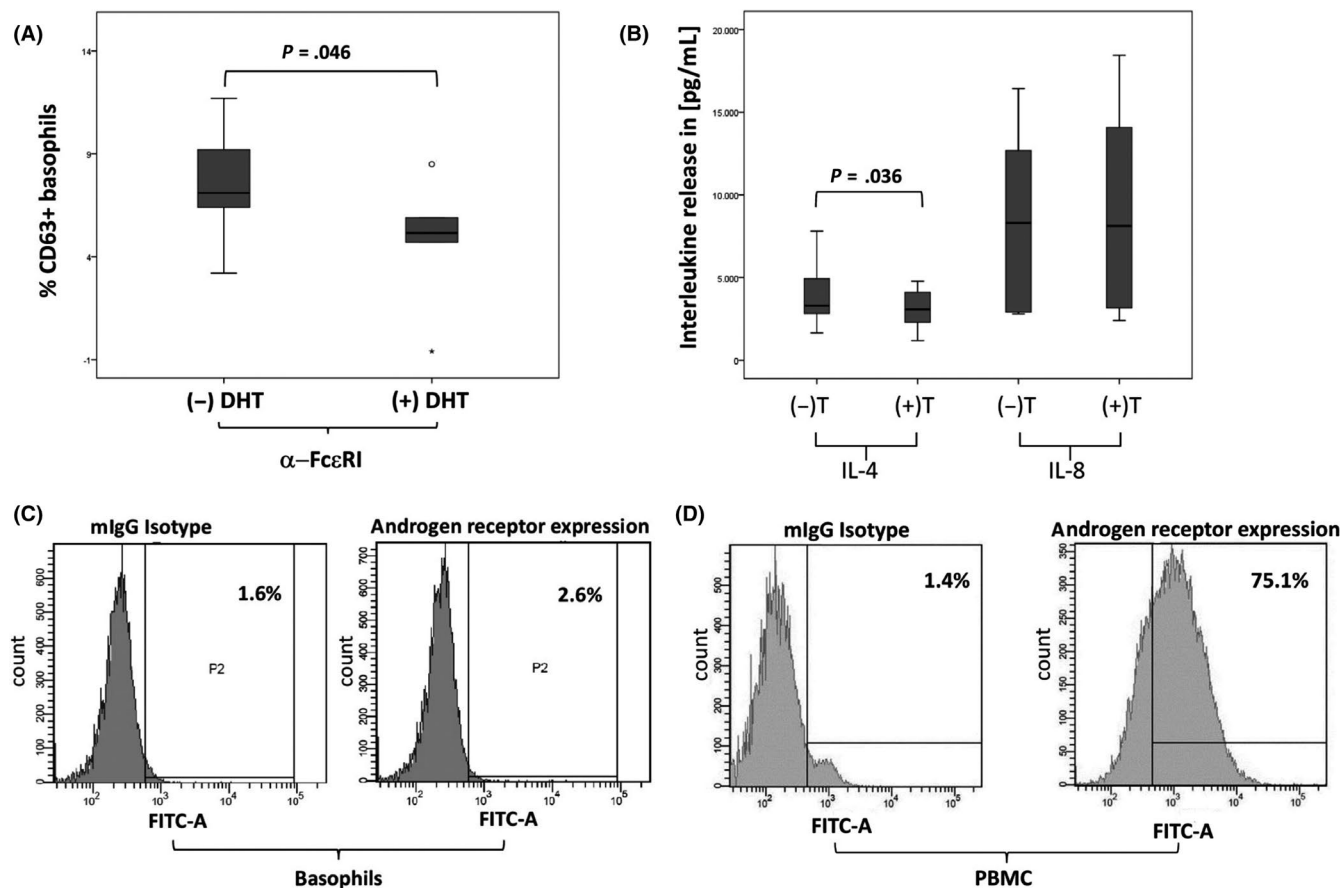


**FIGURE 2** A, Basophil activation test with purified basophils incubated with supernatant of stimulated PBMCs (with anti-FcepsilonRI, with (+)DHT and without (-)DHT) DHT; (n = 6; mean  $\pm$  SEM; Wilcoxon test ( $\chi^2 = P < .05$ )). B, IL-4 and IL-8 release in pg/mL of purified basophils measured with the flex set detection kit after stimulation with anti-FcepsilonRI and with (+)T and without (-)T testosterone (T); (n = 7; mean  $\pm$  SEM; Wilcoxon test ( $\chi^2 = P < .05$ )). C, Intracellular staining of human basophils which indicates the AR is not expressed on human basophils. From left to right: Isotype mIgG, Anti-Androgen-Receptor antibody. D, Intracellular staining of human PBMCs to confirm AR on protein level. From left to right: Isotype mIgG, Anti-Androgen-Receptor antibody

in patients allergic to pollen, also after stimulation with DHT in a concentration of  $10^{-8}$  mol/L (Figure 1B). Addition of the AR-inhibitor cyproterone acetate in a concentration of  $10^{-8}$  mol/L reversed the effects on the basophils (Figure 1C). We were able to reproduce these findings in basophil activation tests performed with isolated basophils incubated with the supernatant of PBMCs, which were incubated with DHT in a concentration of  $10^{-8}$  mol/L and ethanol (as control) before (Figure 2A). To evaluate, whether testosterone influences secretion of pro-inflammatory and Th2 cytokines, the release of IL-4 and IL-8 in purified basophils after FcepsilonRI mediated allergen challenge with and without testosterone incubation (in a concentration of  $10^{-8}$  mol/L) was measured using the flex set detecting kit from BD Biosciences and the BDArray (BD Biosciences). Testosterone attenuated significantly IL-4 release whereas the IL-8 release did not change. These data demonstrate that testosterone can suppress secretion of Th2 cytokines by basophils (Figure 2B). In our experiments, it was not possible to show the androgen receptor on basophils (Figure 2C). To evaluate, how basophils are responsive to testosterone and DHT via its respective receptor, AR mRNA expression of human PBMCs was measured with the help of RT-PCR

(Table S1). AR mRNA expression by PBMCs was detected and was similar in PBMC from female or male donors (data not shown). Additionally, we were able to show the expression of AR on protein level in human PBMCs with the help of flow cytometry (Figure 2D).

In this study, we could demonstrate that androgens modulate the activity as well as the function of basophils. We were also able to show that the effects of androgens on basophils are most likely mediated through soluble factors secreted by PBMCs, which express the androgen receptor. With basophils being one of the effector cells in the allergic cascade, our data could provide explanations for the observation that both frequency of allergic diseases and severity of allergic reactions are gender-dependent and change profoundly with age.<sup>7</sup>

The suppressive effects of testosterone and DHT may besides other factors as a reduced capacity to tolerate hypotension, arrhythmia and hypoxia, also contribute to the findings that fatal anaphylaxis is observed more often in elderly and male patients.<sup>7</sup> These groups are known to have low levels or less testosterone than (other) adults.

Oestrogens have been demonstrated to increase allergic immune responses, whilst testosterone is known to exert rather suppressive

effects on the immune response.<sup>5</sup> It has also been shown that androgens could play a role in the induction and perpetuation of tolerance regarding autoimmune diseases by inducing regulatory T cells.<sup>8</sup> It has been shown that besides the direct impact of testosterone and its derivatives to various reactions, the effects can be altered by the activity of the androgen receptor, for example through CAG-repeat variations.<sup>9</sup>

The data indicate that the effect testosterone has on basophil activation is an indirect one and is mediated through soluble factors. On the other hand, we were able to show that the secretion of inflammatory interleukins is attenuated by testosterone in isolated basophils.

It has been assumed in many places that androgens influence the immune system as a whole. Our recent study gives evidence that androgens could have an influence on allergic reactions and atopic diseases via basophils. There has to be further investigation how androgens have an influence on basophils and if androgens have an influence on allergic reactions and atopic diseases in vivo.

#### CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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#### SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

Supplemental table 1

| specificity | description | Primer-sequence                         | temperature | time                          | cycles |
|-------------|-------------|-----------------------------------------|-------------|-------------------------------|--------|
| AR          | forward     | 3'-AC GAC CAG ATG GCT GTC<br>ATT CAG-5' | 94°C        | 4min                          | 40     |
| AR          | reverse     | 3'-TG AAG GAT CGC CAG CCC<br>ATG-5'     | 58,5°C      | 40sec                         |        |
|             |             |                                         | 72°C        | 1min /10 min<br>as final step |        |
|             |             |                                         |             |                               |        |
|             |             |                                         |             |                               |        |
|             |             |                                         |             |                               |        |

## **Appendix S1**

### **Material and methods**

#### *Patients*

After local ethical committee approval and written informed consent were obtained, peripheral blood was taken from 12 patients (9 male, 3 female) with allergic diseases at the Department of Dermatology and Allergy of the University of Bonn. The mean patient age was 40.7 years.

#### *Isolation of PBMC*

Peripheral blood mononuclear cells were isolated by the Lymphoprep gradient technique, as described in the manufacturer's protocol (Axis Shield, Oslo, Norway).

#### *Basophile Activation*

The Flow CAST® highsens (BÜHLMANN Laboratories AG) was performed with PBMCs diluted in stimulation buffer, mixed 1 part to 2 parts in 10mM Hepes buffer (Biochrom, Berlin, Germany) and incubated for 30 minutes at 37°C followed by 15 minutes at room temperature with testosterone (Applichem, Darmstadt, Germany), Dihydrotestosterone (DHT) (Sigma Aldrich, St. Louis, Missouri, USA), cyproterone acetate (CPA) (Sigma Aldrich, St. Louis, Missouri, USA) and ethanol (Merck, Darmstadt, Germany) as control and the different allergens (yellow jacket venom, bee venom, timothy grass and birch pollen) all from BÜHLMANN Laboratories AG (Schönenbuch, Switzerland) according to the manufactures guidelines. Testosterone, DHT and CPA were all in a concentration of  $10^{-8}$ mol/l. The Flow CAST® highsens (BÜHLMANN Laboratories AG) was also performed with isolated basophils incubated with the supernatant of PBMCs, which were incubated with DHT also in a concentration of  $10^{-8}$ mol/l and ethanol.

### *Isolation of basophils*

Enrichment of basophils was performed as described elsewhere (1). Briefly, 45 ml of K3-ethylenediaminetetraacetic acid (EDTA) blood was mixed with 6 ml Hetasep (StemCell Technologies, Vancouver, Canada), split into two tubes and centrifuged to separate red blood cells from leucocytes. The upper phase containing leucocytes was washed with Dulbecco's phosphate-buffered saline (DPBS) with 1mm EDTA and 2% fetal bovine serum (purification buffer) and centrifuged again. Afterwards, the pellet was resuspended in purification buffer, transferred to another tube and Human basophil enrichment cocktail (EasySep Human Basophil Enrichment Kit, StemCell Technologies) containing monoclonal antibodies to the cell surface antigens CD2, CD3, CD4, CD15, CD16, CD19, CD24, CD34, CD36, CD45RA and Glycophorin A was added. After incubation, purification buffer and EasySep Magnetic Nanoparticles were added to the antibody-cell suspension followed by another incubation period. Cells were transferred to an EasySep Magnet and incubated again. Unbound cells were collected, washed and resuspended in Hepes-buffered Tyrode's solution without calcium. Purity and viability of the cells were determined by flow-cytometric staining using a FACS-Canto (BD Biosciences, Heidelberg, Germany) and analyzed by FACSDiva (BD Biosciences, Heidelberg, Germany) and FlowJo (TreeStar Inc., Ashland, OR, USA) software.

### *Androgen receptor staining*

Isolated PBMCs and basophils were stained with Mouse mAb (IgG1) against Androgen receptor was from Abcam (clone AR441) (Cambridge, UK) according to the manufactures guidelines.

### *Measurement of IL-4 and IL-8 in basophils*

Purified Basophils or PBMCs were resuspended in T-Cell Medium (Biochrom, Berlin, Germany) without phenolred and with added 10% FBS, 1% AB/AM, 1% L-Glutamin and

10 $\mu$ l ME. The cells were incubated with testosterone at a concentration of 10<sup>-8</sup>mol/l or DHT at a concentration of 10<sup>-9</sup>mol/l or ethanol for 1 hour. Afterwards the cells were incubated with 2 $\mu$ g/ml polyclonal anti IgE (Agilent Dako, Sanata Clara, USA)) for 20 min. After a washing step new medium was added. Then the cells were incubated with 10ng/ml rec. IL-3 (Peprotech, Rocky Hill, USA) for 6 hours. Afterwards the supernatant was collected and IL-4 and IL-8 were measured with the Human IL-4 Enhanced Sensitivity Flex Set (BD Biosciences, Heidelberg, Germany) respectively the Human IL-8 Enhanced Sensitivity Flex Set (BD Biosciences, Heidelberg, Germany) according to the manufactures guidelines.*Reverse transcription polymerase chain reaction (RT-PCR)*

To analyze androgen receptor  $\alpha$  mRNA expression in cultured PBMCs, primers were synthesized (Microsynth, Balgach, Switzerland) (supplemental Table 1) and RT-PCR was performed (as seen elsewhere). The amount of human template cDNA in different PCRs was determined with a parallel PCR of a 114bp fragment of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene with following primers, GAPDH\_forward: CCA CAT CGC TCA GAC ACC AT and GAPDH\_reverse: GGC AAC AAT ATC CAC TTT ACC AGA GT (13). The accuracy of the performed PCR was confirmed by DNA sequencing of the entire coding domain sequence (GATC Biotech).

### *Statistical analysis*

Statistical analysis was performed using SPSS 24.0 for Windows (IBM, Armonk, USA). Quantitative values were compared by nonparametric testing (Wilcoxon). Results are given in mean  $\pm$  standard error of the mean (SEM), respectively. Any P-values are two-sided and subjected to a significance level of 5%.

## References

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