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Regulatory Cytokine Expression and Interstitial Fluid Formation in the Normal and Inflamed Rat Testis Are Under Leydig Cell Control

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ABSTRACT: Leydig cells have been implicated in several inflammation-related responses of the testis. Specifically, these cells produce the proinflammatory cytokines interleukin-1 (IL-1) and IL-6, stimulate macrophage recruitment, and promote interstitial fluid formation. In addition, the immunoregulatory cytokines macrophage migration inhibitory factor (MIF), transforming growth factor-β1 (TGFβ1), and interferon-γ (IFNγ) are constitutively expressed by testicular cells, including the Leydig cells. In the present study, the contribution of the Leydig cell to testicular inflammatory responses was examined in adult male rats treated with the Leydig cell–specific toxin, ethane dimethane sulfonate (EDS). Intratesticular testosterone levels were modulated by subcutaneous testosterone implants. After 10 days, animals received an injection of lipopolysaccharide (LPS) to induce an inflammatory response, or saline alone, and were killed 3 hours later. Both depletion of Leydig cells by EDS and LPS treatment caused a decrease in collected testicular interstitial fluid to about 35% of control levels, but the effects were not additive. Maintenance of intratesticular testosterone reversed the interstitial fluid decline following EDS treatment and partially prevented the LPS-induced effect. MIF, TGFβ1, and IFNγ were expressed in both the normal and inflamed testis at similar levels. In contrast, EDS treatment caused a significant decline in expression of all 3 cytokines, which was prevented by the testosterone implants. These data indicate that 1) expression of TGFβ1, MIF, and IFNγ in the testis is not dependent on the presence of intact Leydig cells but is under direct testosterone control and 2) the decline in testicular interstitial fluid during inflammation involves the Leydig cells, acting via both androgens and nonandrogenic secretions. These data provide further support for a significant role for the Leydig cell in modulating the testicular response to inflammation.

Key words: Transforming growth factor-β, interferon-γ, macrophage migration inhibitory factor, ethane dimethane sulfonate, testosterone.

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There is now compelling evidence that cytokines play an important regulatory role in the development and function of the testis (Hedger and Meinhardt, 2003). The proinflammatory cytokine tumor necrosis factor-α (TNFα), for example, modulates Leydig cell steroidogenesis (Xiong and Hales, 1993) and inhibits germ cell apoptosis by regulating the level of FasL (Pentikainen et al, 2001), while members of the transforming growth factor-β (TGFβ) family are implicated in testicular development (Mullaney and Skinner, 1993; Teerds and Dorrington, 1993). Several regulatory cytokines, including macrophage migration inhibitory factor (MIF) and interferon-γ (IFNγ) are produced at significant levels within the testis even in the absence of inflammation or immune activation events (Dejucq et al, 1995; Meinhardt et al, 1996). A better understanding of the production and regulation of these cytokines in the testis may provide the key to unlocking the processes responsible for causing testicular damage during inflammation and disease, as well the maintenance of immune privilege in the testis (Head and Billingham, 1985; O'Bryan et al, 2000b).

While the local mechanisms that control the expression of cytokines in normal and diseased testes are poorly understood, evidence points to an important role for the Leydig cells. There is clear evidence that the Leydig cells are responsible for recruitment and control of the testicular macrophage population (Hedger, 2002). Moreover, the Leydig cells and Sertoli cells contribute to the production of proinflammatory mediators, such as the 2 IL-1 isoforms, IL-6, and inducible NO synthase (iNOS), during testicular inflammation (Meinhardt et al, 1996; Stephan et al, 1997; O'Bryan et al, 2000a; Soder et al, 2000). Finally, the immunoregulatory cytokines, TGFβ1, MIF, and IFNγ, are produced by the Leydig cells either during testicular development or in the adult (Teerds and Dorrington, 1993; Dejucq et al, 1995; Meinhardt et al, 1996).

Leydig cells also are responsible for the local regula-

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tion, formation, and content of the testicular interstitial fluid (IF), which is of considerable importance to normal testis function (Setchell, 1990). A number of studies have established that IF formation is maintained by androgen-dependent regulation of testicular blood pressure and flow, mediated principally via the seminiferous epithelium (Maddocks and Sharpe, 1989; Collin et al, 1993). Consequently, disruption of the germ cells results in an increase in IF volume, whereas ablation of the Leydig cells by ethane dimethane sulfonate (EDS) causes a decrease in IF volume at least in the short term (Maddocks and Sharpe, 1989; Sharpe et al, 1991). This latter response is quite similar to the effect of lipopolysaccharide (LPS)-induced inflammation on testicular fluid formation, which decreases during inflammation, in direct contrast to the edema response that normally is observed in other tissues (O'Bryan et al, 2000a,b).

It is quite evident, therefore, that the Leydig cell may play an important role in regulating local inflammatory responses in the testis, but direct studies have not yet been performed. Consequently, the following experiments were designed to examine the role of the Leydig cells and testosterone in controlling immunoregulatory cytokine production and IF formation in the normal testis and during inflammation by comparing the responses elicited by the application of the proinflammatory agent lipopolysaccharide (LPS) in the presence and absence of functional Leydig cells in vivo. This model uses a relatively low dose of LPS to induce moderate systemic inflammation in the animals, producing a specific inflammatory response within the testis, which is characterized by a decline in the ability of the Leydig cells to produce testosterone and a unique cascade of inflammatory events, including a transient increase in testicular macrophages without intravasation of neutrophils, attenuated production of the proinflammatory mediators IL-1β and nitric oxide, and reduction in fluid volume (O'Bryan et al, 2000a,b; Gow et al, 2001; Gerdprasert et al, 2002a,b).

Materials and Methods

Animals and Experimental Design

Adult male Sprague-Dawley rats (350–375 g body weight) were obtained from Charles River (Sulzfeld, Germany) and housed in the animal house of the Department of Anatomy and Cell Biology, Philipps University of Marburg, for the duration of the experimental procedure under conditions of controlled day length (12:12 h light:dark) with unlimited access to food and water. Experimental procedures were approved by the Regierungspraesidium Giessen and conformed to the local Code of Practice for the Care and Use of Animals for Experimental Purposes.

Treatment with EDS causes complete destruction of the Leydig cells within 3 days (Kerr et al, 1985; Wang et al, 1994). The transient increase in intratesticular macrophages that accompanies this destruction is back to normal within 10 days, leaving an interstitium that is completely devoid of Leydig cells. In numerous studies it has been established that low-dose subcutaneous testosterone implants (3 cm long; T3 implants) cause a reduction in serum luteinizing hormone (LH) levels, a subsequent decline in intratesticular testosterone, and loss of the developing spermatogenic cells in the testis, while high dose testosterone implants (3 × 8 cm = 24 cm long; T24 implants) similarly cause a reduction in LH but provide sufficient testosterone to maintain intratesticular testosterone levels adequate to support qualitatively normal seminiferous tubule function (Sun et al, 1989; Wang et al, 1994). Rats were separated into the following groups (n = 11 or 12 rats/group): 1) no treatment (controls); 2) T3 subcutaneous implant; 3) dimethyl sulfoxide (DMSO):water (1:3) (1.0 mL/kg) intraperitoneal (DMSO vehicle controls); 4) EDS (75 mg/kg) in DMSO:water intraperitoneal; 5) vehicle plus T24 implant; 6) EDS in DMSO:water plus T24 implant. Ten days later, rats were injected (intraperitoneally) with either endotoxin-free saline or 0.1 mg/kg LPS from *Escherichia coli*, serotype 0127:B8 (Sigma, Deisenhofen, Germany). Three hours after injection rats were euthanized and tissues were collected. Testis and seminal vesicle weights were determined. One testis was taken for collection of IF as previously described (Wang et al, 1994), and one for isolation of total RNA. Samples of liver were also collected from the animals for controls.

Measurement of Testosterone

Testicular IF was assayed for testosterone without extraction using a direct ^{125}I-testosterone radioimmunoassay as described previously (Meinhardt et al, 1998).

RNase Protection Assay for Cytokines

Ribonuclease protection analysis of cytokine expression in rat testes was performed using a commercially available kit (RiboQuant Multi-Probe, BD Biosciences, Heidelberg, Germany) according to the manufacturer's instructions. The custom template set contained the following DNA templates: TGFβ1, IL-6, IL-10, TNFα, MIF, and IFNγ. In addition, templates for the ribosomal protein L32 and GAPDH housekeeping probes were included to allow assessment of total RNA levels and normalize sampling. Total RNA was isolated with the single-step acid phenol-guanidinium thiocyanate-chloroform extraction method (Chomczynski, 1993) using Trizol reagent (Invitrogen, Karlsruhe, Germany). Briefly, ^{32}P radiolabeled antisense riboprobes were generated by multiplex in vitro transcription using [α-^{32}P]UTP (Amersham, Braunschweig, Germany) and T7 RNA polymerase. The RNase protection assay was performed according to the manufacturer's protocol. Radiolabeled probes were hybridized with 5 μg of total testicular or liver RNA samples at 56°C overnight. After RNase A/T1 treatment for digestion of single-stranded RNA, samples together with undigested probes and in vitro transcribed 100 bp RNA markers (Century Marker, Ambion, Huntingdon, UK) were resolved on a 50 × 30 cm 5%–6% polyacryamide/7M urea gel (Table). The gel was dried for 1 hour at 80°C under vacuum (Bio-Rad, München, Germany), exposed to a phosphorimage screen overnight, and analyzed on a Fuji PhosphorImager FLA3000G (Raytest, Straubenhardt, Ger-

Probe lengths and lengths of the protected fragments of the rat cytokine template set used

Template	Probe Length in Nucleotides	Protected Fragment in Nucleotides
TGFβ1	285	256
IL-6	231	202
IL-10	210	181
TNFα	189	160
MIF	171	142
IFNγ	156	127
L32	141	112
GAPDH	126	97

many). Subsequently, RNase protected bands were quantified using Fuji Image Gauge software. For each treatment group analyzed, RNA was prepared from 4 randomly selected animals. The probe set was labeled 3 to 4 separate times for analyses of samples from each testis examined. Liver RNA (±LPS) was included as control for cytokine expression. All cytokine data were normalized for variation in loading against L32 and GAPDH. Both normalizations yielded very similar results, but only the L32 normalized data are presented.

Statistics

Data were analyzed by 2-way analysis of variance (ANOVA) after appropriate transformation to normalize the data and equalize variance, in conjunction with the Student Newman-Keuls multiple-range test, using SigmaStat version 1.0 software (Jandel Scientific Software, San Rafael, Calif). Statistical comparisons were limited to groups receiving the same injection vehicle, that is, either saline alone or DMSO:water and saline, to avoid complications due to the minor effects of DMSO on testicular IF volume and permeability characteristics (Hedger and Hettiarachchi, 1994; Wang et al, 1994). Comparisons between experimental groups were considered statistically significant at the $P < .05$ level.

Results

Testicular Weight and Endocrine Parameters

Inhibition of serum LH by short testosterone implants (T3) caused a slight but significant (10%) reduction in testis weight, caused a large decrease in IF testosterone concentrations, and had no effect on seminal vesicle weights, thereby confirming the maintenance of close to normal serum testosterone levels (Figure 1). In contrast, destruction of Leydig cells by EDS caused a 35% decrease in testis weight, which was entirely prevented by the T24 implants (Figure 1A). T24 implants alone had no effect on testis weight. Seminal vesicle weights were reduced in the EDS-treated group and increased in T24 implanted rats, the latter due to the expected elevated peripheral levels of serum testosterone (Figure 1B). Interstitial fluid testosterone levels were reduced likewise in EDS treatment and partially restored in T24 implanted rats (Figure 1C). Treatment with 0.1 mg/kg LPS had no significant effect on any of the above parameters in any group when measured 3 hours after injection (Figure 1).

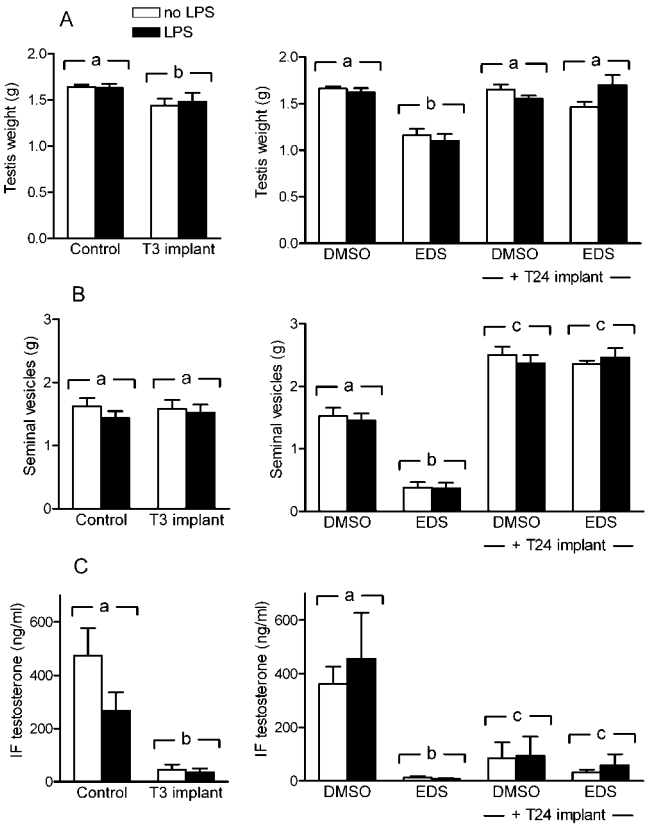

Figure 1. Androgen-dependent organ weights (testis and seminal vesicle) and testicular interstitial fluid (IF) testosterone values from adult rats treated with saline only (□) or 0.1 mg/kg LPS intraperitoneally (■)—experimental groups were normal controls (control), DMSO-treated controls (DMSO), EDS treatment (EDS), 3-cm testosterone implants (T3), and 24-cm testosterone implants (T24) in combination with DMSO or EDS. **(A)** testis weight; **(B)** seminal vesicle weight; and **(C)** IF testosterone. All values are mean ± SEM of 5–6 animals. Statistical comparisons were limited to groups receiving the same injection vehicle, either saline alone (left-hand graph) or DMSO:water and saline (right-hand graphs). Experimental group values with same letter superscript are not significantly different ($P > .05$). Significant changes in organ weights are a reflection of circulating testosterone concentrations. There were no significant differences between values for the saline-injected and LPS-treated testes in any experimental group.

IF Volume Regulation

Neither the T3 nor T24 implants on their own had a significant effect on the volume of recovered IF, but EDS caused a 65% decline in volume, which was prevented by the T24 implants (Figure 2). Treatment with LPS caused a decrease of about 70% in IF volume in control rats by 3 hours after treatment (Figure 2). In contrast to the suppression by EDS, which led to a consistent reduction of between 31% and 56% (range: 27–49 μL) compared with controls, the variation in the degree of suppression by LPS was very large (1–86 μL). In fact, the

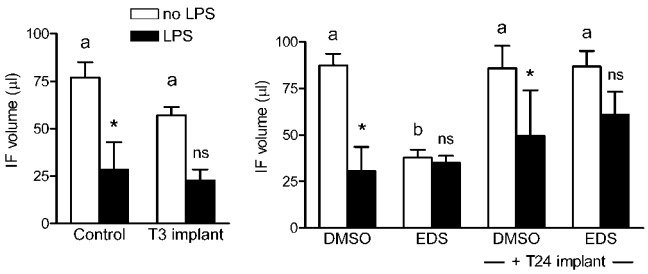

Figure 2. IF volume data from testes of adult rats treated with saline only (□) or 0.1 mg/kg LPS intraperitoneally (■)—experimental groups were normal controls (control), DMSO-treated controls (DMSO), EDS treatment (EDS), 3-cm testosterone implants (T3), and 24-cm testosterone implants (T24) in combination with DMSO or EDS. All values are mean ± SEM of 5–6 animals. Statistical comparisons were limited to groups receiving the same injection vehicle, either saline alone (left-hand graph) or DMSO:water and saline (right-hand graphs). Saline treatment grouped values with the same letter superscript were not significantly different ($P > .05$). Comparisons between values for the saline-injected and LPS-treated testes were significantly different (*) or not significantly different (ns) at the $P < .05$ level.

response to LPS did not actually achieve significance in the T3-implanted animals due to the variation in IF volumes and a slight, but not statistically significant, reduction in IF volume of the corresponding saline-injected controls (Figure 2). However, LPS clearly had no effect on IF volume in EDS-treated rats, and the effects of LPS

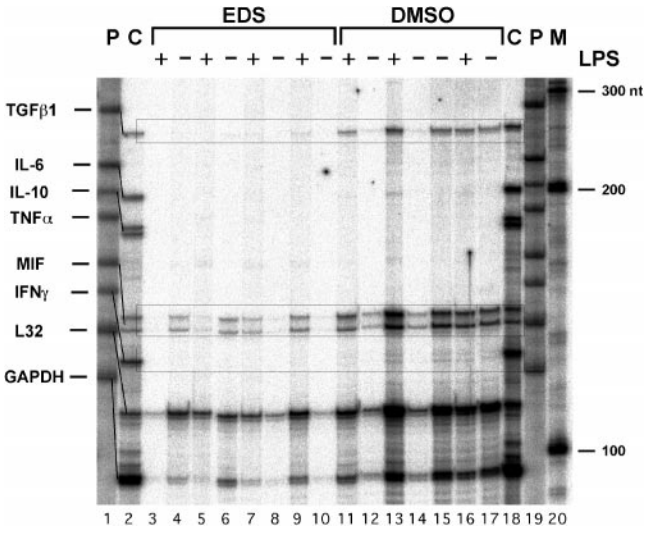

Figure 3. Expression of cytokines in Leydig cell–depleted rat testis. Representative ribonuclease protection analysis using ^{32}P-labeled antisense riboprobes for the indicated cytokines in animals treated with EDS or DMSO as solvent control. Each lane represents 1 animal treated or not treated (±) with LPS for 3 hours. Lane P: undigested probe set. Lane C: digested probe set after hybridization to control RNA provided by the kit, hairlines indicate corresponding bands. The MIF signal always consisted of a double band with the lower band of the doublet being distinct to the undigested L32 signal. Lane M: century RNA marker (Ambion). 10.000 counts per minute were loaded in each lane. The bands ribosomal protein L32 and GAPDH were used for data normalization (see "Materials and Methods"). Reproducibility was verified by labeling the probe set 3 to 4 separate times for each sample analyzed. The signals for TGFβ1, MIF, and IFNγ are highlighted by a box.

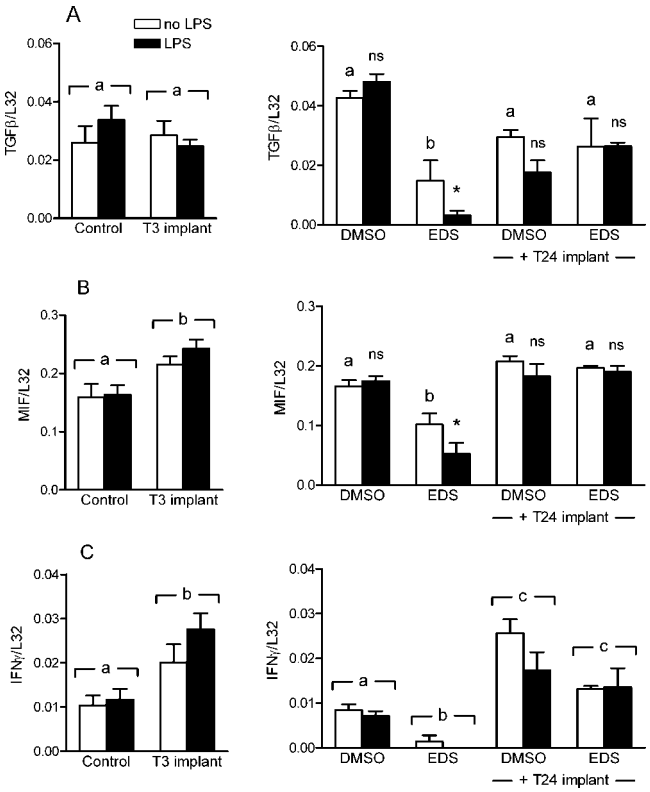

Figure 4. (**A**) Quantification of TGFβ, (**B**) MIF, and (**C**) IFNγ mRNA expression in testes from adult rats treated with saline only (□) or 0.1 mg/kg LPS intraperitoneally (■)—experimental groups were normal controls (control), DMSO-treated controls (DMSO), EDS treatment (EDS), 3-cm testosterone implants (T3), and 24-cm testosterone implants (T24) in combination with DMSO or EDS. Expression of cytokines was normalized against L32 housekeeping gene expression. Values in the histograms are mean ± SEM (n = 4 rats selected at random from each experimental group). Statistical comparisons were limited to groups receiving the same injection vehicle, either saline alone (left-hand graphs) or DMSO:water and saline (right-hand graphs). Experimental group values with same letter superscript were not significantly different ($P > .05$).

on IF volume were at least partially prevented in the T24 implanted rats compared with controls (Figure 2).

From each experimental group 3 to 4 samples were randomly selected for RNase protection assay (Figure 3). Signals were detected for TGFβ1 and MIF in all testes samples. Expression levels for IL-6, IL-10, TNF-α, and IFNγ were either very weak or not detectable even in the LPS treatment groups and therefore not quantifiable (Figure 3). In contrast, all cytokines examined were detected either in liver (not shown) or in the control RNA provided by the kit (Figure 3). T3 implants caused a slight but significant increase in MIF and IFNγ expression, but not in TGFβ1 mRNA levels compared with untreated control (Figure 4A through C). LPS had no significant effect on these cytokines in control and T3 testes. EDS, however, caused a moderate reduction in MIF expression and a large decrease in both TGFβ1 and IFNγ expression (Figure 4A through C). LPS potentiated this effect of EDS

on MIF, TGFβ1, and IFNγ mRNA levels, while T24 implants prevented the decrease. IFNγ also revealed a slight increase in T24 implanted rats (Figure 4C).

Discussion

In this study, the observed changes in testis and seminal vesicle weights of the EDS-treated and testosterone implanted rats were entirely consistent with the corresponding expected and measured levels of testosterone. Total ablation of the Leydig cells by EDS caused a 35% fall in testis weight, attributable to the loss of spermatogenic cells, which was prevented by T24 implants. It previously has been shown that T24 implants, although unable to restore normal intratesticular testosterone levels, maintain intratesticular testosterone at concentrations that support close to normal spermatogenesis as confirmed by the maintenance of testis weights (Sun et al, 1989; Meinhardt et al, 1998). T3 implants maintain normal serum testosterone levels, but intratesticular concentrations fall below the critical concentration necessary to support normal germ cell development. This is consistent with the minor decrease in testis weight observed over the 10-day study period together with unchanged seminal vesicle weights in the present study.

The volume of IF collected from the rat testis overnight via an incision in the testicular capsule is directly proportional to the volume of fluid present in the extratubular space, with only minor contributions from the other testicular compartments (Setchell and Sharpe, 1981; Sharpe and Cooper, 1983). As has been observed previously, treatment with EDS caused a consistent 60% to 70% fall in the volume of IF recovered (Sharpe et al, 1990; Hedger et al, 1998). LPS treatment alone caused a similar average fall in IF volume (O'Bryan et al, 2000b), but it should be noted that LPS induced a much more variable suppression of IF volumes than did EDS treatment, at least within the first 3 hours after LPS injection. This response is in contrast to that of other tissues where inflammation normally causes an increase in blood flow and vascular permeability, followed by edema. That there is a local inflammatory response in the testis following treatment with LPS at this time point has been established by the fact that several proinflammatory mediators, including IL-1β, monocyte chemoattractant protein-1, and iNOS, are up-regulated (O'Bryan et al, 2000a; Gow et al, 2001; Gerdprasert et al, 2002b) and there is neutrophil accumulation in the testicular blood vessels and a small increase in endothelial cell leakage in spite of the overall fall in IF volume (O'Bryan et al, 2000b). The large variability in the suppression of fluid volume from animal to animal is consistent with our previous observations (O'Bryan et al, 2000b) and suggests that this is a characteristic feature of the inflammatory response in the rat testis. In the EDS-treated testis, the mean and variability of IF volumes were almost identical in saline-treated and LPS-treated testes, indicating that in the absence of the Leydig cells, LPS was no longer able to cause the very large suppression in IF volume responses that was observed in some control animals.

The effects of LPS also appeared to be partially, but not completely, reduced in testes of rats in which testicular androgen levels are maintained at a level that supports close to normal spermatogenesis by the use of T24 implants. A slightly different result was observed in the T3-implanted animals, with a minor, but not significant, reduction in pretreatment IF volume, which might be attributable to the reduced intratesticular testosterone levels. Although LH has stimulatory effects on IF volume in vivo and there is some evidence that LH binds to receptors on the testicular endothelium (Veijola and Rajaniemi, 1986; Bergh et al, 1990; Ghinea et al, 1994), the changes in IF volume observed in the present study were not consistent with any significant direct role for this hormone; that is, IF volume was largely unaltered by T implants that completely suppress circulating LH levels, while EDS treatment reduced IF volume even though LH levels would be dramatically elevated in these animals. Altogether, these data indicate that the inhibitory effect of LPS on IF volume is dependent on the presence of intact Leydig cells, and although changes in intratesticular androgen levels influence this response, other products of the Leydig cells also appear to be involved. This response may involve disruption of the putative Leydig cell–mediated vascular regulatory agent(s) (Veijola and Rajaniemi, 1986; Damber et al, 1992; Collin and Bergh, 1996), which has not yet been identified. Physiological responses that could cause a decline in testicular IF volume include an increase in intratesticular pressure from swelling of the seminiferous tubules or contraction of the capsule, increased lymph flow or venous resorption, or a reduction in blood pressure or flow (Setchell et al, 1994). It is possible that the up-regulation of iNOS and increased local production of the vasodilator NO, leading to reduced blood pressure and flow in the testis, may be involved, since the Leydig cells are the earliest and most effective producers of iNOS in the LPS-treated rat testis (O'Bryan et al, 2000a). Whatever the mechanism, the data clearly indicate that in spite of increases in vascular permeability in the inflamed testis (O'Bryan et al, 2000b), which in other tissues result in edema, the inflammatory response of the testis causes additional vascular changes leading to a range of vascular responses, but generally producing an overall reduction in the normally large volume of testicular IF.

As expected, LPS had no significant effect on testis or seminal vesicle weights over the short time frame of the

treatment (3 hours). LPS appeared to have no significant effect on IF testosterone levels in the control groups over this time frame as well. This lack of a significant effect on IF testosterone at this time is consistent with a previous observation that, while Leydig cell testosterone production is obviously affected much earlier, maximal reduction of testosterone levels in the testicular IF does not occur until 6 hours after treatment with LPS in the rat testis (O'Bryan et al, 2000b). It should be noted that there are distinct species differences in the dynamics of inhibition of steroidogenesis by LPS, since Leydig cell inhibition responses in mice generally have a more rapid onset and are larger and more prolonged than those of rats (Bosmann et al, 1996; Sewer and Morgan, 1998; Hales et al, 2000; O'Bryan et al, 2000b; Gow et al, 2001).

With a ribonuclease protection assay, expression of TGFβ1, IFNγ, and MIF was clearly demonstrated in the testis, whereas IL-6, TNFα, and IL-10 mRNAs were barely detectable using this approach, in spite of the fact that the production of at least IL-6 and TNF-α has been described in the adult testis (De et al, 1993; Syed et al, 1993). However, previous studies either used extremely sensitive methods such as reverse transcription–polymerase chain reaction or investigated synthesis and production in isolated cells or a combination of both rather than total testis (De et al, 1993; Xiong and Hales, 1993; Kern et al, 1995; Huang et al, 2003), suggesting that the sensitivity of the assay was insufficient to detect expression of low copy number cytokines in total testes RNA. Conversely, this also suggests that TGFβ1, IFNγ, and MIF are relatively highly expressed cytokines in the testis.

Ablation of the Leydig cells by EDS treatment did not completely eliminate production of TGFβ1, IFNγ, or MIF before inflammation, indicating that this cell type is not required for their expression in the testis. However, expression of all cytokines investigated was significantly reduced by EDS treatment and restored by T24 implants, strongly suggesting that their expression is androgen regulated. Both these observations implicate the Sertoli cells and peritubular cells as the main source of these cytokines in the Leydig cell–depleted testis (Teerds and Dorrington, 1993; Dejucq et al, 1995; Meinhardt et al, 1999). The fact that the reduced intratesticular androgen levels caused by T3 implants did not cause a similar decline in the expression of these cytokines suggested that the level of testosterone required may actually be quite low or that the extent of the damage to spermatogenesis may be an important determinant as well. In fact, 2 of the cytokines (MIF, IFNγ) were elevated in T3-implanted animals, suggesting that testosterone may have a biphasic effect on their expression. Inflammation did not increase the expression of MIF, TGFβ1, and IFNγ mRNA. On the contrary, an additive down-regulatory effect of LPS for these cytokines was observed in the EDS-treated testes. The mechanism by which LPS further inhibits expression of these cytokines in the EDS-treated testis remains to be determined. It appears unlikely that a direct effect of LPS on the Sertoli or peritubular cells acting via the LPS receptor signaling pathway is involved, and it is possible that the reduction is due to an immunomodulatory intermediate produced by the testicular macrophages. Overall, the data suggest that the complete lack of androgens suppresses production of these cytokines. However, treatment with T3 implants showed that only very low levels of intratesticular testosterone are required to maintain normal expression levels, clearly indicating that it is testosterone, and not an indirect effect of androgens mediated via the seminiferous epithelium, that is responsible for this maintenance. Moreover, the failure of these cytokines to display acute regulation by inflammation in the testis may be related both to the fact that they are produced constitutively by testicular cells that are relatively insensitive to LPS stimulation (ie, testicular somatic cells and germ cells) and the unique regulatory phenotype of the testicular macrophage (Hedger, 2002).

These data also confirm that Leydig cells are not the sole source of any of the above cytokines in the testis. IFNγ has been localized to Sertoli cells and postmeiotic germ cells in addition to Leydig cells (Dejucq et al, 1995). The 3 mammalian TGFβ isoforms (1–3) are very highly expressed by Sertoli cells, peritubular cells, and Leydig cells in the fetal and immature testis, although production declines dramatically postpuberty (Mullaney and Skinner, 1993; Avallet et al, 1994; Konrad et al, 2000). In the postpubertal testis, they have been localized to the developing germ cells in a developmentally specific pattern of expression (Teerds and Dorrington, 1993; Caussanel et al, 1997). In addition, immature germ cells secrete bioactive TGFβ (Haagmans et al, 2003). Although MIF has been localized exclusively to the Leydig cells in the normal adult rat testis (Meinhardt et al, 1996), as noted previously, MIF production is not lost after depletion of the Leydig cells because compensatory MIF production by Sertoli cells occurs in the Leydig cell–depleted testis (Meinhardt et al, 1999). It is possible that similar compensatory production may occur for other cytokines in the testis. Finally, activation of the testicular macrophages or recruitment of leukocytes in the EDS- and LPS-treated testis could also influence the pattern of production that was observed (Wang et al, 1994). The data also suggest that, although a role in maintaining IF volume in the normal testis cannot be excluded, none of the 3 cytokines appears to be responsible for the changes in IF volume during LPS-induced inflammation.

Taken together, these data are consistent with a direct effect of androgen on the expression of several cytokines in the testis that are produced at a high constitutive level. The pattern of expression for these cytokines (ie, appar-

ently high level of endogenous production, stimulation by androgens or spermatogenic cells, absent or limited response to inflammation) is unique to the testis and may have important implications for the maintenance of the unique immune environment of the testis.

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