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Androgens alter T-cell immunity by inhibiting T-helper 1 differentiation

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The hormonal milieu influences immune tolerance and the immune response against viruses and cancer, but the direct effect of androgens on cellular immunity remains largely uncharacterized. We therefore sought to evaluate the effect of androgens on murine and human T cells in vivo and in vitro. We found that murine androgen deprivation in vivo elicited RNA expression patterns conducive to IFN signaling and T-cell differentiation. Interrogation of mechanism showed that testosterone regulates T-helper 1 (Th1) differentiation by inhibiting IL-12–induced Stat4 phosphorylation: in murine models, we determined that androgen receptor binds a conserved region within the phosphatase, Ptpn1, and consequent up-regulation of Ptpn1 then inhibits IL-12 signaling in CD4 T cells. The clinical relevance of this mechanism, whereby the androgen milieu modulates CD4 T-cell differentiation, was ascertained as we found that androgen deprivation reduced expression of Ptpn1 in CD4 cells from patients undergoing androgen deprivation therapy for prostate cancer. Our findings, which demonstrate a clinically relevant mechanism by which androgens inhibit Th1 differentiation of CD4 T cells, provide rationale for targeting androgens to enhance CD4-mediated immune responses in cancer or, conversely, for modulating androgens to mitigate CD4 responses in disorders of autoimmunity.

immunomodulation | cancer immunotherapy | prostate neoplasm

The sex-specific hormones, testosterone and estrogen, have a number of immuno-modulatory effects. Women almost universally respond as well or better than men to antibody-inducing vaccinations (1). For example, healthy women treated with the trivalent inactivated influenza vaccine generate a greater antibody titer than men (2). Findings like these have led to the suggestion that estrogen promotes T-helper 2 (Th2) differentiation and antibody production (3). Further supporting an increased antibody response caused by estrogen, more than 80% of patients suffering from antibody-driven autoimmunities such as systemic lupus erythematosus, Sjörgren syndrome, and Hashimoto thyroiditis are women (4). In contrast to estrogen, how testosterone affects the immune system is less clear, but its role in immunity against viruses and host antigens is certainly immunosuppressive.

Recently, it was reported that testosterone levels negatively correlated with the antibody response to the trivalent inactivated seasonal influenza vaccine by interfering with lipid metabolism (5). Testosterone levels are also positively correlated with the viral load of Venezuelan equine encephalitis virus in macaques (6). In addition to the response to viruses, testosterone regulates the response to host antigens in many biological systems. Elevated levels of testosterone following colonization with commensal microbes correlated with reduced islet inflammation and protection from type 1 diabetes in nonobese diabetic mice (7). Also, tolerance to tumor antigens is regulated by testosterone, as androgen ablation in a mouse model of prostate cancer reversed CD4 T-cell tolerance to a prostate restricted tumor antigen (8). Similarly, castration of male mice before vaccination with prostate-specific antigens enhanced CD8 T-cell vaccine response in many studies (9, 10). Patients undergoing androgen deprivation in prostate cancer had increased infiltration of T cells into benign and malignant prostate tissue (11). Based on these findings, clinical trials are currently underway to test the combinatorial efficacy of androgen deprivation and immunotherapy in prostate cancer patients (12). Together, these observations suggest a critical role for testosterone in maintaining T-cell tolerance toward not only viruses, but also host and tumor antigens.

Despite these observations that testosterone inhibits immunity, the precise molecular mechanisms by which testosterone achieves this effect are poorly understood. Here, we sought to address this question by performing gene expression profiling of CD4 T cells isolated from castrated mice. Gene expression analysis revealed a critical effect of testosterone on CD4 T-cell differentiation and identified protein tyrosine phosphatase nonreceptor type 1 (Ptpn1) as a mediator of androgen-induced suppression of CD4 T-cell differentiation. The research presented here highlights a previously unreported molecular mechanism by which testosterone suppresses immunity and allows a better understanding of sex differences in the response to viruses, autoimmunity, and immune escape in prostate cancer.

Results

Gene Expression Profiling of CD4 T Cells Isolated from Castrated Mice Reveals Altered Differentiation and Signaling. To investigate the molecular mechanisms by which androgen withdrawal affects the

Significance

Testosterone has been implicated as a regulator of the immune response to viruses, vaccines, host tissue, and cancer. Despite this pleiotropic effect on the immune system, the mechanisms underlying this effect are not well understood. In this study, we investigated how testosterone altered gene expression and signaling mechanisms in CD4 T cells in mouse models and prostate cancer patients undergoing androgen deprivation therapy. We found that testosterone inhibited T-helper 1 differentiation by up-regulating the phosphatase, *Ptpn1*, in both mice and humans. Additionally, the androgen receptor bound a highly conserved region of the Ptpn1 gene, suggesting an evolutionarily important purpose of this mechanism. This study provides a mechanism to explain recent discoveries regarding the role of testosterone-mediated inhibition of the immune response.

Author contributions: H.T.K., M.G.S., L.K.D., and M.S.A. designed research; H.T.K., L.K.D., K.L.P., S.T.O., J.K.N., and M.S.A. performed research; H.T.K. and M.S.A. contributed new reagents/analytic tools; H.T.K., L.K.D., K.L.P., S.T.O., J.K.N., and M.S.A. analyzed data; and H.T.K., M.G.S., L.K.D., and M.S.A. wrote the paper.

The authors declare no conflict of interest.

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Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE54945).

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T-cell response to self-antigens, gene expression of CD4 T cells isolated from the spleens of surgically castrated or control mice was profiled using Affymetrix microarrays. Of the 1,037 twofold differentially expressed genes, 637 were down-regulated and 400 were up-regulated following castration. Using the Ingenuity Pathway Analysis (IPA) software package, we found that the two most significantly altered pathways within those differentially regulated were IFN signaling and T-helper cell differentiation (Fig. 1*A*). About half of the genes that constitute these two pathways were differentially expressed. In particular, numerous genes related to Th1 differentiation such as T-bet, IL-12R, and IFN-γ were up-regulated (Fig. 1*B*). To confirm these microarray findings, expression of key CD4 T-cell differentiation genes was measured by RT-PCR. Consistent with the microarray analysis, a significant increase in expression of T-bet and IFN-γ was observed, whereas no change was observed for the other master transcription factors involved in CD4 T-cell differentiation, Gata-3, Foxp3, and Ror-γt (Fig. 1*C*), suggesting that differentiation of CD4 T cells into the Th1 phenotype was preferentially affected. Expression of the Th1 surface markers CXCR3, CCR5, and IL-12Rb2 were analyzed. CXCR3 was significantly increased in CD4 cells in castrated mice, whereas the other two markers were unchanged (Fig. S1). Additionally, analysis of upstream transcription factors responsible for the observed gene changes predicted significant activation of the Th1-related transcription factors IRF-1, -3, and -7 and signal transducer and activator of transcription 1 (STAT1) in castrated CD4 T cells (Table S1). These data suggest that important signaling pathways and gene expression regulatory networks orchestrating Th1 differentiation are altered following castration.

Testosterone Inhibits Th1 Differentiation In Vitro. T-helper cell differentiation is a critical junction of an immune response, and Th1 differentiation is particularly important in the case of the response to self- and tumor antigens (13). Following the in silico identification of the major disruptions to Th1 differentiation by androgen deprivation, we sought to further investigate the effect of androgen on this differentiation pathway in vitro. CD4 T cells isolated from spleens of male mice were incubated overnight in androgen-free culture medium. These cells were then cultured under Th1 polarizing conditions in the presence of the androgen analog R1881 or vehicle control and then restimulated with phorbol myristate acetate/ionomycin 3 d later. We found that CD4 T cells treated with androgen produced significantly less IFN-γ as measured by intracellular flow cytometry (Fig. 2 *A* and *B*). To determine whether this was due to inhibition of Th1 differentiation or the recall response, IFN-γ production was measured in cells differentiated without androgen but restimulated in its presence. These cells produced the same amount of IFN-γ as cells that were not exposed to androgen, indicating that androgen inhibited IFN-γ production before restimulation. Similarly, IFN-γ production by cells differentiated for 3 d in the presence of androgen, but restimulated overnight in androgen-free media produced the same amount of IFN-γ as cells both differentiated and restimulated in the presence of androgen, suggesting that androgen inhibition of IFN-γ production by CD4 T cells occurs during the Th1 differentiation phase. To further investigate the effect of androgen on T-cell differentiation, RNA was isolated from cells differentiated in androgen or vehicle control before PMA/ionomycin stimulation. Consistent with the in silico analysis, CD4 T cells differentiated in the presence of androgen had significantly reduced levels of T-bet and IFN-γ (Fig. 2*C*). Together, these data suggest that androgen exerts its effect on T cells during the early events involved in Th1 differentiation.

Testosterone Inhibits IL-12–Induced STAT4 Phosphorylation and Downstream Gene Expression. Because Th1 differentiation critically relies on IL-12–induced phosphorylation of STAT4, we analyzed this protein in CD4 T cells treated with IL-12. STAT4 phosphorylation was reduced in CD4 T cells treated with androgen 30 min following IL-12 exposure. The androgen-treated cells still

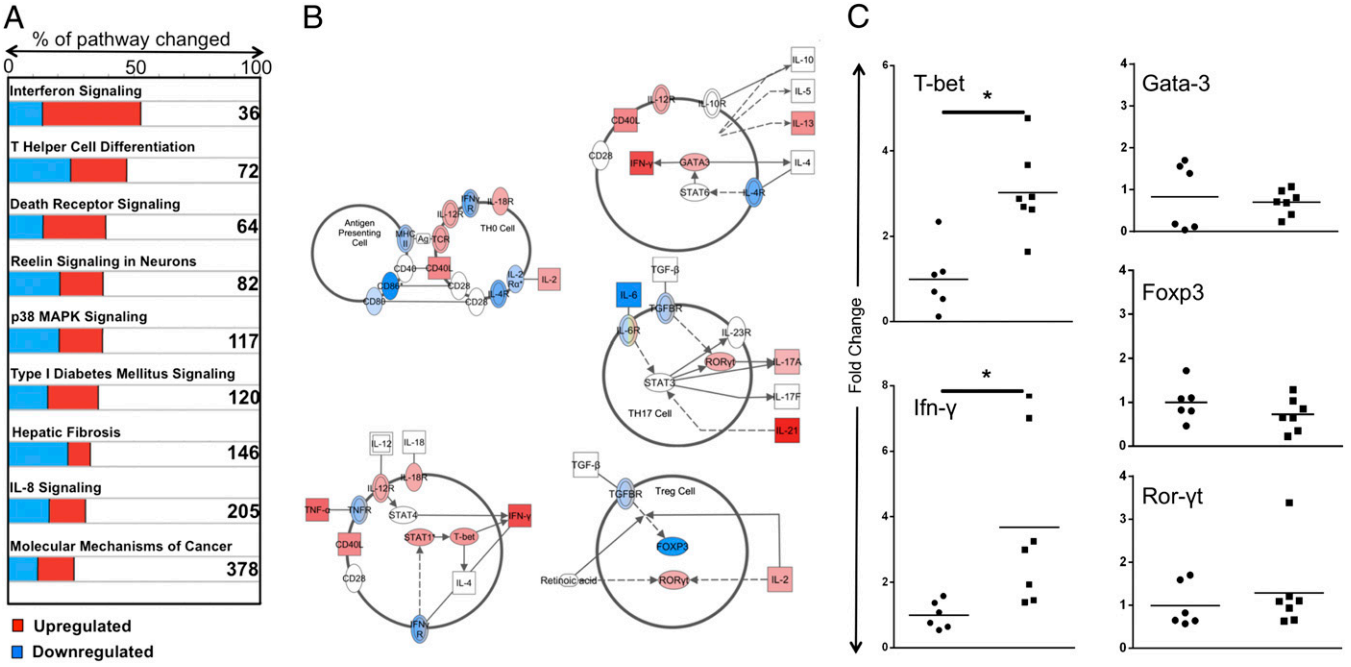

Fig. 1. Th1 differentiation is enhanced in castrated mice. (*A*) Significantly altered pathways in CD4 cells isolated from castrated mice. Genome-wide gene expression profiling of CD4 T cells isolated from the spleen of male mice 1 mo following castration was performed and significantly altered signaling pathways determined using the Ingenuity Pathway Analysis package. (*B*) Genes changed in CD4 T-cell differentiation pathways. CD4 T-cell differentiation pathway was found to be significantly altered in castrated mice and differentially expressed genes associated with these pathways are shown. (*C*) PCR confirmation of important differentiation genes. Gene expression of important differentiation regulators/markers in CD4 T cells isolated from sham (●) or castrated mice (■) was measured by RT-PCR. Data combined from two separate experiments are shown.

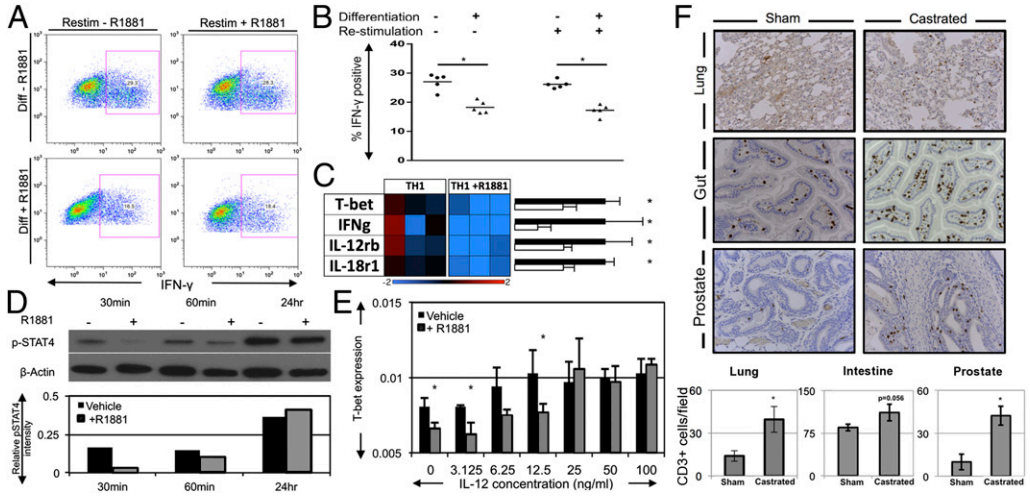

Fig. 2. Th1 differentiation is inhibited by testosterone in vitro. (*A* and *B*) IFN-γ production by CD4 cells differentiated with testosterone. Spleen CD4 T cells were cultured under Th1 polarizing conditions and treated with the androgen receptor agonist R1881 or vehicle control. Representative plots of IFN-γ production measured by flow cytometry after restimulation with PMA/Ionomycin. Mean ± SD of IFN-γ production after Th1 differentiation and restimulation in the presence of R1881 or vehicle for five samples. (*C*) Gene expression in testosterone treated Th1 cells. Fold change of gene expression of Th1-related genes from CD4 T-cell differentiation ($n = 3$). (*D*) Testosterone effect on Stat4 phosphorylation. CD4 T cells were analyzed for levels of phospho-STAT4 at different time points following treatment with 5 μg/mL IL-12 in the presence or absence of R1881. (*E*) Testosterone effect on T-bet expression. T-bet expression in CD4 T cells after 4 d of culture under Th1 polarizing conditions in the presence or absence of R1881 ($n = 5$). (*F*) Castration induces T-cell infiltration into various tissues in mice. Prostate, lung, and gut specimens were collected from castrated male mice 1 mo following surgical castration and stained for CD3. Data show mean counts from 10 random fields of view from each sample ± SD. Each sample has three to four mice per group.

showed a slight reduction in STAT4 phosphorylation 60 min following IL-12 treatment. However, after 24 h, cells treated with androgen or vehicle had equal levels of pSTAT4 (Fig. 2*D*). Because insufficient induction of pSTAT4 during CD4 T-cell activation can lead to T-cell anergy or induction of regulatory T cells (14), we hypothesized that testosterone may increase the threshold of IL-12 required to induce Th1 differentiation. CD4 T cells were cultured under Th1 polarizing conditions with increasing concentrations of IL-12 either in the presence of 1 ng/mL R1881 or vehicle control, and the expression of T-bet was assessed 3 d later. R1881 treatment resulted in significantly less T-bet expression in CD4 T cells receiving 0, 3.125, and 12.5 ng/mL of IL-12 (Fig. 2*E*). However, at the higher concentrations of IL-12 (25–100 ng/mL), androgen had no significant effect on T-bet expression. These data support the notion that Th1 differentiation is inhibited in the presence of androgen and also that CD4 Th1 polarization is specifically impaired in low IL-12 conditions via a reduction in STAT4 phosphorylation.

Castration of Mice Leads to Increased T-Cell Infiltration in Lung, Prostate, and Intestine. Androgen deprivation therapy increases lymphocyte infiltration in the prostate gland of both mice and men, and this infiltration is predominantly CD4 T cells (10, 11). This observation was made in the context of prostate cancer and omitted evaluating nonprostatic tissue inflammation. Given our finding that testosterone reduced the amount of IL-12 required to induce CD4 cell differentiation, we hypothesized that testosterone's effect might extend beyond the prostate. To investigate this, liver, lung, prostate, and intestine samples were collected from male mice 1 mo following surgical castration and stained for CD3 expression. We found a significant increase in the number of $CD3^+$ cells in the prostate gland (4-fold) and lungs (2.5-fold). Infiltration in the prostate was mostly found in the peripheral zone (Fig. S2). Additionally, a nonsignificant increase was observed in the small intestine ($P = 0.056$; Fig. 2*F*). The presence of some sparse and sporadic T-cell infiltrates in tissues of sham castrated mice indicates the onset of a minimal, spontaneous "autoimmune" phenotype, and the significantly increased presence of T cells in nonprostatic tissues of castrated animals suggests that testosterone may limit the reactivity of T cells to host tissue.

Testosterone Up-Regulates the Tyrosine Phosphatase Ptpn1 and Inhibits Tyk2 Phosphorylation. Many molecules regulate phosphorylation of STAT4 following IL-12 exposure. Therefore, we sought to examine the expression of signal transduction molecules upstream of STAT4 in an attempt to understand how testosterone inhibits IL-12 signaling. To identify signal transduction components that may be regulated by testosterone, we searched for genes with opposite expression in CD4 T cells exposed to high or low testosterone conditions by comparing CD4 T cells isolated from castrated mice to testosterone treated CD4 T cells in vitro. We found that the genes encoding protein tyrosine phosphatase nonreceptor 1 and 11 (Ptpn1 and Ptpn11) were significantly decreased in castrated mice and significantly up-regulated in androgen treated CD4 T cells, suggesting that the expression of these phosphatases is regulated by testosterone (Fig. 3 *A* and *B*). Candidate genes from the SOCS, PIAS, and Jak family of molecules were affected in one condition but not the other (Dataset S1). These data suggest that, although androgen seems to exhibit diverse effects on T-cell signaling, only Ptpn1 and Ptpn11 were directly controlled by the presence of androgen under our experimental conditions.

To investigate whether androgen directly affects expression of Ptpn1 and Ptpn11, CD4 T cells were treated overnight with androgen, and Ptpn1 and Ptpn11 gene expression was analyzed. We found that Ptpn1 was significantly up-regulated (1.7-fold), whereas no significant difference in Ptpn11 expression was seen (Fig. 4*C*). To further understand how androgen regulates expression of Ptpn1, we investigated publically available gene expression profiling datasets involving testosterone treatment to find how Ptpn1 expression was affected in other cell types and conditions. Ptpn1 was found to be unchanged or up-regulated by testosterone exposure in all datasets examined, including human prostate cancer cell lines, mouse adipose tissue, and mouse lacrimal and submandibular glands (Fig. 3*D*). These data show that Ptpn1 expression is regulated by androgen in numerous cell types, including lymphocytes.

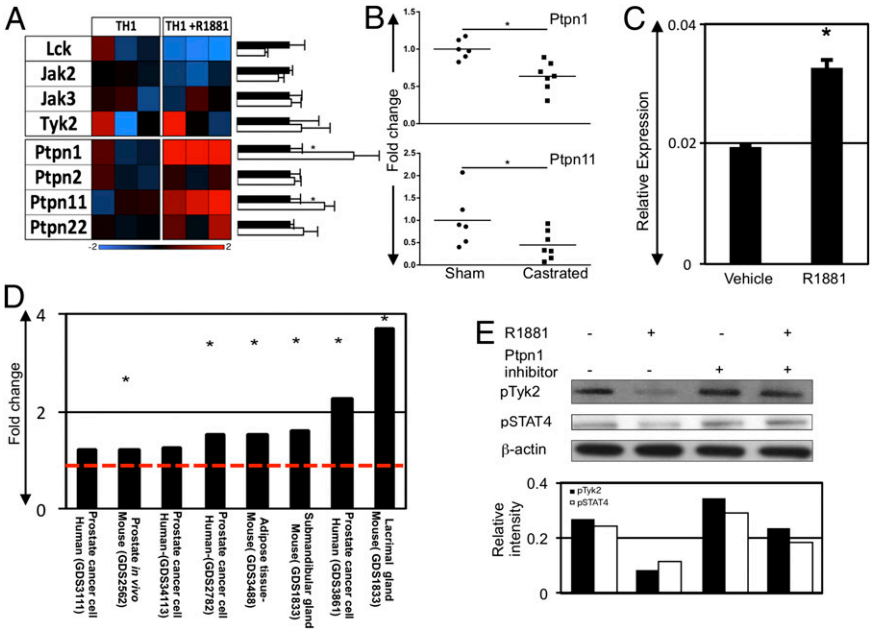

Fig. 3. Expression of Ptpn1 is regulated by androgens. (*A*). Gene expression of various cell signaling components in Th1 CD4 T cells. CD4 cells were cultured in the presence or absence of androgen under Th1 conditions for 3 d. Gene expression was measured by quantitative PCR. (*B*) Effect of testosterone on Ptpn1 and Ptpn11 in vivo. Expression of Ptpn1 and Ptpn11 in CD4 T cells isolated from spleens of control or surgically castrated male mice 4 wk post-surgery ($n = 6$ and 7, respectively, from two separate experiments). (*C*) Direct effect of testosterone on Ptpn1. Expression of Ptpn1 in murine CD4 T cells treated with R1881 overnight ($n = 3$). (*D*) Effect of testosterone on Ptpn1 expression in other cell types. Expression of Ptpn1 from various datasets available on the GEO where cells had been treated with androgen. Numbers in parentheses indicate dataset GEO identification numbers. (*E*) Effect of Ptpn1 on Tyk2 and STAT4 phosphorylation after IL-12 exposure. CD4 T cells were treated with 5 μg/mL IL-12 in the presence or absence of R1881, with and without treatment with a Ptpn1-specific inhibitor. CD4 cells were analyzed for levels of phosph-Tyk2 5 min after treatment. Blots are representative of two experiments.

Ptpn1 is a phosphatase that dephosphorylates insulin-like growth factor 1 receptor (IGFR), Jak2, and Tyk2, thereby inhibiting downstream signals triggered by these molecules (15). Our IPA analysis of CD4 T cells from castrated mice predicted that the kinase Tyk2 was significantly activated in castrated mice (Table S1). The up-regulation of Ptpn1 by testosterone, leading to the dephosphorylation of Tyk2, could explain how androgens inhibit Th1 differentiation. To investigate this, CD4 T cells were treated overnight with androgen, and Tyk2 phosphorylation was measured following administration of IL-12. Strong Phospo-Tyk2 signal was detected 5 min after IL-12 treatment under androgen-free conditions. In contrast, androgen-treated cells had a much lower level of Tyk2 and STAT4 phosphorylation (Fig. 3*E*). To investigate the contribution of Ptpn1 to this inhibition, cells were treated with a specific Ptpn1 inhibitor for 2 h before the administration of IL-12. The inhibitor restored the phosphorylation of Tyk2 and STAT4, suggesting that Ptpn1 induction by testosterone inhibits IL-12 signaling (Fig. 3*E*). This finding supports our hypothesis that up-regulation of Ptpn1 by testosterone leading to the inhibition of Tyk2 phosphorylation is a mechanism by which testosterone inhibits Th1 polarization.

Androgen Receptor Binds a Conserved Region Within Intron 3 of Ptpn1. Following testosterone binding, the androgen receptor (AR) is translocated to the nucleus where it associates with androgen response elements (AREs) in the DNA. When bound to DNA, AR can regulate gene expression by directly initiating transcription or indirectly altering the epigenetic environment of the target gene (16). AR was detectable in CD4 cells isolated from both sham and castrated mice (Fig. S3). To investigate how androgen affects Ptpn1 expression, we created a search algorithm to identify potential AR binding sites in the *Ptpn1* gene and upstream promoter region. Five putative AR-binding sites were found in and around the Ptpn1 gene (Fig. 4*A*). To determine whether AR bound any of these regions, chromatin from CD4 T cells treated with R1881 or vehicle was immunoprecipitated (ChIP) with an anti-AR antibody, and potential binding sites were tested by PCR. Interestingly, no significant enrichment for the predicted AR binding site in the promoter region was observed. However, the predicted binding site in the intron between exon 3 and 4 (intron 3) was significantly enriched after R1881 treatment (Fig. 4*A*). Because we have observed androgen-specific down-regulation of Ptpn1 expression in both human and mouse cells, we assessed the homology of mouse intron 3 among humans, mice, and three other species. Although the exonic regions of *Ptpn1* were highly conserved across many species, human and mouse introns rarely showed homology above 50% (Fig. 4*B*). However, regions of microhomology, defined as 20- to 50-bp regions of very high homology (>80%) conserved across multiple species, were present in some introns (highlighted in blue in Fig. 4*C*). One of these regions contained the AR binding site that we identified in intron 3, and a similar site in the same intron was also present in rats, Macaca monkeys, and elephants, suggesting that an evolutionarily conserved role of androgen regulation of Ptpn1 might exist (Fig. 4*C*). Together, these data show that androgen regulates expression of Ptpn1 in numerous cell types, in both humans and mice, and that a conserved androgen binding site in the third intron of the Ptpn1 gene may be the critical regulator of this effect.

CD4 Cells Isolated from Patients Undergoing Androgen Deprivation Therapy Have Decreased Ptpn1 Expression. Prostate cancer patients undergoing androgen derivation therapy (ADT) have increase T-cell infiltration into the prostate gland, and castrated mice have an improved response to vaccination against tumor antigens (8–10). Given the homology of the AR binding site between the mouse and human Ptpn1 gene, we investigated whether Ptpn1 was regulated in a similar way in human T cells in vivo. To do this, we isolated CD4 T cells from the peripheral blood of prostate cancer patients undergoing ADT. CD4 T cells from patients on ADT showed a twofold reduction in Ptpn1 expression compared with control patients (Fig. 5). In comparison with mice, there was no significant change in IFN-γ or T-bet expression in the human CD4 T cells. Additionally, there was no difference in any other CD4 T-cell transcription factors (Dataset S1). These data support the conserved function of androgen receptor regulation of Ptpn1 and may explain the improved T-cell response to the tumor in ADT patients.

Discussion

Here, we investigated the effect of testosterone on CD4 T-cell function. Our data suggest that testosterone inhibits CD4 T-cell differentiation by up-regulating the phosphate Ptpn1. Ptpn1 is an enzyme that is expressed in many cell types, including lymphocytes, and importantly, it dephosphorylates both Jak2 and Tyk2, the upstream kinases responsible for Stat4 phosphorylation (15, 16). Because Ptpn1 is known to dephosphorylate Tyk2, our data

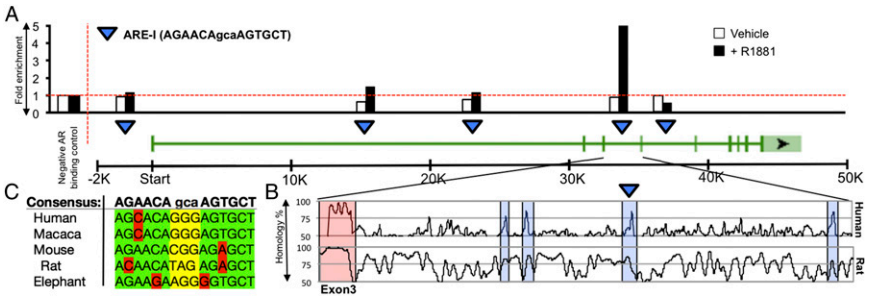

Fig. 4. Androgen receptor binds to a region in intron 3 of the Ptpn1 gene. (*A*) ChIP and quantitative PCR quantification of AR binding to Ptpn1. Male mouse spleen CD4 T cells treated overnight with R1881 or vehicle control. ChIP for androgen receptor was performed, and fold enrichment was determined by qRT-PCR of putative AR binding regions within the Ptpn1 gene. (*B*) Homology between species of intron 3 of Ptpn1. Homology of Ptpn1 between mouse and human or rat. Exon is shown in red and regions of high homology conserved between species are shown in blue. (*C*) DNA sequence of AR binding region in different species.

showing testosterone reduced Tyk2 phosphorylation support this role of Ptpn1. Additionally, Tyk2 phosphorylation could be partially restored by coadministration of a specific Ptpn1 inhibitor, further suggesting that androgen inhibition of Stat4 phosphorylation and Th1 differentiation is mediated through up-regulation of Ptpn1. This role of Ptpn1 has not been previously reported; however, other tyrosine phosphatases, Ptpn6 and Ptpn11, inhibit Th1 differentiation (17–19). Inhibited Th1 differentiation by Ptpn1 has clear implications to the immune response and helps explain previous observations that testosterone limits viral vaccine effectiveness, viral clearance, the response to host antigens, and the T-cell response to cancer (5–9).

Beyond de-phosphorylating Tyk2, Ptpn1 has many other known roles and likely contributes to suppression of the immune system beyond what we have reported. For example, we found that androgens could also inhibit Th17 differentiation (Fig. S4). Stat5 is also a substrate for Ptpn1, and Ptpn1 deficiency increases Stat6 phosphorylation in B cells (20, 21). Additionally, Ptpn1-deficient mice suffer from systemic inflammation, increased leukocyte migration, and is a potential molecule in regulating the allergic response (22). In addition to regulating known pathways affecting immunity, Ptpn1 has a critical role in regulating metabolism. Ptpn1 regulates both insulin-like growth factor (IGF) signaling by inhibiting receptor kinases associated with the receptors of these molecules (23, 24). In addition, there are a number of SNPs found in Ptpn1 that are associated with hypertension and obesity (25). One of the critical findings of a recent report demonstrating the negative correlation of testosterone and influenza vaccine efficacy was that testosterone altered lipid metabolism in the immune system (5). Given that Ptpn1 is a regulator of IGF signaling, it is rational to expect that testosterone induced expression of Ptpn1 may be associated with regulation of lipid metabolism, and therefore, could be one of the mechanisms that leads to reduced influenza vaccine efficacy by testosterone.

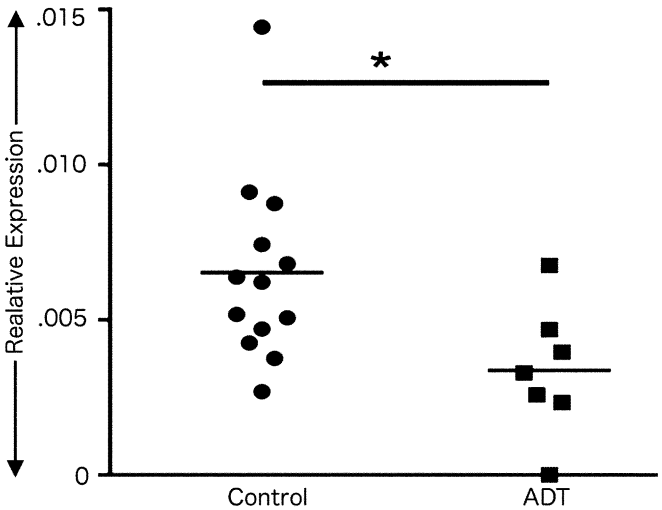

Fig. 5. Ptpn1 expression is reduced in CD4 cells isolated from prostate cancer patients undergoing androgen deprivation. CD4 cells were isolated from patients undergoing ADT by FACS. Ptpn1 expression in CD4 T cells isolated from PBMC of prostate cancer patients on androgen deprivation therapy and control patients; $n = 13$ and 7.

The mechanism of androgen-induced regulation of Ptpn1 expression was also addressed in this work. Various studies have demonstrated the presence of AR both in the cytosol and on the membrane of T lymphocytes (26, 27). We found that there was an AR binding site in the intron between exon 3 and 4 of the Ptpn1 gene. However, how the binding of AR to this region increased Ptpn1 expression of the gene is unclear. One possibility is chromatin modification of this region by factors that associate with AR (28). The effect of this has been shown recently in genome wide investigations where AR binding to DNA has been found to be associated with increased accessibility of the location and this could be how AR regulates Ptpn1 expression (29). Others have recently found that AR binds Ptpn1 in human prostate cancer cells, leading to Ptpn1 expression, although the binding was at a different location from the one we found (30). Although these observations implicate AR-mediated expression of Ptpn1, the question remains of why androgen regulates Ptpn1. We found that the AR binding site was highly conserved among numerous species within a region of very low homology, indicating that the regulation clearly has some evolutionary importance. Why males need to be more protected from autoimmunity is unclear. Others have offered plausible speculation that the evolutionary role of testosterone-mediated immune suppression may be to protect men from pathogen exposure during traumatic injury, for which in many species the males are more likely to suffer (5). Our data suggest an additional possibility: that because the role of Ptpn1 is important in metabolism regulation and IGF1 signaling (31), testosterone regulation of Ptpn1 is more important for these processes, and the effect of testosterone on the immune system either directly or by altered lipid metabolism may be a bystander effect. Nonetheless, testosterone does contribute to expression of Ptpn1 in multiple cell types and species, and in T cells, this increase in Ptpn1 expression reduces the response of the cells to IL-12.

The findings presented here show a potential mechanism to explain how testosterone alters CD4 T-cell function. We found that down-regulation of Ptpn1 following castration increased Th1 differentiation in sterile inflammatory conditions. Further investigation in vitro found that testosterone limited Stat4 phosphorylation and Th1 differentiation. We propose that these findings may help explain why testosterone helps alleviate Th1-driven autoimmune conditions. Furthermore, these findings support the idea of using ADT as an adjuvant for immunotherapeutic intervention in prostate cancer and suggest that Ptpn1 targeting could be investigated as an adjuvant for cancer vaccine against tumor antigens.

Methods

Surgical Castration. C57BL/6J mice were castrated as previously described (9). Mice were used in experiments 28 d after castration, which was the earliest

time point we previously found to be when androgen-dependent gene expression is lost (9).

Histology. Trained pathologists at the Beth Israel Deaconess Medical Center histology core performed tissue sections and immunohistochemistry staining. Slides were stained using anti-CD3 (134096; Abcam) at a 1:500 dilution and a secondary Biotin SP-conjugated anti-rabbit IgG diluted 1:250 (711-065-152; Jackson ImmunoResearch). A microscope operator blinded to the experimental design and staining procedure acquired 10 random fields of view from each slide. $CD3^+$ cells were counted using an automated counting algorithm in ImageJ.

Microarray Analysis. $CD4Foxp3^{GFP}$ cells were isolated from mice using FACS. RNA was extracted, and gene expression was assessed using Affymetrix's GeneChip Mouse Genome 430 PM arrays over two separate experiments. Data can be accessed from the Gene Expression Omnibus (GEO) database, accession no. GSE54945.

Pathways and Network-Based Analysis. Interactive networks, pathways, and functions analysis were performed on this list of genes using the commercial system biology oriented package IPA (www.ingenuity.com).

T-Cell Isolation and Th1 Differentiation. The CD4 T cells isolated from human and mouse samples used for gene expression analysis were sorted by FACS. A detailed gating strategy to isolate these cells is shown in Fig. S5. CD4 T cells used for in vitro experiments were isolated from the spleen of male C57BL/6J mice using StemCell Technologies CD4 T-cell isolation kit (cat# 19765). To eliminate the effect of endogenous testosterone, T cells were incubated overnight in charcoal-stripped media. For Th1 differentiation experiments, CD4 cells were incubated with anti-CD3/anti-CD28 stimulation beads (Life Technologies), 5 ng/mL IL-12 (Peprotech), and 10 ng/mL IL-2 (Peprotech). To investigate the role of androgen, T cells were treated with either 2 ng/mL R1881 (Perkin Elmer) or DMSO as a vehicle control. Restimulation of T cells was performed using eBioscience cell stimulation mixture (PMA/ionomycin, cat#: 00-4979-93). IFN-γ production by CD4 T cells was assessed by flow cytometry using eBioscience antibodies.

Western Blot. CD4 cells were isolated as described above and incubated overnight in charcoal-stripped androgen-free media. T cells were treated with 5 ng/mL IL-12, 2 ng/mL R1881, or vehicle control. Experiments investigating the role of Ptpn1 used the inhibitor 3-(3,5-dibromo-4-hydroxy-benzoyl)-2-ethyl-benzofuran-6-sulfonicacid-(4-(thiazol-2-ylsulfamyl)-phenyl)-amide at a concentration of 4 μM (cat# 539741; Millipore). Protein was extracted using cell lysis buffer (Cell Signaling) as described by the manufacturer. Western blot analysis of pSTAT4 (D2E4) and PTyk2 (Cell Signaling #9321) was performed following the manufacturer's protocol. Figures shown are representative of at least three separate experiments.

Androgen Receptor Binding Site Prediction. Putative androgen receptor binding sites in the Ptpn1 gene were determined by searching the mouse Ptpn1 gene for regions of high similarity to the androgen response element I sequence AGAACANNNAGTGCT.

Ptpn1 Homology. Homology between species was determined by comparing the similarity of the mouse Ptpn1 gene to the human Ptpn1 gene using a custom python script. Briefly, the mouse and human Ptpn1 genes were subdivided into 300-bp frames. These fragments of the mouse gene were then paired with the fragments derived from the human gene based on their homology. Within the 300-bp matching frames, 50-bp regions from the mouse gene were compared with 50-bp regions in the human gene, and the location with the highest percentage homology was reported. The highest percentage homology of a region is reported in Fig. 4*B*. The same approach was used for comparing the mouse and rat copies of Ptpn1.

ChIP. CD4 T cells were isolated as described above and incubated overnight in testosterone-free media. T cells were then treated in triplicate with 2 ng/mL R1881 or DMSO as a vehicle control for 12 h. Samples were pooled after 12 h, and ChIP was performed using the Cell Signaling enzymatic preparation kit (cat# 9003) (see Table S1 for specific information).

Statistical Analysis. Statistical analysis was performed using the Student *t* test. $P < 0.05$ was considered significant.

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