

*Research paper*

## **Modulation of monocytic lipopolysaccharide-induced tissue factor expression and tumor necrosis factor alpha release by estrogen and calcitriol**

George Vlotides,<sup>1</sup> Roland Gaertner,<sup>2</sup> Matthias W.A. Angstwurm<sup>2</sup>

<sup>1</sup>Department of Internal Medicine II, Standort Grosshadern, Ludwig-Maximilians-Universität, Munich, <sup>2</sup>Department of Internal Medicine, Medizinische Klinik, Standort Innenstadt, Ludwig-Maximilians-Universität, Munich, Germany

### **ABSTRACT**

**OBJECTIVE:** Modulatory effects of estrogens on both the immune and the coagulation system are only partially understood. In severe infections high estrogen levels have been observed both in men and postmenopausal women and are associated with increased mortality. Monocyte-derived tissue factor (TF) expression can activate the coagulation system and worsen the course of severe infection. The aim of the current study was to evaluate the *in vitro* effect of estrogens on differentiation, TF expression and Tumor Necrosis Factor alpha (TNF $\alpha$ ) release in human monocytes. **DESIGN:** Isolated peripheral blood monocytes, MM6- and THP-1 cells were cultured and stimulated by lipopolysaccharides (LPS) in the presence of 17 $\beta$ -estradiol (E2) and/or calcitriol. Proliferative responses were evaluated by determining the proliferation rate and by cell cycle analysis. Cell surface expression of CD14 and TF was determined by flow cytometry. TNF $\alpha$  was determined by ELISA. **RESULTS:** Although calcitriol induced the expression of the differentiation marker CD14 and decreased the expression of TF in both immature monocytic cell lines and primary monocytes, the LPS stimulation of TF expression was not significantly increased in immature monocytic cells and was decreased in mature monocytes. Calcitriol-treatment increased LPS-induced TNF $\alpha$  release in MM6 cells but inhibited TNF $\alpha$  release from peripheral blood monocytes. Treatment with E2 did not alter the phenotype or cell proliferation of resting monocytic cells. However, E2-treated monocytic cells and monocytes responded to LPS by increased TF expression and decreased TNF $\alpha$ . **CONCLUSIONS:** The results suggest that estrogens may modulate TF expression and cytokine production by monocytes and may thus be involved, at least in part, in the pathophysiology of acute inflammatory processes associated with high estrogen levels.

**Key words:** Calcitriol, Estrogens, Lipopolysaccharides, Monocytes, Tissue factor, Tumor Necrosis Factor  $\alpha$

*Address for correspondence:*

George Vlotides, MD, Cedars-Sinai Medical Center, 8700 Beverly Blvd., Los Angeles, CA 90048, Tel: (310) 423 7669, Fax: (310) 423 0119, E-mail: George.Vlotides@cshs.org

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## INTRODUCTION

The impact of gender and sex hormones on patients with severe infections is still controversial. Although there is evidence of protective effects of estrogens in critical diseases such as trauma-hemorrhage associated sepsis,<sup>1,2</sup> persistent elevation of estrogens during the course of severe inflammation/sepsis<sup>3-7</sup> may be associated with increased mortality.<sup>4,7</sup>

The biological actions of estrogens are mediated by the specific estrogen receptors (ER) ER $\alpha$  and ER $\beta$ , which are ligand-regulated transcription factors that influence the transcription of estrogen-responsive genes. Activated ERs regulate gene transcription through direct DNA binding to estrogen response elements (EREs) located within the regulatory regions of target genes, as well as by interacting with other transcription factors, such as NF $\kappa$ B, AP-1 or Sp1.<sup>8,9</sup> Such molecular interactions of ERs with other transcription factors can regulate cytokine activity in a cell and gene-specific manner.<sup>9</sup>

In addition to interactions between E2 and cytokines at the molecular level, reciprocal interactions between the hypothalamic-pituitary-adrenal (HPA) axis, gonadal axis and immune-mediated inflammatory processes may contribute to changes in inflammation parameters, particularly during stress.<sup>10</sup> E2 directly enhances corticotropin releasing hormone (CRH) gene expression and stimulates the central noradrenergic system, thereby affecting the two principal components of the stress system,<sup>10</sup> which in turn influence the secretion of regulatory cytokines such as IL-10 and IL-12<sup>11</sup> and modulate lymphocyte TH1/TH2 balance and/or monocyte activation.

Monocytes/macrophages are the only circulating cells that significantly synthesize Tissue Factor (TF), the main initiator of the extrinsic coagulation protease cascade.<sup>12</sup> In various pathologic conditions, monocytes and endothelial cells may express surface TF with subsequent activation of the coagulation system.<sup>12</sup> Indeed, TF exposure to the circulation is an important mechanism underlying the initiation of disseminated intravascular coagulation (DIC),<sup>13</sup> a severe complication of sepsis. Although monocytes constitute targets of E2 action,<sup>14-17</sup> little is known of the potential impact of E2 on monocyte TF expres-

sion, particularly during inflammation.

Monocytic cell lines like U-937, THP-1 or MM6 are often used as monocytic models, although they represent relatively immature cells of the monocyte-macrophage cell lineage.<sup>18</sup> To simulate the *in vivo* process, monocytic cells are often treated with specific agents which block their proliferation and induce differentiation in distinct patterns.<sup>19,20</sup> A well established differentiation inducing agent for myelomonocytic leukemia cells is the activated form of vitamin D3 (calcitriol).<sup>21,22</sup>

Upon stereo-specific ligand interaction with the vitamin D receptor (VDR) and heterodimerization of VDR with the retinoid X receptor, VDR is translocated to the nucleus. Binding of VDR to vitamin D response elements (VDRE) as well as interaction with coactivators regulate target gene expression.<sup>23</sup> In addition to its classical target tissues, VDR is also expressed in monocytic cells<sup>24</sup> and systemic as well as locally produced calcitriol can influence both the immune and coagulation systems.<sup>25,26</sup>

In the present study, we evaluated the differentiation induced by calcitriol in two human monocytic cell lines (THP-1 and MM6) as well as in freshly isolated primary monocytes. In order to understand the impact of high E2 levels on monocyte activation during inflammation, we examined the effect of E2 on monocyte TF expression and TNF $\alpha$  production in an *in vitro* sepsis model.

## MATERIALS AND METHODS

### Reagents

RPMI 1640 without phenol red, non-essential amino acids, L-glutamine and fetal calf serum (FCS) were purchased from Gibco Life Technologies (Karlsruhe, Germany), penicillin and streptomycin from Boehringer (Mannheim, Germany) OPI media supplement, 1 $\alpha$ ,25-dihydroxyvitamin D3, 17 $\beta$ -estradiol and lipopolysaccharide (LPS, Salmonella Minnesota) from Sigma Chemical Co (St. Louis, MO) and Ficoll-Paque Plus from Amersham Biosciences (Freiburg, Germany). Fluorescein (FITC)-conjugated TF monoclonal antibody (IgG1) were obtained from American Diagnostica Inc. (Pfungstadt, Germany), phycoerythrin (PE)-conjugated anti-CD14 antibody

and all conjugated isotype controls (IgG1-FITC, IgG1-PE) from Coulter (Krefeld, Germany) and FcR blocking reagent from Miltenyi Biotec (Bergisch Gladbach, Germany). CycleTEST™ PLUS DNA Reagent Kit and DNA Quality Control Particles were purchased from Becton Dickinson (Heidelberg, Germany).

### ***Mono Mac 6 cells***

Monocytic Mono Mac 6 (MM6) cells were cultured in phenol red-free RPMI 1640 medium supplemented with 2mM L-glutamine, 100U/ml penicillin, 100µg/ml streptomycin, 1mM sodium pyruvate, 1% non-essential amino acids, 1mM oxalacetic acid and 10µg/ml insulin. After the addition of supplements, the medium was ultra-filtered through a 0.2µm MediaKap filter (Spectrum, Huntingdon, England) followed by the addition of FCS (10%, heat inactivated). All cultures were seeded at a density of 200,000 cells/ml in 24-well plates (Costar, USA) and cultured for 2 to 3 days at 37°C in a 5% CO<sub>2</sub> atmosphere.

### ***THP-1 cells***

VDR and ER positive monocytic THP-1 cells<sup>27,28</sup> were cultured in the aforementioned medium and supplemented with 2mM L-glutamine and 10% heat inactivated FCS. The cells were cultured in tissue culture flasks (NUNC) for 3-4 days at 37°C in a 5% CO<sub>2</sub> atmosphere and for experiments they were seeded at a density of 200,000 cells/ml in 24-well plates and cultured for 2 to 3 days.

### ***Peripheral blood monocytes***

Heparin (Heparin Novo) anti-coagulated peripheral blood cells were received from apparently healthy male donors, who had given informed consent after full explanation of the purpose and nature of all procedures used. PBMCs were separated by density centrifugation over Ficoll-Paque density gradient. Isolated cells from the interphase were washed twice in cold phosphate-buffered saline (PBS) and re-suspended in RPMI 1640 medium without phenol red, supplemented with 10% heat-inactivated FCS, 2mM L-glutamine, 100U/ml penicillin and 100µg/ml streptomycin.

In order to obtain a monocyte-enriched cell population (MECs), PBMCs were briefly incubated in plastic culture flasks as previously described.<sup>29</sup> Non-

adherent cells were removed by medium aspiration and adherent cells were intensively rinsed and removed with cold PBS. This procedure yielded monocytes of ~80% purity, as confirmed by flow cytometric CD14 expression. The MECs were re-suspended in fresh medium (supplemented as described above) and, in order to prevent further cell adherence and thus possible activation, they were cultured in petri-erm culture plates with a hydrophobic culture surface (Vivascience, Hannover, Germany). They were then incubated for 1 to 3 days in a fully humidified air atmosphere containing 5% CO<sub>2</sub> at 37°C.

### ***Flow Cytometry***

The cell surface expression of TF and CD14 on MM6 and THP-1 cells were determined as a single parameter measurement. A minimum of 500,000 cells diluted in 60µl PBS in Falcon 2052 tubes were incubated for 5 minutes with 20µl of FcR blocking reagent. The cells were then incubated with primary antibody (human FITC-conjugated anti-TF, 11µg/ml, PE-conjugated anti-CD14, 25µg/ml) or isotype control for 10 minutes at room temperature. They were then washed twice in PBS and subsequently analyzed in a flow cytometer (EPICS XL, Coulter, Krefeld, Germany). The cells were gated according to their appearance in the forward scatter (FS)/side scatter (SS)-histogram in order to exclude debris and dead cells. Background staining was determined with the use of isotype control antibody and mean channel fluorescence intensity (MCFI)/percentage of positive cells were determined for each sample.

The detection of cell surface expression of TF on MECs was performed as a dual parameter measurement. TF-FITC was detected on the first channel and CD14-PE on the second in order to specifically determine TF expression on CD14-positive cells (monocytes). Specific isotype controls-containing samples were prepared for each measurement in order to compensate spectral overlapping of fluorescence, as required by multi-parametric flow cytometric analysis.

The cells were removed from the culture plates, washed twice in cold PBS and fixed in 2ml of cold 1% formaldehyde buffer for 30 min at 4°C and then washed in cold PBS. Prior to antibody incubation (30 minutes at 4°C), the cellular pellet containing

approximately 500,000 cells was re-suspended in 60 $\mu$ l PBS and was incubated for 5 minutes with 20 $\mu$ l of FcR blocking reagent as described above. The fixed cells were then washed in cold PBS and were kept at 4°C until flow cytometric analysis.

### Cell viability and proliferative responses

Cell viability was determined with acridin-orange/ethidium-bromide staining as previously described<sup>30</sup> and cell counts were performed with a coulter-counter (Coulter, Krefeld, Germany).

For cell cycle analysis, the EPICS XL was set up using the Becton Dickinson DNA Quality Control Particles and these settings were saved to be used for the acquisition of human nuclear preparations with CycleTEST™ PLUS DNA Reagent Kit as described by the supplier.

### TNF $\alpha$

Determination of TNF $\alpha$  was performed with a commercial enzyme-linked immunosorbent assay (ELISA) kit (Endogen, Woburn, MA) according to the manufacturer's instructions.

### Statistical analysis

Statistical analysis was performed with JMP 5.0.1 software. The data are expressed as the mean  $\pm$  SE of independently performed experiments. The mean of a single treatment group was compared with the mean of a single control group using the Student's t-test and a p-value of <0.05 was considered statistically significant.

## RESULTS

### Effect of calcitriol on CD14 expression

MM6 cells constitutively expressed high levels of CD14. 87.8  $\pm$  2% of cells were CD14 positive. Culture of the cells with calcitriol (40ng/ml) for 48 hrs further up-regulated CD14 expression; 97.3  $\pm$  1% of cells were CD14 positive and a  $\sim$ 7.5-fold MCFI increase ( $p < 0.001$ ) was observed (Figure 1A).

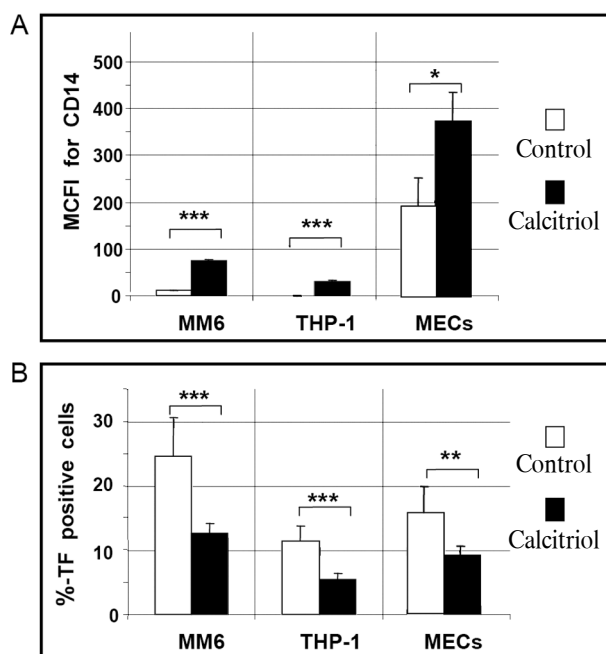
THP-1 cells constitutively expressed lower levels of CD14. On average, 33.2  $\pm$  1% of cells were CD14 positive. Treatment with calcitriol also up-regulated CD14 expression; 94.5  $\pm$  1% were CD14 positive and a  $\sim$ 30-fold MCFI increase ( $p < 0.001$ ) was observed

(Figure 1A).

MECs were isolated as described and cultured in the presence or absence of calcitriol for 48 hrs. Calcitriol-treatment had no effect on the overall percentage of CD14 positive cells ( $\sim$ 20%), but increased CD14 MCFI  $\sim$ 2-fold ( $p$ -value 0.046) (Figure 1A), suggesting up-regulation of the amount of CD14 receptors in already CD14-positive cells.

### Effect of E2 on CD14 expression and proliferation

Treatment of MM6 and THP-1 cells with concentrations of E2 varying from 0 to 1000 pg/ml had no effect on constitutive CD14 expression (not shown). Incubation of MM6 cells for 24 to 72 hrs with the aforementioned E2-concentrations had no significant influence on their GR. Cell cycle analysis of



**Figure 1.** Effect of calcitriol on CD14 and TF expression in MM6, THP-1 and MECs. (MM6, THP-1 and MECs were treated with 40 ng/ml calcitriol for 48 hrs). A: The results represent mean values  $\pm$  SE of at least 3 independently performed experiments (MM6, n=6; THP-1, n=4; MECs, n=3). Significant differences vs. the respective control group are indicated with asterisks. \*:  $P < 0.05$ ; \*\*\*:  $P < 0.001$ . B: The results represent mean values  $\pm$  SE of at least 3 independently performed experiments (MM6, n=9; THP-1, n=7; MECs, n=5). Significant differences vs. the respective control group are indicated with asterisks. \*\*:  $P < 0.01$ ; \*\*\*:  $P < 0.001$ .

MM6 cells under the influence of 1000pg/ml E2 was performed after 24 and 48 hrs and no significant difference was observed compared to controls (data not shown).

### Effect of calcitriol on TF expression

24.6±6% of MM6 cells were positive for TF. Treatment with calcitriol (40ng/ml) for 48 hrs decreased TF expression ~2-fold ( $p<0.001$ ) (Figure 1B). 11.4±2% of THP-1 cells expressed TF and calcitriol also decreased constitutive TF expression ~2-fold ( $p<0.001$ ) (Figure 1B). TF expression was not detected on freshly isolated PBMCs (data not shown). However, 12.3±5% of *in vitro* cultured CD14-positive MECs expressed TF and calcitriol treatment decreased it ~1.7-fold ( $p<0.01$ ) (Figure 1B).

LPS stimulation (1µg/ml; 5hrs) increased MM6 and THP-1 TF expression ~1.4-fold ( $p<0.01$ ), and MECs TF expression ~2.4-fold ( $p<0.001$ ) (Figure 2A).

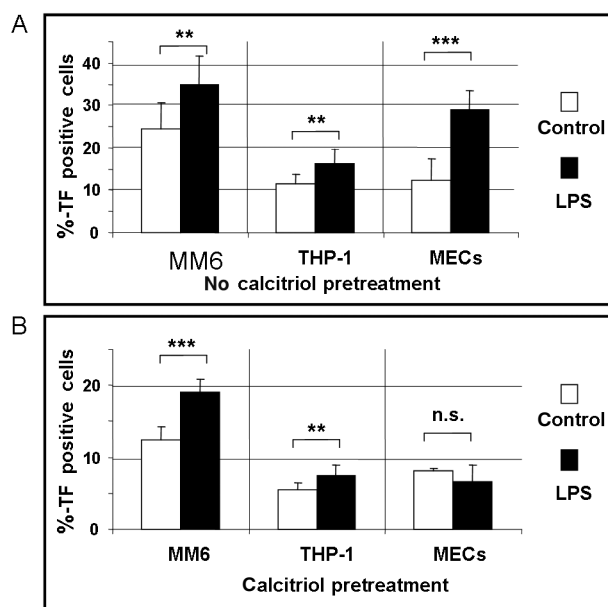
Under calcitriol treatment, LPS induced MM6 and THP-1 TF expression 1.5- ( $p<0.001$ ) and 1.4-fold ( $p<0.01$ ), respectively (Figure 2B). No significant increase of TF expression in response to LPS was observed in calcitriol-treated MECs (Figure 2B).

### Effect of E2 on TF expression

Incubation of MM6 cells with 1000pg/ml E2 for 24 hrs had no effect on baseline TF expression (not shown). However, pre-treatment of all cell types with E2 up-regulated LPS-induced TF expression (Figure 3 and 4).

A preliminary dose-response curve to examine the optimal E2 dose and the effect of additional treatment with calcitriol revealed that pre-incubation of MM6, THP-1 and MECs with increasing E2 concentrations for 24 hrs prior to LPS stimulation (1µg/ml; 5hrs) enhanced LPS-induced TF expression (Figure 3). Calcitriol-treated (40ng/ml; 48 hrs) MM6 cells showed a similar pattern of LPS-induced TF expression under E2 (Figure 3A), in contrast to THP-1 cells and MECs (Figure 3B, 3C).

In order to investigate the significance of this effect, we repeated this experiment at 1000pg/ml E2,



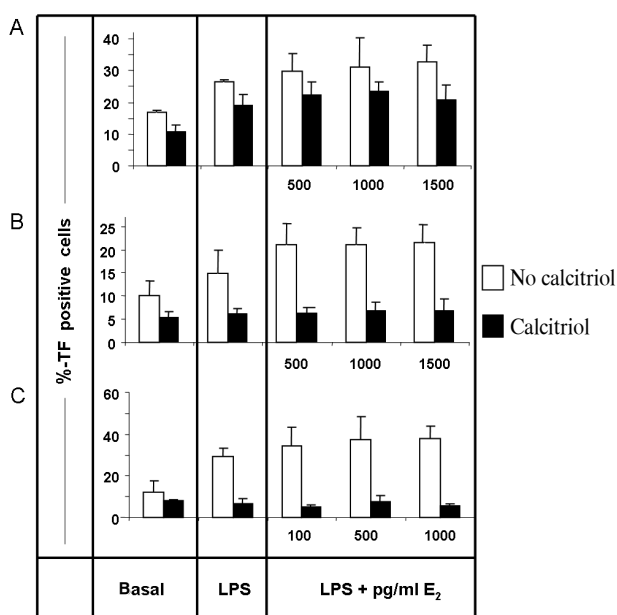
**Figure 2.** Effect of calcitriol on LPS-induced TF expression in MM6, THP-1 and MECs. (MM6, THP-1 and MECs were incubated with or without 40ng/ml calcitriol for 48 hrs and treated with or without 1µg/ml LPS for the last 5 hrs). Shown is the parameter “% TF-positive cells”. 2A: LPS-induced TF expression in MM6, THP-1 and MECs without calcitriol pre-treatment. The results represent mean values ± SE of at least 3 independently performed experiments (MM6, n=9; THP-1, n=7; MECs, n=6). Significant differences vs. the respective control group are indicated with asterisks. \*\*:  $P<0.01$ ; \*\*\*:  $P<0.001$ . 2B: LPS-induced TF expression in MM6, THP-1 and MECs with calcitriol pre-treatment. The results represent mean values ± SE of at least 3 independently performed experiments (MM6, n=9; THP-1, n=7; MECs, n=6). Significant differences vs. the respective control group are indicated with asterisks. \*\*:  $P<0.01$ ; \*\*\*:  $P<0.001$ .

and MM6 cells were placed under calcitriol-treatment (Figure 4). E2-treatment for 24 hrs prior to stimulation significantly enhanced LPS-induced TF in MM6 cells from 20±3% to 24±2.6%, in THP-1 cells from 13.5±3.7% to 19±3.2% and in MECs from 29±4.5% to 37.7±6.1% (Figure 4).

### Effect of calcitriol and E2 on TNFα production

Untreated MM6 cells produced very low levels of TNFα (1.8±4pg/ml). LPS induced TNFα 53.2-fold (95.7±5pg/ml), and pre-incubation with 500 and 1000pg/ml E2 suppressed this effect (Figure 5A).

Calcitriol-treated MM6 cells constitutively produced slightly higher levels of TNFα (12.3±13.3pg/



**Figure 3.** Dose-dependent effects of E2 on LPS-induced TF expression in MM6 (3A), THP-1 (3B) and MECs (3C) with and without calcitriol pre-treatment. MM6, THP-1 and MECs were incubated with or without 40ng/ml calcitriol for 48 hrs. 24 hrs prior to stimulation with LPS (1 $\mu$ g/ml for 5 hrs) they were treated with or without the indicated concentrations of E2. Shown is the parameter “% TF-positive cells”. The results represent mean values $\pm$ SE of 3 independently performed experiments.

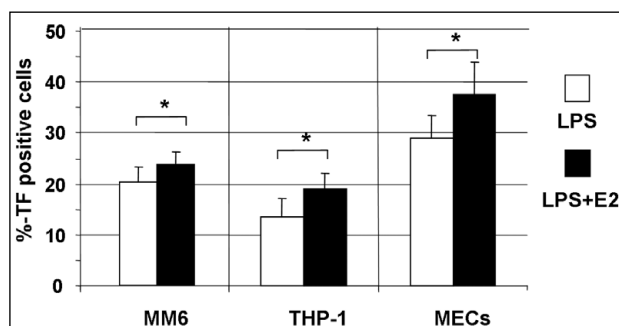
ml) compared to baseline levels. Stimulation with LPS led to a 10.9-fold increase in TNF $\alpha$  (133.7 $\pm$ 10.9pg/ml) and pre-incubation with increasing E2 concentrations suppressed LPS-induced TNF $\alpha$  release (Figure 5A).

TNF $\alpha$  in the supernatants of untreated MECs was not detectable in all experiments. After stimulation with LPS, MECs produced 485 $\pm$ 300pg/ml TNF (Figure 5B) and treatment with increasing E2 concentrations suppressed LPS-induced TNF $\alpha$  ~2-fold.

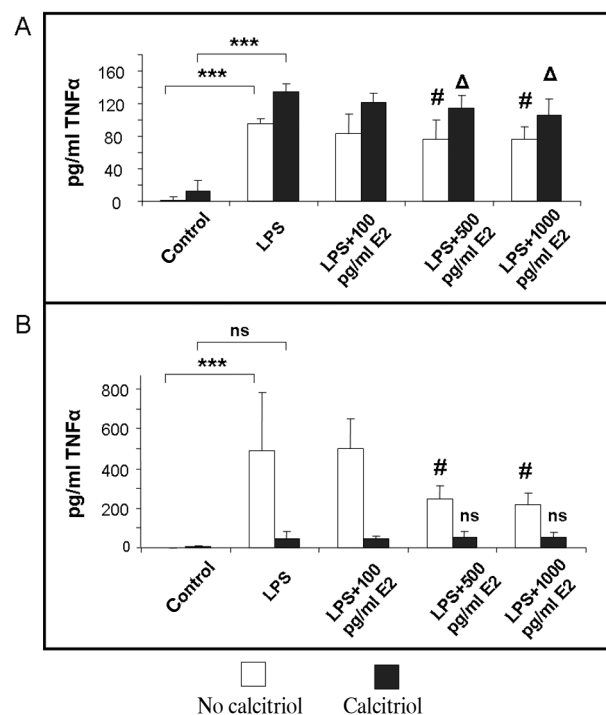
Calcitriol-treated MECs produced low levels of TNF $\alpha$  (6.3 $\pm$ 5.7pg/ml) and LPS stimulation did not significantly induce TNF $\alpha$  above baseline (Figure 5B). E2 pre-treatment had no significant effect on this low LPS-induced TNF $\alpha$  production under calcitriol (Figure 5B).

## DISCUSSION

The impact of gender and sex hormones on pa-



**Figure 4.** Effect of E2 on LPS-induced TF expression in MM6, THP-1 and MECs. MM6, THP-1 and MECs were cultured for 48 hrs in vitro and were treated in the last 24 hrs with 1000pg/ml E2. The MM6 cells were additionally treated during the whole 48 hrs with 40ng/ml calcitriol. Stimulation was performed with 1  $\mu$ g/ml LPS for 5 hrs. Shown is the parameter “% TF-positive cells”. The results represent mean values $\pm$ SE of 6 independently performed experiments. Significant differences vs. the respective control group (LPS+E2 vs. LPS) are indicated with asterisks. \*: P<0.05.



**Figure 5.** Effect of E2 and calcitriol on TNF $\alpha$  production in MM6 (5A) and MECs (5B). MM6 and MECs were incubated with or without 40ng/ml calcitriol for 48hrs. 24 hrs prior to stimulation with LPS (1 $\mu$ g/ml for 5 hrs) the cells were treated with or without the indicated concentrations of E2. Shown are mean values $\pm$ SE of 5 independently performed experiments. Significant differences are indicated. \*\*\*: P<0.001 vs. control; #: P<0.05 vs. LPS no calcitriol; Δ: P<0.05 vs. LPS calcitriol.

tients with severe infections is still controversial. Septic intensive care unit (ICU) patients present a ~10-fold increase of serum estrone and a ~2-fold increase of estradiol levels during the first 24 hrs after admission.<sup>4</sup> Although this early estrogen elevation may initially have a protective role, persistent elevation of estrogens during the course of severe inflammation/sepsis<sup>3-7</sup> is associated with increased mortality.<sup>4,7</sup> A recent study performed in elderly patients with severe infection upon admission to the ICU (208 males and 100 females) did not show gender-specific differences in demographic or infectious characteristics, but showed significantly elevated estrogen levels in non-survivors of either gender.<sup>7</sup>

Possible causes for the elevation of estrogens include adrenal dysfunction, increased aromatase activity, decreased liver metabolism and central stress-mediated HPA-gonadal axis dysregulation.

As monocyte TF is increased in septic patients<sup>31</sup> and monocyte-derived TF is implicated in disseminated intravascular coagulation (DIC),<sup>13</sup> a severe complication of sepsis, we sought to examine a putative role of E2 on monocyte activity and particularly TF expression.

Monocytes are the only circulating cells able to produce TF, the main initiator of the extrinsic coagulation protease cascade, and thus play a pivotal role in the regulation of physiological and pathophysiological reactions involving the coagulation system.<sup>12</sup> In fact, monocyte TF has been reported to be increased in septic patients<sup>31</sup> and in patients with liver cirrhosis,<sup>32</sup> while in several *in vitro* studies monocytes have been induced to express TF upon stimulation.<sup>33-35</sup>

In this context, we first examined the effect of E2 on resting monocytic cells *in vitro*, and treated both native and calcitriol-treated MM6 and THP-1 cells with increasing concentrations of E2 for 24 hrs to determine the expression of CD14 and TF. In this experimental setup, the resting phenotype of both cell lines was not influenced by E2. An inhibitory effect of E2 on proliferation of U-937 cells has been reported, which was associated with a cell cycle arrest and accumulation in the G2/M phase.<sup>36</sup> We treated MM6 cells with various E2 concentrations for 24-72 hrs but detected no significant effect of E2 on cell viability,

cell proliferation rate and DNA phase distribution of these cells. Thus, these results do not suggest a role of E2 in the regulation of phenotype or differentiation status of non-stimulated monocytic cells.

In order to examine the effect of increased concentrations of E2 on activated monocytes, as observed during sepsis *in vivo*, E2-pretreated monocytic cells (with and without additional calcitriol treatment) were stimulated with bacterial LPS. At rather high physiological E2 levels similar to those observed during pregnancy (500-1000pg/ml E2) and in some cases of severe inflammation in males and females, both monocytic cell lines and the primary cells responded to E2-treatment with enhanced LPS-induced TF expression (Figures 3 and 4). Thus, E2-mediated TF induction in activated monocytes and the associated procoagulant activity could contribute to the negative outcome of critically ill patients with persistently high E2 levels.

Although TNF $\alpha$  induces procoagulant activity in cultured human vascular endothelium<sup>37</sup> and has been implicated in monocyte hypercoagulability,<sup>38</sup> it is unlikely that LPS-induced TNF $\alpha$  release in E2-primed monocytes mediates up-regulation of TF expression, as our results showed inhibition of TNF $\alpha$  release in MM6 and primary monocytes (Figure 5) in accordance with previous reports.<sup>14,16,17,39</sup>

Despite seemingly contradictory data, particularly from *in vitro* studies, E2 is generally considered immunosuppressive with respect to secretion of cytokines such as IL-1, IL-6 and TNF $\alpha$  from immunocompetent cells.<sup>9</sup> Mechanisms involved in E2-mediated inhibition of TNF $\alpha$  in monocytes include decreased JNK activity, which results in decreased c-Jun/Fos and JunD/Fos heterodimer binding to the AP-1 binding site in the TNF $\alpha$  promoter.<sup>40</sup> An AP-1 site in the TNF-RE is essential for mainly ER $\beta$ -mediated repression of promoter activity, requires activation function-2 (AF-2) and is enhanced by coactivator proteins.<sup>41</sup>

In contrast to regulation of cytokine secretion, little is known of the effect of E2 on monocyte TF expression. Twelve months (but not 3 months) of hormone replacement therapy (HRT) reduced TF activity in both unstimulated and LPS-stimulated monocytes.<sup>42</sup> On the other hand, increased TF gen-

eration was detected in untreated and LPS-treated monocytes from women using oral contraceptives, with particular involvement of NF $\kappa$ B-regulated transcription,<sup>43</sup> data analogous to the results herein presented. However, the above studies involved long-term exposure of healthy female subjects to a lower concentration of estrogens, compared to the concentrations of estrogens in septic ICU patients. Within the experimental setting of this study, the results do not suggest direct induction of TF transcription by short-term E2 exposure, as no effect was observed on baseline TF expression, in contrast to the potent calcitriol-mediated TF down-regulation. It is also unlikely that E2-mediated TF up-regulation resulted from E2-mediated enhancement of the overall reaction to endotoxin exposure, as E2 had no effect on CD14 expression and exhibited divergent effects on LPS-dependent TNF $\alpha$  and TF expression. The mechanisms involved in LPS-induced TF in THP-1 cells include functional interaction between c-Fos/c-Jun and c-Rel/p65 heterodimers bound to the AP-1 and  $\kappa$ B sites within the LPS response element (LRE),<sup>44</sup> as well as activation of MAPKs p38 and ERK.<sup>44,45</sup> Thus, the effect of E2 on LPS-induced TF could result from E2-mediated modulation of LPS signaling.

Several mechanisms can be involved in counteractive effects between E2 and calcitriol. E2-mediated VDR up-regulation has been observed in several tissues, including colon/duodenum,<sup>46</sup> liver,<sup>47</sup> osteoblast-like<sup>48</sup> and breast cancer cells.<sup>49</sup> In breast cancer cells, direct ER $\alpha$  and ER $\beta$ -mediated activation of human VDR promoter activity has been reported.<sup>50</sup> On the other hand, calcitriol down-regulates ER abundance and suppresses E2 actions in MCF-7 cells<sup>51</sup> which contain a potential VDRE within the ER promoter.<sup>52</sup> In addition to direct transcriptional regulation of VDR by E2 and vice versa, stimulatory or inhibitory effects can also be the result of interaction with the signaling of other molecules, such as suppression of calcitriol-induced differentiation of primary bone marrow-derived hematopoietic precursors into osteoclast-like cells by E2-mediated inhibition of IL-6 signaling.<sup>53</sup>

The counteracting effect of calcitriol on E2-mediated regulation of LPS-induced TF and TNF $\alpha$  in primary monocytes observed in our study is most

probably the result of calcitriol-mediated Toll-like receptor down-regulation and decline in LPS responsiveness, supported by the absence of a counteracting effect of calcitriol on E2-mediated up-regulation of LPS-induced TF and TNF $\alpha$  in MM6 cells.

These results suggest that particularly during inflammation, high estrogen levels may modulate monocyte TF expression and thus be involved, at least in part, in the pathophysiology of sepsis syndrome. Targeting E2-mediated monocyte TF expression in a subset of septic patients with high estrogen levels could decrease procoagulant activity and associated intravascular thrombosis. However, whether monocytic TF induced by other inflammatory stimuli is also modulated by estrogens and the underlying molecular mechanisms still need to be elucidated.

In summary, although calcitriol induces the expression of the monocyte differentiation marker CD14 and decreases the expression of TF, the LPS response with respect to TF expression is not significantly increased in immature monocytic cells and is decreased in mature monocytes. Treatment with E2 does not alter the phenotype or proliferation of resting monocytic cells. However, E2-treated monocytic cells and primary monocytes respond with increased TF expression but decreased TNF $\alpha$  production to stimulation with LPS.

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