Androgen-replacement therapy depresses the *ex vivo* production of inflammatory cytokines by circulating antigen-presenting cells in aging type-2 diabetic men with partial androgen deficiency

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Abstract

Androgens are considered to have immunomodulatory effects but their cellular mechanisms of action remain largely unknown. In the present study we prospectively analyzed the serial effects of androgen-replacement therapy on both the distribution of peripheral blood lymphocytes, monocytes and dendritic cells as well as on the production of interleukin (IL)-1 β , IL-6 and tumor necrosis factor α (TNF α) inflammatory cytokines by circulating monocytes and CD33 myeloid, CD16 and plasmacytoid dendritic cell subsets, the most potent antigen-presenting cells (APCs) in type-2 diabetic men with partial androgen deficiency. Analyses were performed before therapy and at 1, 3, 6 and 12 months after treatment with 150 mg testosterone enanthate every 2 weeks in a group of 13 type-2 diabetic men. Our results show for the first time that testosterone-replacement therapy is associated with a reduction or complete abrogation

of spontaneous *ex vivo* production of IL-1 β , IL-6 and TNF α by APCs. Meanwhile, the *in vitro* production of inflammatory cytokines by these cells after stimulation with lipopolysaccharide plus recombinant human interferon- γ remained unchanged, suggesting that APCs preserve their constitutive machinery to produce inflammatory cytokines under androgen treatment. These results confirm and extend previous observations about the anti-inflammatory effects of androgen therapy on APCs in a new, previously unexplored model of androgen deficiency; namely, aging type-2 diabetic men. A decreased production of inflammatory cytokines by APCs might have important consequences for sex differences in susceptibility to autoimmune diseases, inflammatory response to injury and atheromatosis.

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Introduction

Aging in men is associated with a progressive decrease in serum testosterone levels and an increase in the prevalence of hypogonadism (Harman *et al.* 2001, Feldman *et al.* 2002). Although the age-related decrease in testosterone levels is independent of health status (Harman *et al.* 2001), in diseases such as diabetes mellitus androgen deficiency could be exacerbated since lower testosterone values have been reported in older type-2 diabetic men than in age-matched non-diabetic individuals (Barrett-Connor *et al.* 1990, Anderson *et al.* 1994). The partial androgen deficiency detected in aging men has been related to the development of multiple symptoms and signs, and it has been shown that androgen-replacement therapy may induce improvements of lean body mass (Tenover 1992, Snyder *et al.* 1999, Kenny *et al.* 2001), bone mineral density (Kenny *et al.* 2001), libido (Hajjar *et al.* 1997),

sexual function (Kunelius *et al.* 2002), grip strength (Sih *et al.* 1997) and spatial cognition (Janowski *et al.* 1994), together with a decrease in body fat mass (Snyder *et al.* 1999, Kenny *et al.* 2001). Nevertheless, present information about the benefits and risks of testosterone replacement is limited and there is consensus about the need for further studies in which the effects of androgen replacement in aging males can be evaluated (American Society for Reproductive Medicine 2004, Snyder 2004).

In line with this, little is known about the cellular effects of androgen-replacement therapy on the immune response in men (Jacobson & Ansari 2004). Further insight into this field may contribute to the understanding of the mechanisms involved in the beneficial effects of androgens on the immune system (Malkin *et al.* 2003, Cutolo *et al.* 2004) and their protective effect against the development of autoimmune diseases (Bizzarro *et al.* 1987, Fitzpatrick *et al.* 1991, Bebo *et al.* 1998, Gylling *et al.* 2003). In fact,

Table 1 Clinical features of controls and type-2 diabetic men

Parameter	Controls (n=8)	Type-2 diabetic men (n=13)	P value
Age (years)	64 ± 3	64 ± 6	ns
Weight (kg)	73.2 ± 12.5	77.6 ± 14	ns
Body mass index (kg/m ²)	26.8 ± 1.2	27.3 ± 3.8	ns
Fasting plasma glucose (mg/dl)	85 ± 3	153 ± 11	0.001*
HbA1c (%)	NM	6.9 ± 0.4	
Fasting plasma insulin (μIU/ml)	12.9 ± 3.7	15.4 ± 3.4	ns
Fasting plasma C-peptide (pmol/ml)	0.95 ± 0.2	0.5 ± 0.1	0.04*
Total testosterone (ng/ml)	5.2 ± 0.6	3.9 ± 0.2	0.04†
Free testosterone (pg/ml)	11.1 ± 1.5	9.8 ± 0.5	ns
Luteinizing hormone (mIU/ml)	3.2 ± 0.7	6.1 ± 0.8	0.02*
Follicle-stimulating hormone (mIU/ml)	6.5 ± 1	9.7 ± 1.6	ns
Prolactin (mIU/ml)	194 ± 21	211 ± 16	ns

Results are expressed as means ± S.E.M. *Mann–Whitney U test. †Student’s t test. NM, not measured; ns, not statistically significant.

previous studies have indicated that gender-associated differences in susceptibility to autoimmune diseases may be related to sex differences in cytokine secretion by circulating immunocompetent cells (Bebo *et al.* 1999, Liva & Voskuhl 2001).

Here we report a prospective study on the short- and long-term effects of androgen-replacement therapy on the circulating inflammatory cellular compartment of the immune system in a group of type-2 diabetic men with partial androgen deficiency followed over 1 year of treatment.

Materials and Methods

Patients and control subjects

A total of 13 consecutive men older than 55 years (range, 55–73 years; mean ± S.E.M. 64 ± 2 years) with type-2 diabetes were enrolled in the study. Diagnosis of diabetes was established either from the patient’s history or according to the American Diabetes Association criteria (American Diabetes Association 1998) in the case of patients with recent onset of diabetes. Type-2 diabetes was diagnosed on the basis of a history of initial successful treatment with oral hypoglycemic agents as well as by assessment of residual β-cell function, measuring C-peptide serum values. At the moment of entering the study the duration of diabetes ranged between 2 and 22 years. Four patients were treated with insulin, five with insulin plus oral hypoglycemic agents, and four with oral hypoglycemic agents. None of the patients had clinically evident atherosclerotic complications and all were ambulatory.

Inclusion criteria were: (1) a positive history of fertility (indicating previous testicular health); (2) a healthy partner (to prevent answers on the questionnaire used to obtain

the clinical criteria of andropause from being influenced by exogenous factors); (3) absence of clinically overt hypogonadism, or hepatic, cardiac or renal insufficiency and (4) lack of ingestion of drugs that could induce hypogonadism. Exclusion criteria included diseases in which treatment with testosterone should be contraindicated, such as prostate cancer, active benign prostatic hyperplasia, breast carcinoma, sleep apnea, or chronic lung disease.

In addition to diabetic patients, eight healthy men with neither diabetes mellitus nor any of the components of the metabolic syndrome, of similar age (range, 52–76 years; mean ± S.E.M. 64 ± 3 years), weight and body mass index to the diabetic patients, were studied in parallel as a control group (Table 1). Control individuals were recruited in the same outpatient clinic as the diabetic patients. The patients and controls did not have infection, inflammatory or allergic diseases nor were they receiving treatment with glucocorticoids or nonsteroidal anti-inflammatory drugs, and they all gave written informed consent prior to entering the study. The study protocol was approved by the local research ethics committee of the University Hospital of Salamanca, Spain.

Clinical and hormonal criteria of partial androgen deficiency

Clinical criteria to define partial androgen deficiency were established on the basis of a specific questionnaire consisting of eight items related to androgen deficiency, which has been previously described in detail (Corrales *et al.* 2004). This questionnaire had a sensitivity of 90% and a specificity of 74% when applied to aging type-2 diabetic patients (Corrales *et al.* 2003). The questionnaire was applied by the same person in all but one case and all patients had positive questionnaires and hence met the clinical criteria of androgen deficiency.

Androgen deficiency was confirmed using a biochemical criterion consisting of a total testosterone serum level of ≤ 3.4 ng/ml, according to its normal concentrations in a group of healthy young fertile controls (Corrales *et al.* 2000). This figure is similar or identical to the level used to define biochemical andropause by authorities in the field (Kaufman & Vermeulen 1997) or in recent epidemiological studies (Harman *et al.* 2001). In a few patients who met the clinical criteria for androgen deficiency but displayed total serum testosterone values marginally higher than 3.4 ng/ml, the criterion of free testosterone values of ≤ 11 pg/ml, which represent the lower end of the normal range in young adults, was used. In agreement with our recommendations (Nieschlag *et al.* 2004) symptoms were always considered in the context of the full clinical picture in relation to testosterone serum levels; thus partial androgen deficiency was considered when a patient met both the clinical and biochemical criteria defined above.

Hormone and other laboratory measurements

Blood samples were obtained for all laboratory measurements simultaneously between 8:00 to 9:00 AM. Plasma glucose levels were assayed by a glucose oxidase method. Total and free testosterone concentrations were measured by solid-phase [125 I]RIA (Coat-a-Count; DPL, Los Angeles, CA, USA) and luteinizing hormone (LH), follicle-stimulating hormone (FSH), and prolactin levels were determined with a RIA (RIA-gnost; CIS Bio International, Gif-sur-Yvette, France), as previously described in detail (García-Díez *et al.* 1983, Corrales *et al.* 2000). The RIA-React (CIS Bio International) and the INS-Irma (Biosource, Nivelles, Belgium) RIAs were used to measure prostate-specific antigen (PSA) and insulin serum levels, respectively. C-peptide levels were determined using a commercial RIA (C-PEP; Biosource). HbA_{1c} (Menarini Diagnostics, Florence, Italy) and fructosamine (Fruc; Roche Diagnostics) were measured using commercial kits.

The sensitivities and the intra- and interassay coefficients of variation of the methods were: 0.04 ng/ml, 5.7% and 6.7%, respectively, for total testosterone; 0.15 pg/ml, 5.4% and 8.5% for free testosterone; 0.1 mIU/ml, 2.9% and 3% for LH; 0.1 mIU/ml, 3.4% and 3% for FSH; 2 μ IU/ml, 6.9% and 7.8% for prolactin; 0.04 ng/ml, 2.2% and 4.2% for PSA; 1.5 μ IU/ml, 6.9% and 8.1% for insulin; 0.04 pmol/ml, 8.2% and 9.3% for C-peptide; 2.5%, 5.2%, and 5.2% for HbA_{1c}; 10 μ mol/l, 0.9% and 2.9% for fructosamine.

Treatment with testosterone

Diabetic patients meeting the criteria of androgen deficiency were treated with 150 mg intramuscular testosterone enanthate every 15 days for 1 year. They were

studied prior to therapy, and at months 1, 3, 6 and 12 of testosterone therapy. On each of these visits, the following laboratory safety tests were performed: complete blood count, blood pressure, PSA levels and liver tests; in addition, symptoms of urinary obstruction were also evaluated. During follow-up, measurements of total and free testosterone levels were performed on day 8 after each injection. Evaluation of treatment compliance was performed by monitoring the decrease in serum LH levels induced by exogenously added testosterone.

Identification and enumeration of peripheral blood (PB) monocyte, dendritic cell (DC) and lymphocyte subsets

Identification and enumeration of PB monocytes, DCs and lymphocytes were performed on EDTA-anticoagulated PB samples stained with specific combinations of monoclonal antibodies (mAbs) using a direct immunofluorescence, single-platform flow cytometry technique as previously described (Almeida *et al.* 2001). Briefly, 100 μ l of a pre-mixed PB sample was placed in two separate tubes and stained for 15 min at room temperature (RT) with the Dendritic Cell Exclusion kit, which contains a mixture of FITC-conjugated anti-CD3, CD14, CD19 and CD56 mAbs (Cytognos, Salamanca, Spain), CD16-phycoerythrin (PE; clone 3 G8; Immunotech, Marseille, France), anti-HLA-DR-peridinin chlorophyll protein (PerCP; clone L243; Becton Dickinson Biosciences, San Jose, CA, USA) and CD33-allophycocyanin (clone LeuM9; Becton Dickinson Biosciences) mAb or the LYMPHOGRAM reagent (Cytognos). Then cells were incubated with 2 ml of either Quicklysis (Cytognos) or FACS lysing solution (Becton Dickinson Biosciences) for 10 min at RT. Once this incubation period was finished, the cells stained with the LYMPHOGRAM reagent were centrifuged at 540 g (5 min, RT); following this, the lysing solution was removed, and the cell pellet washed twice in 2 ml PBS (pH 7.6) and resuspended in 0.5 ml PBS. Immediately prior to data acquisition, 100 μ l pre-mixed Perfect-Count beads (Cytognos) were added to the other tube. After gentle mixing samples were run on a FACSCalibur flow cytometer (Becton Dickinson Biosciences) using Cell-QUEST software (Becton Dickinson Biosciences). Data acquisition was performed as previously described, collecting information on $>10^3$ DCs (defined as those cells being of the MHC-II⁺/lineage⁻/^{dim+}) and $>10^4$ lymphocytes. For data analysis, Paint-A-Gate Pro software (Becton Dickinson Biosciences) was used.

In addition to the monocytes, the following subsets of DCs and lymphocytes were identified: (1) myeloid DCs (CD33^{hi}); (2) CD16⁺ DCs (CD33⁺, CD14⁻/^{dim+}); (3) plasmacytoid DCs (CD33⁻/^{dim+}; Almeida *et al.* 1999, 2001); (4) CD4⁺/CD8⁻ T cells; (5) CD8⁺/CD4⁻ T lymphocytes; (6) CD4⁻/CD8⁻ T cells; (7) CD4⁺/CD8⁺ T lymphocytes; (8) CD4⁻/CD8^{dim} T-cells;

(9) CD4⁺/CD8^{dim+} T cells; (10) CD56⁺/CD8⁺ natural killer (NK) cells; (11) CD56⁺/CD8[−] NK cells and (12) CD19⁺ B lymphocytes.

Analysis of cytokine secretion by PB monocytes and DCs

The spontaneous and stimulated secretion of cytokines by monocytes and DCs was analyzed at the single-cell level in heparinized PB samples, using a well-established four-color staining technique that combines the specific identification of monocytes and DCs with the measurement of intracellular cytokine production after short-term *in vitro* culture (Almeida *et al.* 1999, Bueno *et al.* 2001). Briefly, 500 µl heparin-anticoagulated PB was placed in a tube, to which 100 ng/ml lipopolysaccharide (LPS; from *Escherichia coli* serotype 055:B5; Sigma) and 10 ng/ml human recombinant interferon γ (IFN γ ; Promega) were added for the specific stimulation of PB MHC-II⁺/lineage[−] cells (Bueno *et al.* 2001). Then, 500 µl RPMI 1640 culture medium (BioWhittaker, Walkersville, MD, USA) supplemented with 2 mM L-glutamine was added to complete a final volume of 1 ml. In addition, 10 µg/ml brefeldin A (Sigma; Bueno *et al.* 2001) was added to block cytokine secretion by cytokine-producing cells. An unstimulated sample also containing brefeldin A and processed in an identical way in the absence of any exogenous stimuli was used to evaluate spontaneous *ex vivo* cytokine secretion. Then cells were incubated for 6 h at 37 °C in a 5% CO₂ and 95% humidity sterile environment, as previously described in detail (Almeida *et al.* 1999). Once this incubation period had ended, the sample was aliquoted into four different tubes (approximately 200 µl/tube) and cells were stained with the Dendritic Cell Exclusion kit, anti-HLA-DR-PerCP and CD33-allophycocyanin. After a gentle mixing, cells were incubated for 15 min in the darkness (at RT), washed in 2 ml PBS, fixed, permeabilized and stained with mAbs directed against different human inflammatory cytokines using the Fix & Perm reagent kit (Caltag, San Francisco, CA, USA) according to the recommendations of the manufacturer. The mAb reagents used to detect intracellular cytokines were as follows: anti-interleukin (IL)-6-PE (clone MQ2-6A3; Pharmingen, San Diego, CA, USA); anti-tumor necrosis factor α (TNF α)-PE (clone 11; Pharmingen) and anti-IL-1 β -PE (clone AS10; Becton Dickinson Biosciences). Isotype-matched negative mAb reagents conjugated with PE were used as negative controls.

Data acquisition and analysis were performed on a FACSCalibur flow cytometer as described above. For data analysis, cytokine production by specific subsets of DCs and monocytes was calculated in terms of both the percentage of positive cells within each cell subset and the mean fluorescence intensity (MFI) obtained for each specific cytokine after subtracting the MFI of the corresponding isotype-matched negative control.

Statistical analyses

Data were analyzed using SPSS software (SPSS 12.0; Chicago, IL, USA). The normal quantile (Normal Q-Q Plot) test was used to assess whether or not data for each variable had a normal distribution. The statistical significance of the differences observed between healthy controls and diabetic men (unpaired samples) was determined using the Student's *t* and Mann-Whitney *U* tests for variables with a normal and non-parametric distribution, respectively. The statistical significance of the differences observed between distinct treatment time points in diabetic samples (paired samples) was determined using the non-parametric Friedman and Wilcoxon signed-rank tests. The relationship between plasma hormone values (testosterone, LH and FSH) with antigen-presenting cell (APC) counts (monocytes and DCs) was tested by Spearman correlation analysis. *P* values (two-tailed) of ≤ 0.05 were considered to be associated with statistical significance.

Results

The clinical characteristics of the control subjects and type-2 diabetic men are shown in Table 1. Both groups had similar anthropometric features but, as expected, exhibited significantly different fasting plasma glucose ($P=0.001$) and C-peptide ($P=0.04$) levels. In addition, the diabetic patients had lower serum concentrations of total testosterone ($P=0.04$) and higher LH levels ($P=0.02$) than controls. Replacement therapy with testosterone enanthate increased the serum levels of total and free testosterone while the serum values of LH decreased to undetectable levels (Fig. 1).

All but one patient completed the 12-month course of testosterone-replacement therapy. During this period, no patient had any adverse cardiovascular or prostatic events. In one patient, testosterone therapy was withdrawn after completing 6 months of treatment due to an increase in his hematocrit value above 52%. In another two cases, a phlebotomy was performed at 6 and 9 months after starting treatment because of a hematocrit value of between 50 and 52%. The mean PSA levels before and after testosterone treatment were 1.4 and 1.8 ng/ml, respectively. No significant differences in weight were detected before testosterone therapy or at the end of treatment (78 ± 14 compared with 76 ± 14 kg, respectively).

Prior to testosterone-replacement therapy, the type-2 diabetic men showed higher white blood cell ($P \leq 0.05$) and monocyte ($p \leq 0.05$) counts, together with increased numbers of CD4⁺/CD8^{lo} T cells ($P \leq 0.01$) as compared with the control subjects (Table 2). In general, testosterone treatment did not induce significant quantitative changes in most of the subsets of white blood cells studied, in either absolute or relative numbers, which remained stable and similar to those found before treatment.

Figure 1 Serum concentrations of testosterone and LH before (0) and during 12 months of treatment with 150 mg testosterone enanthate administered intramuscularly in 13 type-2 diabetic men. Results are expressed as means $\pm$ s.d. * $P < 0.05$, vs before treatment; ** $P < 0.01$ vs before treatment. TT, total testosterone; FT, free testosterone.

Despite this, some exceptions were noted, including a transient increase in the number of polymorphonuclear leukocytes at month 1 of treatment ($P < 0.05$), as well as both an increase in the number of CD4⁺/CD8⁺ T cells ($P < 0.05$) and a decrease in the proportion of CD4⁺/

CD8⁺ T cells ($P < 0.05$) at the end of therapy (6 and 12 months); these were associated with a transient increase in the percentage of CD56⁺/CD8⁺ NK cells (1 month of therapy) and a decrease in the percentage of CD56⁺/CD8⁺ NK cells (at 1 month), with respect to the data

Table 2 Distribution of monocytes and subsets of lymphocytes and dendritic cells (DCs) in PB samples from type-2 diabetic men with partial androgen deficiency prior to and during testosterone-replacement therapy (TX), as compared to healthy age-matched controls

	Diabetic patients with partial androgen deficiency					
	Controls	Prior to TX	1 month TX	3 month TX	6 month TX	12 month TX
Cell subset						
No. of WBCs/ μ l	5778 $\pm$ 1008	6972 $\pm$ 1939*	7832 $\pm$ 2232**	7259 $\pm$ 1872*	7872 $\pm$ 2273**	8050 $\pm$ 2188**
No. of PMN leukocytes	3617 $\pm$ 818	3940 $\pm$ 1636	4684 $\pm$ 1366*	4448 $\pm$ 1877	4240 $\pm$ 1494	4453 $\pm$ 1746
No. of monocytes/ μ l	335 $\pm$ 89	426 $\pm$ 132*	444 $\pm$ 155	416 $\pm$ 145	422 $\pm$ 134	456 $\pm$ 113*
No. of lymphocytes/ μ l	1331 $\pm$ 482	1636 $\pm$ 549	1539 $\pm$ 663	1623 $\pm$ 556	1738 $\pm$ 737	1582 $\pm$ 450
No. of T cells/ μ l	924 $\pm$ 356	1140 $\pm$ 430	1084 $\pm$ 482	1107 $\pm$ 428	1202 $\pm$ 518	1086 $\pm$ 364
% CD4 ⁺ /CD8 ⁺ T cells	60 $\pm$ 7	61 $\pm$ 9	60 $\pm$ 9	61 $\pm$ 8	58 $\pm$ 11 ⁺	56 $\pm$ 7.4 ⁺⁺
% CD4 ⁺ /CD8 ⁺ T cells	0.36 $\pm$ 0.19	0.56 $\pm$ 1.55	0.30 $\pm$ 0.36	0.27 $\pm$ 0.22	0.31 $\pm$ 0.21	0.70 $\pm$ 1.12
% CD4 ⁺ /CD8 ^{lo} T cells	1.3 $\pm$ 2.9	0.8 $\pm$ 0.9	0.4 $\pm$ 0.8	0.24 $\pm$ 0.56	0.34 $\pm$ 0.7	0.44 $\pm$ 0.8
% CD4 ⁺ /CD8 ^{lo} T cells	0.14 $\pm$ 0.43	2.3 $\pm$ 2.5**	0.58 $\pm$ 2.1	0.19 $\pm$ 0.7	0.49 $\pm$ 1.5	0.56 $\pm$ 1.9
% CD4 ⁺ /CD8 ⁺ T cells	34.7 $\pm$ 7	31.6 $\pm$ 8.2	36.1 $\pm$ 8	35.8 $\pm$ 7.2	37.1 $\pm$ 10.7 [†]	39.3 $\pm$ 8.5 ^{††}
No. of B cells/ μ l	103 $\pm$ 68	128 $\pm$ 80	133 $\pm$ 107	154 $\pm$ 109	144 $\pm$ 71	147 $\pm$ 109
No. of NK cells/ μ l	195 $\pm$ 121	252 $\pm$ 150	246 $\pm$ 212	202 $\pm$ 101	245 $\pm$ 161	218 $\pm$ 104
No. of CD56 ⁺ /CD8 ⁺ NK cells	84 $\pm$ 70	103 $\pm$ 77	84 $\pm$ 57	67 $\pm$ 34	89 $\pm$ 74	76 $\pm$ 40
No. of CD56 ⁺ /CD8 ⁺ NK cells	111 $\pm$ 57	149 $\pm$ 100	162 $\pm$ 167	134 $\pm$ 85	156 $\pm$ 104	142 $\pm$ 93
% of NK cells/WBC	4.1 $\pm$ 2.9	3.7 $\pm$ 2.1	3.4 $\pm$ 3.1	3.0 $\pm$ 1.7	3.2 $\pm$ 2.1	2.9 $\pm$ 1.6
% CD56 ⁺ /CD8 ⁺ NK cells	43 $\pm$ 15	40 $\pm$ 14	36 $\pm$ 14 [†]	35.4 $\pm$ 12	37 $\pm$ 15	37 $\pm$ 16
% CD56 ⁺ /CD8 ⁺ NK cells	57 $\pm$ 15	59 $\pm$ 14	64 $\pm$ 14 [†]	65 $\pm$ 12	63 $\pm$ 15	63 $\pm$ 16
No. of total DCs/ μ l	76 $\pm$ 35	99 $\pm$ 39	112 $\pm$ 49*	100 $\pm$ 49	107 $\pm$ 39	107 $\pm$ 45
No. of CD33 ^{hi} DCs/ μ l	9.9 $\pm$ 3.3	12.9 $\pm$ 5	14.7 $\pm$ 5.6*	14.4 $\pm$ 6.7	14.8 $\pm$ 8	15.6 $\pm$ 5.2**
No. of CD16 ⁺ DCs/ μ l	58 $\pm$ 35	77 $\pm$ 36	88 $\pm$ 46	76 $\pm$ 42	81 $\pm$ 32	80 $\pm$ 43
No. of CD123 ^{hi} DCs/ μ l	7.8 $\pm$ 2.3	9.6 $\pm$ 4.5	9.2 $\pm$ 3.9	9.6 $\pm$ 5.1	10.8 $\pm$ 4* [†]	10.8 $\pm$ 6.1

Results are expressed as means $\pm$ s.d. Statistical analyses were performed using the Mann-Whitney *U* test (for comparisons between healthy controls and diabetic men) and both the Friedman and Wilcoxon signed rank tests (for comparisons between distinct treatment time-points in diabetic samples). * $P \leq 0.05$ and ** $P \leq 0.01$ vs controls; [†] $P < 0.05$ and ^{††} $P < 0.01$ vs prior to TX. WBC, white blood cells; NK, natural killer cells; PMN, polymorphonuclear.

Table 3 Spontaneous *ex vivo* secretion of inflammatory cytokines by peripheral blood monocytes and dendritic cells (DCs) in type-2 diabetic patients with partial androgen deficiency before treatment and after 1, 3, 6 and 12 months of testosterone-replacement therapy (TX), in comparison to control subjects

	Type-2 diabetic men					
	Controls	Prior to treatment	1 month TX	3 month TX	6 month TX	12 month TX
Cells and cytokines						
Monocytes						
% of IL-1 β	0.8 $\pm$ 1.6	0.3 $\pm$ 1.1	<0.01	<0.01	<0.01	<0.01
MFI of IL-1 β	100 $\pm$ 77	104 $\pm$ 32	NE	NE	NE	NE
% of IL-6	3.0 $\pm$ 4.3	2.5 $\pm$ 8.1	1.6 $\pm$ 1.8	2.8 $\pm$ 3.3	4.1 $\pm$ 4.9	<0.01*
MFI of IL-6	275 $\pm$ 132	292 $\pm$ 168	232 $\pm$ 169	315 $\pm$ 288	160 $\pm$ 76	NE
% of TNF α	3.1 $\pm$ 4.8	2.3 $\pm$ 4.5	1.7 $\pm$ 2.3	3.1 $\pm$ 5.6	1.5 $\pm$ 2.4	<0.01†*
MFI of TNF α	673 $\pm$ 440	464 $\pm$ 274	507 $\pm$ 756	417 $\pm$ 290	385 $\pm$ 51	NE
CD33^{hi} myeloid DCs						
% of IL-1 β	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
MFI of IL-1 β	NE	NE	NE	NE	NE	NE
% of IL-6	<0.01	0.6 $\pm$ 2.3	<0.01	2.1 $\pm$ 5	<0.01	<0.01
MFI of IL-6	NE	453 $\pm$ 340	NE	232 $\pm$ 81	NE	NE
% of TNF α	<0.01	1.4 $\pm$ 4	2.6 $\pm$ 6.6	1.1 $\pm$ 2.8	<0.01	<0.01
MFI of TNF α	NE	653 $\pm$ 406	319 $\pm$ 3854	446 $\pm$ 485	NE	NE
CD16+ DCs						
% of IL-1 β	<0.01	0.1 $\pm$ 0.41	<0.01	<0.01	<0.01	<0.01
MFI of IL-1 β	NE	78 $\pm$ 35	NE	NE	NE	NE
% of IL-6	2.3 $\pm$ 4.7	1.5 $\pm$ 5.2	1.3 $\pm$ 2.1	1.5 $\pm$ 2.3	2.9 $\pm$ 3.5	0.4 $\pm$ 1.1
MFI of IL-6	215 $\pm$ 105	249 $\pm$ 152	196 $\pm$ 160	202 $\pm$ 117	186 $\pm$ 78	161 $\pm$ 23
% of TNF α	3.1 $\pm$ 5.2	2.8 $\pm$ 8.2	3.7 $\pm$ 4.1	9.1 $\pm$ 21.8	2.6 $\pm$ 4.4	1.7 $\pm$ 1.4
MFI of TNF α	787 $\pm$ 468	568 $\pm$ 398	327 $\pm$ 267	556 $\pm$ 689	361 $\pm$ 213	203 $\pm$ 59
Plasmacytoid DCs						
% of IL-1 β	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
MFI of IL-1 β	NE	NE	NE	NE	NE	NE
% of IL-6	10 $\pm$ 20	7.6 $\pm$ 13.6	9.5 $\pm$ 13.4	12.4 $\pm$ 17	<0.01	<0.01
MFI of IL-6	49 $\pm$ 8	69 $\pm$ 19	53 $\pm$ 8	56 $\pm$ 9	NE	NE
% of TNF α	6.8 $\pm$ 10.3	6.9 $\pm$ 12.6	8.6 $\pm$ 11.6	4.8 $\pm$ 9.2	3.3 $\pm$ 8.9	<0.01
MFI of TNF α	47 $\pm$ 7	57 $\pm$ 13	51 $\pm$ 12	60 $\pm$ 14	58 $\pm$ 4	NE

Results are expressed as means $\pm$ S.D. Statistical analyses were performed using the Mann–Whitney *U* test for comparisons between healthy controls and diabetic men and both the Friedman and Wilcoxon signed rank tests for comparisons between distinct treatment time points in diabetic samples. * $P \leq 0.05$ vs controls; † $P \leq 0.05$ vs prior to therapy. MFI, mean fluorescence intensity; NE, not evaluated owing to the absence of cytokine-positive cells.

before treatment. In addition, transient increases in the number of CD33^{hi} myeloid DCs (months 1 and 12 of therapy) and CD123^{hi} plasmacytoid DCs (month 6) to values significantly higher than those of the controls were also observed during treatment with testosterone (Table 2).

Small percentages of monocytes and DCs capable of spontaneously secreting detectable amounts of proinflammatory cytokines were detected in both the diabetic men and the control group prior to therapy (Table 3). After testosterone-replacement therapy, the number of PB monocytes capable of spontaneously producing IL-1 β , IL-6 and TNF α became undetectable both at the first follow-up time point (IL-1 β) and at the end of therapy (IL-6 and TNF α). Likewise, the percentage of CD33^{hi} myeloid DCs and that of plasmacytoid DCs capable of spontaneously producing IL-6 and TNF α also became undetectable during testosterone treatment. In contrast, no major changes were observed with testosterone-

replacement therapy in the spontaneous production of proinflammatory cytokines by peripheral blood CD16+ DCs (Table 3).

Prior to therapy, CD33^{hi} myeloid DCs and CD16+ DCs from the diabetic men showed a decreased ability to secrete IL-1 β ($P \leq 0.05$) after *in vitro* stimulation with LPS as compared with the control group (Table 4). Testosterone-replacement therapy was associated with a transient recovery of the percentage of IL-1 β -secreting CD33^{hi} myeloid DCs and CD16+ DCs at months 3 and 12, and month 3 of therapy, respectively. In addition, testosterone-replacement therapy was also associated with a decreased number of TNF α -secreting plasmacytoid DCs at month 12.

Interestingly, analysis of the relationship between plasma hormone levels (testosterone, LH and FSH) and cytokine production by different subsets of PB APCs in diabetic men revealed a statistically significant negative correlation between the levels of LH and the percentage

Table 4 *In vitro*-stimulated secretion of inflammatory cytokines by peripheral blood (PB) monocytes and dendritic cells (DCs) in type-2 diabetic patients with partial androgen deficiency before treatment and after 1, 3, 6 and 12 months of testosterone-replacement therapy (TX), in comparison to control subjects

	Type-2 diabetic men					
	Controls	Before treatment	1 month TX	3 month TX	6 month TX	12 month TX
Cells and cytokines						
Monocytes						
% of IL-1 β	100 $\pm$ 0	90 $\pm$ 25	84 $\pm$ 27*	92 $\pm$ 23†	75 $\pm$ 39	93 $\pm$ 21
MFI of IL-1 β	76 $\pm$ 49	91 $\pm$ 74	79 $\pm$ 54	75 $\pm$ 29	60 $\pm$ 21	62 $\pm$ 20
% of IL-6	96 $\pm$ 11	89 $\pm$ 26	91 $\pm$ 22	83 $\pm$ 31	76 $\pm$ 34	86 $\pm$ 28
MFI of IL-6	2407 $\pm$ 1575	2176 $\pm$ 1486	1838 $\pm$ 1008	2224 $\pm$ 1433	1124 $\pm$ 942	1356 $\pm$ 898
% of TNF α	87 $\pm$ 26	88 $\pm$ 29	87 $\pm$ 23	93 $\pm$ 18	79 $\pm$ 30	84 $\pm$ 29
MFI of TNF α	2371 $\pm$ 1486	2466 $\pm$ 1634	1823 $\pm$ 1044	2268 $\pm$ 1542	1314 $\pm$ 1124	1334 $\pm$ 1215
CD33^{hi} myeloid DCs						
% of IL-1 β	96 $\pm$ 12	86 $\pm$ 20*	80 $\pm$ 22*	93 $\pm$ 10†	78 $\pm$ 23*	95 $\pm$ 9†
MFI of IL-1 β	50 $\pm$ 25	60 $\pm$ 43	56 $\pm$ 40	46 $\pm$ 15	41 $\pm$ 12	42 $\pm$ 14
% of IL-6	89 $\pm$ 24	90 $\pm$ 18	90 $\pm$ 17	90 $\pm$ 20	83 $\pm$ 23	92 $\pm$ 14
MFI of IL-6	957 $\pm$ 755	911 $\pm$ 674	729 $\pm$ 359	831 $\pm$ 572	388 $\pm$ 284	537 $\pm$ 361
% of TNF α	92 $\pm$ 16	80 $\pm$ 28	83 $\pm$ 23	90 $\pm$ 20	86 $\pm$ 18	89 $\pm$ 21
MFI of TNF α	979 $\pm$ 641	1349 $\pm$ 950	1251 $\pm$ 1010	1098 $\pm$ 601	612 $\pm$ 484	726 $\pm$ 608
CD16+ DCs						
% of IL-1 β	97 $\pm$ 7	84 $\pm$ 24*	75 $\pm$ 30**	99 $\pm$ 1††	65 $\pm$ 39*	88 $\pm$ 26
MFI of IL-1 β	82 $\pm$ 52	86 $\pm$ 60	78 $\pm$ 72	59 $\pm$ 16	58 $\pm$ 18	59 $\pm$ 20
% of IL-6	91 $\pm$ 26	88 $\pm$ 23	90 $\pm$ 21	89 $\pm$ 19	77 $\pm$ 34	88 $\pm$ 24
MFI of IL-6	2049 $\pm$ 1255	1685 $\pm$ 1190	1354 $\pm$ 868	1672 $\pm$ 1047	915 $\pm$ 563*	1092 $\pm$ 742
% of TNF α	89 $\pm$ 22	86 $\pm$ 26	84 $\pm$ 26	92 $\pm$ 20	82 $\pm$ 25	85 $\pm$ 28
MFI of TNF α	3961 $\pm$ 2055	3215 $\pm$ 2002	2866 $\pm$ 1742	3333 $\pm$ 1559	1780 $\pm$ 1320**	2478 $\pm$ 1567
Plasmacytoid DCs‡						
% of IL-6	17 $\pm$ 19	9 $\pm$ 18	22 $\pm$ 21	17 $\pm$ 19	9 $\pm$ 16	12 $\pm$ 21
MFI of IL-6	60 $\pm$ 12	79 $\pm$ 23	62 $\pm$ 9	70 $\pm$ 14	57 $\pm$ 7	56 $\pm$ 6
% of TNF α	14 $\pm$ 19	11 $\pm$ 19	18 $\pm$ 16	15 $\pm$ 19	8 $\pm$ 20	4 $\pm$ 13†
MFI of TNF α	62 $\pm$ 13	67 $\pm$ 15	54 $\pm$ 10	64 $\pm$ 13	53 $\pm$ 2	<0.01

Results are expressed as means $\pm$ s.d. Statistical analyses were performed using the Mann–Whitney *U* test for comparisons between healthy controls and diabetic men and both the Friedman and Wilcoxon signed rank tests for comparisons between distinct treatment time points in diabetic samples. * $P \leq 0.05$ and ** $P \leq 0.01$ versus controls. † $P \leq 0.05$ and †† $P \leq 0.01$ versus before treatment. ‡No plasmacytoid DCs capable of producing IL-1 β were detected in either controls or diabetics. MFI, mean fluorescence intensity.

of both monocytes ($r^2 = -0.329$; $P = 0.02$) and CD16+ DCs ($r^2 = -0.311$; $P = 0.03$) spontaneously producing IL-6 in untreated patients. In turn, LH serum levels correlated positively with both the mean amount of IL-6 spontaneously secreted per cell (MFI) by CD16+ DCs ($r^2 = 0.40$; $P = 0.03$) in both untreated and treated diabetic men and the mean amount of IL-6 produced by CD33^{hi} myeloid DCs after stimulation with LPS plus IFN γ ($r^2 = 0.419$; $P = 0.01$) in type-2 diabetic men during treatment with testosterone.

Discussion

In the present study we show for the first time that testosterone-replacement therapy in type-2 diabetic men with partial androgen deficiency is associated with an almost complete abrogation of spontaneous *ex vivo* secretion of the IL- β , IL-6 and TNF α inflammatory cytokines by both circulating PB monocytes and DCs. Interestingly, similar effects were observed for the various subsets of

circulating APCs and the different inflammatory cytokines analyzed.

To date, few reports have analyzed the effects of androgen-replacement therapy on cytokine production in hypogonadal subjects. In this regard, it has been shown that androgen therapy in patients with Klinefelter's syndrome decreased the levels of serum IgA, IgG, IgM, IL-2 and IL-4 (Koçar *et al.* 2000). Likewise, testosterone-replacement therapy in hypogonadal men has been shown to induce reductions in TNF α and IL-1 β serum levels, together with increased IL-10, in the absence of significant changes in the serum concentrations of IL-6 (Malkin *et al.* 2004). In turn, gonadotropin treatment in patients with idiopathic hypogonadotropic hypogonadism, significantly decreased the production of IL-1 β and TNF α by stimulated PB mononuclear cells; this effect was associated with decreased serum levels of IL-2 and IL-4 and reduced PB counts of total lymphocytes, CD3+ and CD4+ T cells, CD19+ B cells and the CD4+/CD8+ ratio (Yesilova *et al.* 2000, Musabak *et al.* 2003). In addition, it has been shown that acute hypogonadism induced in

normal elderly men by administration of gonadotropin-releasing hormone (GnRH) has the opposite effect, increasing the circulating levels of TNF α and IL-6. In turn, in these subjects subsequent transdermal testosterone therapy reduced the concentrations of TNF α but not those of IL-6 (Kosla *et al.* 2002). Our results confirm and expand these observations in that they show, in another model of human androgen deficiency, that androgen-replacement therapy depresses spontaneous *ex vivo* production of inflammatory cytokines by PB APCs. Interestingly, our results also indicate that depression of the production of proinflammatory cytokine associated with androgen therapy is not due to an exhaustion of the ability of these cells to produce these cytokines in diabetic men since stimulation of PB monocytes and DCs with LPS plus IFN γ induced fairly similar patterns of response to those observed in a group of age-matched healthy controls.

Little is known about the effects of androgen treatment on DC in humans. In this sense, Galasso *et al.* (1996) investigated the effects of topical application of testosterone propionate on the epidermal density of CD1+ DCs in a group of women, detecting a clear reduction of these cells. Likewise, in the mouse, systemic and topical application of testosterone significantly reduced the density of epidermal Langerhans cells (Koyama *et al.* 1989).

In our patients, testosterone-replacement therapy could induce immunosuppressive effects through either direct or indirect mechanisms. Androgen receptors or their mRNA have been detected in both CD4+ and CD8+ T cells as well as in macrophages (Liva & Voskuhl 2001, Tanriverdi *et al.* 2003) and it has been shown that testosterone may directly induce cytokine gene expression on CD4+ T lymphocytes via androgen receptors (Liva & Voskuhl 2001). Monocyte-derived macrophages also express androgen receptors (McCrohon *et al.* 2000) and these cells may metabolize testosterone to 5 α -dihydrotestosterone (Araneo *et al.* 1991). In this regard, treatment of mice with flutamide, an androgen-receptor blocker, restores previously depressed cellular immunity after brain injury (Messingham *et al.* 2001). However, to the best of our knowledge no study has been reported so far in which the expression of androgen receptors is analyzed in DCs. Therefore, the key premise for a direct action of androgens on these cells is currently lacking. In turn, GnRH has immunostimulatory effects (Tanriverdi *et al.* 2003) and it has been suggested that the immunosuppressive effects of androgens could be indirectly mediated by GnRH (Jacobson & Ansari 2004). Thus, whereas androgens have suppressive effects on GnRH and gonadotropin secretion, estrogens enhance their secretion through a positive-feedback effect (Jacobson & Ansari 2004). In line with this notion, plasma levels of LH showed a significant correlation with IL-6 secretion by both CD16+ DCs and CD33^{hi} myeloid DC in diabetic patients during treatment with testosterone. Another possibility could be related to the fact that the secretion of IL-1 β and TNF α is under the

positive influence of IFN γ , as also seen in this study, and it has been shown that the production of IFN γ by immune cells is reduced by testosterone (Araneo *et al.* 1991).

The anti-inflammatory effects of androgen therapy observed in our model may have both beneficial and deleterious effects. Regarding the former, a depression in proinflammatory cytokine secretion induced by androgens in DC may in turn alter the patterns of cytokine secretion by T cells and alter their ability to direct T-helper responses (Cua & Stohlman 1997, Chang *et al.* 2000). In this regard, it has been reported that sex steroids induce changes in cytokine secretion by APCs in mice (Wilcoxon *et al.* 2000); these changes polarize cytokine secretion by T cells towards a Th1 or Th2 pattern (Wilcoxon *et al.* 2000, Lanzavecchia & Sallusto 2001). A decrease in the production of Th1-associated cytokines (IL-1 β , IL-6, IFN γ and TNF α) by immune cells may bias the balance towards a Th2 pattern (IL-4, IL-10, IL-12), which could protect subjects from autoimmune diseases (Dalal *et al.* 1997, Liva & Voskuhl 2001), whereas a predominance of Th1 cytokines would enhance susceptibility to developing autoimmune disorders (Bao *et al.* 2002).

Since monocytes, DCs and the inflammatory cytokines they secrete play an important role in both the development of atheroma plaque and its stability (Hansson 2005) it could be speculated that a decrease in cytokine production might have anti-atheromatous effects. Therefore, the anti-inflammatory role of androgen-substitutive therapy in aging type-2 diabetic men with partial androgen deficiency, a condition associated with clinically evident atherosclerosis in humans (Fukui *et al.* 2003), could have atheroprotective effects (Malkin *et al.* 2003). In turn, a depressed immune response by APCs may be detrimental in terms of host defence. In this sense, it has been shown that females tolerate trauma hemorrhage and subsequent sepsis better than males (Schroder *et al.* 1998).

In summary, our findings indicate that androgen-replacement therapy in aging type-2 diabetic men with clinical and biochemical androgen deficiency has immunosuppressive and anti-inflammatory effects on both circulating PB monocytes and DCs. The anti-inflammatory effects of androgens may contribute to our understanding of the well-known relative resistance of males to autoimmune diseases and the outcome of atherosclerosis, and at the same time they could contribute to explain the depressive effect of androgens on host cellular immunity. Our results add new information to a promising field related to autoimmune diseases, gender responses to inflammation/sepsis, atheromatosis and plaque stability.

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