Dual-specificity phosphatase 3 deletion protects female, but not male mice, from endotoxemia- and polymicrobial-induced septic shock

Running title: Sex-dependent DUSP3's role in sepsis

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Abstract

DUSP3, is a small dual specificity phosphatase of poorly known physiological functions and for which only few substrates are known. Using DUSP3-deficient mice, we recently reported that DUSP3 deficiency confers resistance to endotoxin- and polymicrobial-induced septic shock. We showed that this protection was macrophage-dependent. In this work, we further investigate the role of DUSP3 in sepsis tolerance and show that the resistance is sex-dependent. Using adoptive transfer experiments and ovariectomized (OVX) mice, we highlighted the role of female sex hormones in the phenotype. Indeed, in OVX female and male mice, the dominance of M2-like macrophages observed in DUSP3$^{-/-}$ female mice was reduced suggesting a role of this cell subset in sepsis tolerance. At the molecular level, DUSP3 deletion was associated with oestrogen-dependent decreased phosphorylation of ERK1/2 and Akt in peritoneal macrophages stimulated *ex vivo* by LPS. Our results demonstrate that oestrogens may modulate M2-like responses during endotoxemia in a DUSP3-dependent manner.

Key words: DUSP3, sepsis, endotoxemia, LPS, female sex hormones, oestrogen, macrophages

Introduction

Sepsis and septic shock are complex clinical syndromes that arise when the local body response to pathogens becomes systemic and injures its own tissues and organs (1). When infection occurs, bacterial components such as LPS, are recognized by the host and inflammation is initiated. TLR4 pathway is activated and triggers the release of cytokines, chemokines and nitric oxide (NO) (2, 3). Systemic release of pro-inflammatory cytokines causes large-scale of cellular and tissue injuries, leading to microvascular disruptions, severe organ dysfunctions and eventually death (4). Sepsis occurrence and outcome depend on pathogen characteristics but also on risk factors such as age or sex (1). Indeed, women are better protected against infection and sepsis compared to men. Women younger than 50 years show a lower incidence of severe sepsis and a better survival compared to age-matched men. This may be explained by the influence of female sex hormones on the immune system responses (5).

DUSP3, or *Vaccinia*-H1-related (VHR), is an atypical dual specificity phosphatase of 21kDa. The phosphatase contains one catalytic domain but lacks a binding domain (6). DUSP3 broader catalytic site allows the protein to dephosphorylate both phospho-Tyr and phospho-Thr residues (7). The MAPK ERK1/2 and JNK were the first reported DUSP3 substrates (8–10). Other substrates such as the EGFR and ErbB2 tyrosine receptors (11) and STAT5 transcription factor (12) were also reported. DUSP3 physiological functions started to be elucidated thanks to the knockout mouse we have generated. Studies from our laboratory using $DUSP3^{-/-}$ mice showed that DUSP3 plays an important role in platelets biology, in monocytes and macrophages and in endothelial cells (13–15). In platelets, DUSP3 plays an important role in arterial thrombosis and platelet activation through GPVI and CLEC-2

signalling pathways (14). DUSP3 plays also an important role in endothelial cells and angiogenesis and seems to act as a pro-angiogenic factor (16). Surprisingly, this function was not correlated with reduced tumour or metastatic growth. Indeed, in an experimental metastasis model using Lewis lung carcinoma cells (LLC), we found that DUSP3 plays rather an anti-tumour role since DUSP3$^{-/-}$ mice were more sensitive to LLC metastatic growth when compared to WT littermates. This enhanced tumour growth in DUSP3$^{-/-}$ mice was associated with higher recruitment of M2-like macrophages (Vandereyken et al, under revision). Previous studies from our laboratory and others showed that DUSP3 was downregulated in some human cancers and upregulated in others (reviewed in (16, 17)). Further studies are required to better understand the role of this phosphatase in cancer biology.

DUSP3 plays also an important role in immune cell functions. In T cells, DUSP3 can be activated by ZAP-70 tyrosine kinase after TCR triggering (18). This activation, through tyrosine phosphorylation of DUSP3, allows the targeting of the MAPK ERK1/2 and the activation of its downstream signalling pathway. Moreover, in Jurkat leukemia T cells, DUSP3 targets ERK and JNK, but not p38. Together, these data suggest that DUSP3 controls T cell physiological functions at least partially through the MAPKs ERK and JNK (8). In innate immune cells, we recently showed that DUSP3 is the most highly expressed atypical DUSP in human monocytes. This was also true in mice (15). These findings suggested to us that DUSP3 could play an important role in innate immune responses. Indeed, using DUSP3$^{-/-}$ mice, we found that DUSP3 deletion conferred resistance of female mice to LPS-induced endotoxemia and to polymicrobial infection-induced septic shock. This protection was macrophage dependent since a higher percentage of M2-like macrophage subset was found in DUSP3$^{-/-}$ mice. Moreover, the resistance was also associated with a decreased phosphorylation of the tyrosine kinases ERK1/2 and a subsequent decrease in TNF-α production (15).

In this study, we report that DUSP3 deletion does not protect male mice from LPS-induced endotoxemia and CLP-induced septic shock and that this protection was female sex hormones dependent. Furthermore, we report that sepsis resistance was associated with a higher percentage of M2-like macrophages in peritoneal cavity of DUSP3$^{-/-}$ female mice but not with decreased pro-inflammatory cytokines production. We also showed that sepsis resistance in females, but not in males or in OVX females, was associated with decreased ERK1/2, PI3K and Akt activation.

Material and methods

Mice and ethic statement

C57BL/6 (CD45.2)-DUSP3$^{-/-}$ mice were generated by homologous recombination as previously reported (13). These mice were backcrossed with C57BL/6-CD45.2 mice (Charles River) to generate heterozygotes that were mated to generate DUSP3$^{+/+}$ and DUSP3$^{-/-}$ littermate colonies used for experimentation. Age matched male and female DUSP3$^{+/+}$ and DUSP3$^{-/-}$ mice were used in all the experiments. Mice were kept in ventilated cages under 12-hours dark/12-hours light cycle in an SPF animal facility and received food and water and libitum. Health status was evaluated every 3 months and mice were always found free of specific pathogens.

All mouse experiments and procedures were approved by the animal ethics committees of the Universities of Ghent and Liege and were carried out according to their guidelines.

Cecal ligation and puncture and in vivo LPS challenge

Cecal ligation and puncture (CLP) was performed as previously described (19). For LPS challenge, mice were i.p. injected with 6mg/kg of LPS. Body temperature was monitored using a rectal thermometer at various times after LPS injection and after CLP. Death of mice was recorded and the data were analysed for statistical significance of differences between the experimental groups.

Mice irradiation and bone marrow transplantation

10-12 weeks old C57BL/6 (CD45.2) donor mice were killed by cervical dislocation. Tibiae and femurs were collected and BM cells were flushed with PBS. BM cells ($10x10^6$) were immediately i.v. injected to 6-8 weeks old lethally irradiated (866, 3cGy) C57BL/6 (CD45.1)

recipient mice. 4 weeks later, transplantation efficiency was evaluated on the basis of the ratio of CD45.2 to CD45.1 cells in the blood of transplanted mice.

Female ovariectomy and in vivo oestrogen complementation

4 weeks old females were anesthetized using ketamine/xylazine (150 mg/kg and 20 mg/kg). A vertical incision of 2-3 cm was performed in the middle of the back. 1 cm lateral of the midline, another incision of 2-3 mm was performed in the fascia. Adipose tissue surrounding ovary was pulled out and ovary was removed after clamping. The same operation was realized for contralateral ovary. The incision in fascia was closed with stitches and the skin incision with clips. Shame operated mice were used as a control. All above procedures were applied to these mice except the removal of ovaries. For *in vivo* oestrogen complementation, 2 weeks after surgery, subcutaneous implants for controlled release of 17β-oestradiol (1.5µg/day) (Belma technologies) were applied to OVX mice and were kept for 3 weeks before sacrifice.

Antibodies and reagents

The following materials were from Cell Signalling Technology Inc: anti-phospho-Akt (Ser473), anti-Akt, anti-phospho-ERK1/2 (Thr202/Tyr204), anti-ERK, anti-phospho-PI3K p85 (Tyr458)/p55 (Tyr199), anti-PI3K p85, anti-phospho-GSK3α/β (Ser21/9). Anti-GSK3α/β was from Santa Cruz. Anti-GAPDH antibody was from Sigma. HRP-conjugated anti-goat antibody was from Dako. HRP-conjugated anti-mouse antibody was from GE healthcare. HRP-conjugated anti-rabbit antibody was from Merck Millipore. APC-anti-CD45.1 (A20) and PerCp-Cy5.5-anti-CD45.2 (104), FITC-anti-CD11b, APC-Cy7-anti-Ly6G, PE-anti-CD3, PerCp-anti-CD8, FITC-anti-CD4, Biotin-anti-B220 and streptavidin-PE-Cy7 were all from BD Biosciences. APC–anti-F4/80, PerCp-Cy5-anti-NK1.1, and PerCP–Cy5.5–anti-CD11b

were from eBiosciences. PE-Cy-anti-Ly6G antibody was from BioLegend. LPS from Escherichia coli serotype O111:B4 was from Sigma and was diluted in pyrogen-free PBS.

Animal blood sampling and plasma preparation

Peripheral blood was drawn in EDTA-coated tubes (BD Microtainer K2E tubes; BD Biosciences) by puncturing the heart with 26G needle. Centrifugation was performed twice at 800g for 15 min at RT. Plasma samples were separated in sterile Eppendorf tubes, aliquoted in small volumes, and stored at -80°C until used.

Meso Scale Discovery electrochemiluminescence assay

MSD assay was performed according to manufacturer's instructions (Mesoscale Discovery). Briefly, plasma was diluted 15 and 15.000 times for TNF and IL-6 respectively. For IL-10 and IFN-γ, samples were diluted twice. Samples were loaded on 96 well plates, incubated 2h at RT and washed. Detection antibodies were added for 2h at RT. Signal detection was measured within 15 minutes after read buffer addition using MSD instrument.

Isolation and stimulation of thioglycollate elicited peritoneal macrophages

Peritoneal washes were performed 4 days after intraperiteonal injection of 1 mL of 4% thioglycollate broth (Sigma). 5 mL of PBS-EDTA 0.6 mM were injected twice in the peritoneal cavity using an 18G needle and then collected. Peritoneal macrophages were selected by adherence to tissue culture plastic dishes in complete RPMI 1640 medium. Peritoneal macrophages were stimulated with LPS 1µg/mL during 15, 30 or 60 minutes or during 8 and 24h hours, depending on the experiment performed

Phenotyping and flow cytometry.

Peritoneal washes were centrifuged 10 min at 350g and the pellet was re-suspended in PBS. For surface cell staining, cells were incubated for 15 min with anti-CD16/CD32 (Fcγ III/IIR) before labelling for 30 min with specific antibodies for 30 min at 4°C. Cells were then washed and fixed with 1% paraformaldehyde solution. Cells were next analysed on FACSCanto II (Becton Dickson) using FlowJo (Tree Star).

Protein extraction and Western blot

For Western blot experiments, cells were stimulated for the indicated time points and lysis was performed with RIPA buffer (50 mM Tris-HCl (pH = 8.0), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM orthovanadate, complete protease inhibitor cocktail tablets EDTA free and 1 mM phenylmethylsulfonyl fluoride) on ice during 20 min. Lysates were next clarified by centrifugation at 19.000g during 20 min at 4°C. The resulting supernatants were collected and protein concentrations were determined using the colorimetric Bradford reagent (Bio-Rad). Proteins were next denatured at 95°C in Laemmli buffer (40% glycerol; 8% SDS 5%; 20% B-mercaptoethanol; 20% Tris-HCl 0.5 M pH6.8; 0.05% bromophenol blue and water) during 5 min.

Denatured samples were run on 10% SDS-PAGE gel and transferred onto nitrocellulose membranes. To block the non-specific binding sites, membranes were incubated for one hour at room temperature in Tris-buffered saline-Tween 20 containing 5% of non-fat milk or 3% BSA (bovine serum albumin). Membranes were incubated overnight with primary antibody at 4°C. Membranes were next washed thrice in Tris-buffered saline-Tween and incubated with HRP-conjugated secondary antibody during one hour at room temperature. The blots were developed by enhanced chemiluminescence (ECL kit, Amersham) according to the manufacturer's instructions.

RNA purification, reverse transcription, and real-time PCR

RNA was extracted from PMs using the miRNeasy Mini Kit (Qiagen) and cDNA was synthesized using Expand reverse transcriptase (Roche) according to the recommendations of the manufacturer. cDNA was amplified using Sybr Green PCR Master Mix (Roche) and 0.3 mM specific primers for Arginase 1 (Arg1), iNOS, and β2-microglobulin (β2M). All quantitative PCR were performed on a LightCycler System for RealTime PCR (Roche). The ratio between the expression level of the gene of interest and b2M in the sample was defined as the normalization factor. Relative mRNA quantities for Arg1 and iNOS were determined using the ΔCq method. All primers were from Eurogentec. Sequences were as follow: inducible NO synthase (iNOS): FW, 59-GCTTCTGGTCGATGTCATGAG-39, RV, 59-TCCACCAGGAGATGTTGAAC-39; Arg1: FW, 59-CAGAAGAATGGAAGAGTCAG-39, RV, 59-AGATATGCAGGGAGTCACC-39; and b2M: FW, 59-CACCCCACTGAGACTGATACA-39, RV, 59-TGATGCTTGATCACATGTCTCG-3.

Statistical analysis

The student t-test was used to assess statistical differences between different groups. Survival differences after LPS challenge and CLP were analysed by Kaplan-Meier analysis with log rank test. Results were considered as significant if p-value < 0.05. Results are presented as mean ± SEM. Prism software (GraphPad) was used to perform statistical analysis. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

Results

DUSP3$^{-/-}$ female, but not male, mice are resistant to LPS-induced endotoxemia and to CLP-induced septic shock

In a previous study, we showed that DUSP3 deletion protected mice from LPS-induced endotoxemia and polymicrobial infection-induced septic shock (15). Only female were used in the first study. To investigate whether the protection observed is a general feather of DUSP3 deletion or sex dependent, we challenged DUSP3$^{-/-}$ males with a lethal dose of LPS (i.p. injection of 6 mg/kg) and compared their survival to females and to WT control littermates of both sex. Body temperature was also monitored. As expected and previously reported, 90% of DUSP3$^{-/-}$ female mice were resistant to LPS while only 5% of DUSP3$^{+/+}$ female mice survived the challenge (15). Interestingly, DUSP3$^{+/+}$ and DUSP3$^{-/-}$ male mice were equally sensitive to LPS-induced death (**Fig. 1A**). Body temperature of all groups of mice, but not DUSP3$^{-/-}$ females, decreased after LPS injection. 24h later, almost all DUSP3$^{-/-}$ females recovered while the other groups remained hypothermic (**Fig. 1B**). These results were further confirmed in the cecal ligation and puncture (CLP) model performed on DUSP3$^{+/+}$ and DUSP3$^{-/-}$ males and females. As expected, only 10% of DUSP3$^{+/+}$ and DUSP3$^{-/-}$ male mice and DUSP3$^{+/+}$ female mice were still alive by the end of the experiment whereas 70% of DUSP3$^{-/-}$ female mice survived (**Fig. 1C**.). The body temperature of each group dropped after surgery and only DUSP3$^{-/-}$ female mice recovered (**Fig. 1D**). These results indicate a sex specific response to septic shock in DUSP3$^{-/-}$ mice.

Ovariectomized DUSP3$^{-/-}$ mice are sensitive to LPS-induced death

Male and female sex hormones receptors have been identified on immune cells suggesting direct effects of androgen and oestrogen on these cells (20). Sexual steroid hormones have

been recognized to influence numerous immune pathophysiological processes (21). To elucidate the effect of female sex hormones, we ovariectomized (OVX) 4 weeks old DUSP3$^{+/+}$ and DUSP3$^{-/-}$ mice (OVX mice). As controls, another group of 4 weeks old DUSP3$^{+/+}$ and DUSP3$^{-/-}$ were sham operated. To assess the ovariectomy's efficiency, we checked the presence and the size of the uterus. Successful OVX mice were deprived of normal uterus development whereas sham operated mice presented a normally developed uterus (**Fig. 1E**). 6 weeks after surgery, sham and OVX mice were challenge with 6 mg/kg of LPS and survival and temperature were monitored (**Fig. 1F** and **1G**). Ovariectomy impaired the observed endotoxemia resistance of DUSP3$^{-/-}$ mice, whereas sham operated DUSP3$^{-/-}$ mice were still fully protected from endotoxin-induced death. These data demonstrate that female sex hormones are involved in the observed resistance of DUSP3$^{-/-}$ female mice to LPS-induced lethality.

DUSP3$^{-/-}$ female bone marrow cells rescue DUSP3$^{+/+}$ female, but not male mice from LPS-induced lethality

We previously showed that adoptive transfer of DUSP3$^{-/-}$ female bone marrow cells or monocytes to DUSP3$^{+/+}$ female mice was sufficient to transfer resistance to LPS-induced lethality (15). We therefore investigated whether this is also true when recipient mice are males. To generate chimeric mice, 10x10^{6} bone marrow cells (BM) from DUSP3$^{-/-}$ C57BL/6-CD45.2 female mice were intravenously injected into lethally irradiated DUSP3$^{+/+}$ C57BL/6-CD45.1 recipient male and female mice (DUSP3$^{-/-}$ > M-DUSP3$^{+/+}$ and DUSP3$^{-/-}$ > F-DUSP$^{+/+}$, respectively). As a control, DUSP3$^{+/+}$ females BMs were transplanted into lethally irradiated DUSP3$^{+/+}$ male or female mice (DUSP3$^{+/+}$ > M-DUSP3$^{+/+}$ and DUSP3$^{+/+}$ > F-DUSP3$^{+/+}$, respectively). Successful hemato-lymphoid reconstitution was verified by flow cytometry 3 to 4 weeks after the transplantation. 95% of peripheral blood cells were CD45.2

positive (**Fig. 1H** and **1I**). Moreover, in recipient mice, the expression of DUSP3 in peritoneal macrophages was abolished in the recipient mice transplanted with $DUSP3^{-/-}$ BM cell suspension, as showed by DUSP3 immunoblotting (**Fig. 1J**). 4 weeks after BM transplantation, 6 mg/kg of LPS were i.p. injected into recipient mice and survival was monitored during 8 days (**Fig. 1K**). Interestingly, more than 70% of the chimeric $DUSP3^{-/-}$ > F-$DUSP^{+/+}$ mice survived up to the end of the experiment compared to 9% of $DUSP3^{+/+}$ > F-$DUSP3^{+/+}$ mice. On the other hand, all $DUSP3^{-/-}$ > M-$DUSP3^{+/+}$ and $DUSP3^{+/+}$ > M-$DUSP3^{+/+}$ mice died within 4 days after LPS injection (**Fig. 1K**). These data suggest that, in the absence of DUSP3, both female sex hormones and myeloid cells are required for resistance to LPS shock.

DUSP3-deletion-induced LPS shock resistance in female mice, but not in male, OVX and wild type mice, is associated with increased M2-like macrophages in the peritoneal cavity.

We have previously reported that DUSP3 is expressed in several immune cells where it plays an important role in macrophage and in T cell functions (15)(18). Since sepsis involves the participation of both innate and adaptive immune cells (22), we investigated whether DUSP3 deletion-associated survival to shock, in females, was linked to unbalanced contribution of one cell type or another in LPS-resistant compared to LPS-sensitive mice. We found that, at basal levels as well as after LPS injection, percentage of $CD19^{pos}$B, $CD4^{pos}$T, $CD8^{pos}$T, macrophages ($Ly6G^{neg}CD11b^{pos}F4/80^{pos}$), Neutrophils ($F4/80^{neg}/CD11b^{pos}Ly6G^{pos}$), NK ($CD3^{neg}NK1.1^{pos}$) and NKT ($CD3^{pos}NK1.1^{pos}$) cells were equal between males and females of both genotypes (**Fig. 2A**). LPS injection induced a significant reduction of T cells and macrophages, increased neutrophils infiltration on the peritoneal cavity and had no significant impact on the percentage of NK, NKT and B cells (**Fig. 2A**).

We previously reported that increased survival of DUSP3$^{-/-}$ female mice after LPS and CLP was associated with a higher percentage of M2-like macrophages in the peritoneal cavity of these mice compared to DUSP3$^{+/+}$ females (15). To investigate if this is associated to DUSP3-deficient female survival, we phenotyped DUSP3$^{+/+}$ and DUSP3$^{-/-}$ peritoneal macrophages from male and female mice (both sham operated and OVX) challenged with LPS based on the characterisation previously reported by Ghosn et al (23). M1 macrophages are F4/80^{int}CD11b^{int}Ly6G^{neg}, whereas M2-like macrophages are F4/80^{hi}CD11b^{hi}Ly6G^{neg} (**Fig. 2B** and **2C**). We confirmed previous findings showing that the percentage of M2-like macrophages was higher in the peritoneal cavity of DUSP3$^{-/-}$ female mice compared to littermate controls 2h and 24h after LPS injection (**Fig. 2B** and **2C**). Interestingly, we observed that the percentage of M2-like macrophages in male mice was slightly lower compared to DUSP3$^{-/-}$ female mice 2h after LPS challenge. This difference was exacerbated at 24h after LPS injection. There was not significant difference for the percentage of M2-like macrophages between DUSP3$^{+/+}$ and DUSP3$^{-/-}$ male mice. Similarly, there was no difference in the percentage of M1-like macrophages at 2h and 24h after LPS injection between DUSP3$^{+/+}$ and DUSP3$^{-/-}$ female mice. However we noticed a slight increase in the percentage of M1-like macrophages in males compared to female mice 2h after LPS injection. This difference was accentuated, though not significantly, at 24h (**Fig. 2B** and **2C**). For the OVX mice, 2h after LPS injection, the percentage of M1-like macrophages (F4/80^{int}CD11b^{int}) was higher in DUSP3$^{+/+}$ and DUSP3$^{-/-}$ OVX mice compared to DUSP3$^{-/-}$ sham mice. The difference was maintained at 24h, although not significantly (**Fig. 2B** and **2C**). M2-like macrophages percentage was equal in DUSP3$^{+/+}$ and DUSP3$^{-/-}$ OVX mice compared to DUSP3$^{+/+}$ and DUSP3$^{-/-}$ sham mice 2h after LPS injection. However 24h after LPS challenge, the percentage of M2-like macrophages in the peritoneal cavity of OVX mice decreased, but did not reach statistical significance when compared to DUSP3$^{-/-}$ sham mice (**Fig. 2B** and

370 **2C**). These data suggest that M2-like macrophages could be involved in the resistance to LPS-
371 induced endotoxemia. To further characterise these cells, we measured the relative expression
372 of genes associated with M1-like and M2-like PMs, namely, *Nos2* and *Arg1*. At basal levels,
373 none of the transcript was detected (data not shown). 2h after LPS challenge, *Arg1* expression
374 increased significantly in DUSP3$^{-/-}$ sham compared to DUSP3$^{+/+}$ sham (**Fig. 2D**). In males and
375 OVX groups, *Arg1* was detected but at significantly lower levels compared to sham operated
376 female mice. 24h after LPS injection, level of *Arg1* increased dramatically in DUSP3$^{-/-}$ sham
377 group compared to all the other groups (**Fig. 2D**). *Nos2* levels were low 2h after LPS injection
378 but increased significantly 22h later in sham operated female mice of both genotypes, though,
379 the increase was more significant in DUSP3$^{+/+}$ female mice (**Fig. 2D**). Altogether, these data
380 suggest that M2-like macrophages and female hormones could be involved in DUSP3-
381 induced resistance to LPS-induced endotoxemia.
382
383 *DUSP3-KO female mice survival to LPS is not due to a modification in pro-inflammatory*
384 *cytokines production*
385 We previously reported that DUSP3$^{-/-}$ female survival to LPS was associated with decreased
386 systemic TNF level compared to DUSP3$^{+/+}$ mice (15). Therefore, we wanted to know whether
387 the susceptibility of DUSP3$^{-/-}$ male and OVX mice to LPS-induced death could be linked to
388 differential expression of TNF or to other pro-inflammatory cytokines such as IL6, IFNγ and
389 IL10. We measured and compared plasma levels of these four cytokines at basal levels, at 2h
390 and 24h after LPS challenge in all group of mice, using MSD assay. For TNF, there was no
391 difference between DUSP3$^{+/+}$ and DUSP3$^{-/-}$ males. However and as previously reported (15)
392 there was a significant decrease of this cytokine in DUPS3$^{-/-}$ females compared to DUSP3$^{+/+}$
393 female mice 2h and 24h after LPS challenge (**Fig. 3A**). Compared to DUSP3$^{+/+}$ mice, DUSP3^{-}
394 $^{/-}$ mice of both sex had a slight, but not significant decrease of IL6 2h after LPS injection (**Fig.**

3B). These differences were maintained in OVX mice groups (**Fig. 3A** and **3B**). For IFNγ, secretion was equal in all groups of mice 2h after LPS challenge. However, at 24h after LPS injection, IFNγ levels were lower in DUSP3$^{-/-}$ females sham and OVX compared to DUSP3$^{+/+}$ females sham and OVX. There was however, a 10-fold decrease of IFNγ in all OVX mice, regardless of their genotype. In males, the level of IFNγ was significantly higher in DUSP3$^{-/-}$ than in the littermates controls at 24h but not at 2h after LPS injection (**Fig. 3C**). Finally, the level of IL10 was lower in DUSP3$^{-/-}$ mice compared to controls regardless of sex or type of surgery (**Fig. 3D**). Altogether, these data strongly suggest that DUSP3 deletion-induced female mice resistance to LPS-induced shock is not a consequence of the observed modifications of the measured cytokines.

DUSP3-deletion alters ERK1/2 and PI3K/Akt phosphorylation magnitudes and kinetics in oestrogen-depend manner.

We have previously reported that, although DUSP3 is ubiquitously expressed protein, the level of expression vary significantly between cell types (15)(14) and during cell cycle progression (24). We therefore investigated whether its expression vary between males and females and if it changes in response to LPS or after ovariectomy. As shown in figure 5A, DUSP3 expression level was similar in males and females and was not influenced by LPS or OVX (**Fig. 5A**).

We have previously reported that DUSP3 deletion in female mice macrophages was associated with decreased ERK1/2 phosphorylation levels after *ex vivo* LPS stimulation (15). To investigate if this alteration was also associated with the sex-specific resistance to septic choc, DUSP3$^{+/+}$ and DUSP3$^{-/-}$ peritoneal macrophages from sham or OVX mice were stimulated *ex vivo* with LPS (1 μg/mL) at different time points and cell lysates were probed with phospho-specific ERK1/2 antibodies. As expected, ERK1/2 phosphorylation was

significantly lower in DUSP3$^{-/-}$ sham peritoneal macrophages at all time points compared to DUSP3$^{+/+}$ macrophages. Interestingly, in OVX mice, LPS stimulation led to an equal ERK1/2 activation in both DUSP3$^{-/-}$ and DUSP3$^{+/+}$ peritoneal macrophages as demonstrated by the observed phosphorylation levels. There was no difference of ERK1/2 phosphorylation in male mice from both genotypes (**Fig. 4B** and **4C**).

The observed reduced phosphorylation of ERK1/2 in DUSP3$^{-/-}$ sham mice suggests that DUSP3 could be targeting either ERK1/2 upstream kinase or one of ERK1/2 phosphatases. Therefore we analysed MAPKK MEK1/2 activation following *ex vivo* LPS stimulation (1 µg/mL) of peritoneal macrophages. MEK1/2 kinetic phosphorylation was equal between DUSP3$^{+/+}$ and DUSP3$^{-/-}$ sham mice of both sex (**Fig. 4D** and **4E**), suggesting that MEK1/2 is not targeted by DUSP3.

The PI3K/Akt pathway is another important pathway activated after TLR4 triggering (25). We therefore investigated whether DUSP3 deletion could impact this pathway after activation with LPS and whether the kinetic and magnitude of this activation could be sex dependent. PI3K and Akt activations were evaluated using phospho-specific antibodies and Western blot after *ex vivo* LPS stimulation (1 µg/mL) of peritoneal macrophages at different time points. Interestingly, PI3K and Akt activations decreased in DUSP3$^{-/-}$ sham peritoneal macrophages compared to DUSP3$^{+/+}$ peritoneal macrophages at all time points. This difference was abolished in OVX mice since the phosphorylation level of PI3K and Akt remained equal between DUSP3$^{+/+}$ and DUSP3$^{-/-}$ peritoneal macrophages. The activation of GSK3 downstream target of Akt was, however, not affected by DUSP3 deficiency neither in sham nor OVX mice (**Fig. 5A** and **5B**). There was no difference in PI3K and Akt activations in male peritoneal macrophages after LPS stimulation. PI3K and Akt were equally activated at

all time points in DUSP3$^{+/+}$ and DUSP3$^{-/-}$ LPS- stimulated peritoneal macrophages. GSK3 activation was not affected by DUSP3 deficiency (**Fig. 5A** and **5B**).

These data suggest that DUSP3 affects ERK1/2, PI3K and Akt activation probably in concert with estrogens. To investigate this hypothesis, DUSP3$^{-/-}$ and DUSP3$^{+/+}$ female mice were ovarictomized at the age of 4 weeks. 2 weeks later, half of the mice from each group were complemented with estrogen using subcutaneous implant for controlled release of 17β-oestradiol (1.5µg/day). Mice were kept for 3 weeks before sacrifice. Peritoneal macrophages were stimulated ex vivo with LPS (1 µg/mL) at different time points and cell lysates were probed with anti-phospho-ERK1/2, anti-ERK, anti-phospho-PI3K, anti-PI3K, anti-phospho-Akt and anti-Akt antibodies. As shown in figure 6, oestrogen complementation reduced significantly the phosphorylation levels of ERK1/2 and Akt in DUSP3$^{-/-}$, but not in DUSP3$^{+/+}$, peritoneal macrophages (**Fig.6A** and **6B**). These data clearly suggest that DUSP3-dependent reduced phosphorylation of ERK1/2 and Akt are oestrogen dependent.

Discussion

It is well recognized that immune responses to infection are sex dependent. Indeed stronger immune responses confer to women protection against infections and sepsis (26). Several epidemiological studies have been performed and showed a greater incidence of sepsis in males compared to females (27). Consequently, compared to males, there are less female hospitalizations associated with infections. In addition, male sex, and presence of comorbidities were commonly reported independent predictors of post-acute mortality in sepsis survivors (28). Interestingly, many of the differences between males and females in response to infections become apparent at puberty (29). In line with this, women younger than 50 years show lower incidence of severe sepsis and better survival compared to age-matched men (30). Altogether, these observations suggest a role for sexual hormones in the protection from severe infections and sepsis. This hypothesis has been supported by the finding that receptors for reproductive hormones are present in a variety of immune cell types (31). On the other hand, estrogen have been demonstrated to increase resistance to several bacterial infections whereas the removal of endogenous estrogens have been shown, for example, to markedly increase the severity of *Mycobacterium avium* infections, an effect that can be reversed after 17β-estradiol replacement (32, 33). The role of female reproductive hormones in susceptibility to acute infection and sepsis is still however poorly understood.

In the present study, we report that DUSP3 deletion confers resistance to LPS-induced lethality and to polymicrobial-induced septic shock in female mice but not in males. We demonstrated that this protection is female sexual hormone and monocyte/macrophage dependent. Indeed, ovariectomy induced a loss of resistance. On the other hand, DUSP3$^{-/-}$ monocytes transfer to WT females was sufficient to transfer the resistance to WT recipient mice (15). This protection was, however, not due to decreased TNF production as suggested

by our previous study (15). To our knowledge, this is the first report demonstrating a signalling molecule-induced synergistic immunoprotective effect of monocytes/macrophages and female sexual hormones against sepsis.

The observed resistance to LPS-induced septic shock of DUSP3$^{-/-}$ female mice was associated with a modest increase of M2-like macrophages in the peritoneal cavity of mice. This observation was strengthened by the increase of *Arg1* gene expression in DUSP3$^{-/-}$ females but not in males or ovarictomized mice. *Arg1* is indeed a known marker for M2-like macrophages (34). DUSP3-deficient mice ovariectomy induced a loss of resistance to LPS-induced death with no difference in M2-like macrophage percentage between control groups and OVX-DUSP3$^{-/-}$ mice. Together with the fact that the percentage of M2-like macrophages was also equal in both DUSP3$^{+/+}$ and DUSP3$^{-/-}$ male mice, it suggests that female sex hormones may influence macrophage alternative activation. Our observations are in line with studies showing that oestrogens influence numerous immunological processes, among which monocytes and macrophages physiological functions (35). Indeed, ovarian sex hormones modulate monocyte adhesion and chemotaxis, TLR expression, cytokines production as well as phagocytosis activity (36). Moreover several evidences suggest that oestrogens also influence macrophage polarization. ER-α knockout mice undergo a decrease of alternative activated macrophages (36). ER-α-deficient macrophages are indeed refractory to IL-4-induced alternative activation as demonstrated by a decrease of IL-4R and STAT6 phosphorylation in these cells (37). Oestrogens have also been reported to increase the expression of the transcription factor IRF4 (*interferon regulatory factor-4*) involved in alternative activation of macrophages (38). Using transcriptomic assay, we did not observe differences in IL4, IL4R or IRF4 expression levels between DUSP3-KO males and females neither at basal levels nor after LPS challenge (data not shown). On the other hand, TNF production does not seem to play a role in the observed phenotype since ovariectomy of

DUSP3$^{-/-}$ mice did not influence the level of this pro-inflammatory cytokine, although mice succumb to endotoxemia. These data were rather surprising since sex steroids are known to regulate pro- and anti-inflammatory cytokine levels released by macrophages. On the other hand, female sex hormones are known to negatively regulate TNF production (39), one of the most important cytokines in sepsis (40, 41). The change of TNF production, as well as the observed change in IFNγ, IL6, IL-10 and perhaps other cytokines upon DUSP3 deletion should be therefore considered as an independent phenomenon not related to DUSP3$^{-/-}$ female mice survival to sepsis.

How does DUSP3 regulate macrophage alternative activation in a female sexual hormone dependent manner is a complex question to answer. The molecular mechanisms involved are probably linked to the observed decrease of ERK1/2 and Akt/PI3K activations. Upon *ex vivo* LPS stimulation, DUSP3$^{-/-}$ female peritoneal macrophages showed reduced phosphorylation of both ERK1/2 and Akt when compared DUSP3$^{+/+}$ female macrophages. These differences were not observed in macrophages from OVX DUSP3$^{-/-}$ mice but were maintained in DUSP3$^{-/-}$ OVX mice under oestrogen complementation. Together, these data suggest that, under inflammatory conditions, oestrogen controls macrophage polarization through DUSP3-ERK1/2-Akt signalling pathway axis.

ERK1/2 has been previously reported to play a role in macrophage polarization through mTOR signalling pathway (42). Indeed, ERK1/2 phosphorylates and dissociates the tuberous sclerosis protein (TSC) complex leading to its inactivation and subsequent activation of mTOR (42), constitutive activation of which leads to decreased IL-4-induced M2 polarization in TSC-deficient mice (42)(43). The role of sex hormones has not been investigated in these studies. In our model, it would be interesting to investigate whether the observed lower phosphorylation of ERK1/2 found in DUSP3$^{-/-}$ female peritoneal macrophages could lead to

TCS activation and consequently to M2 polarization. On the other hand, it has been reported that, upon TLR4 stimulation, PI3K engagement is followed by Akt and mTORC1 activation due to TSC inactivation by Akt (44). This may lead to M1 macrophages polarization (44)(45). Similarly to ERK decreased phosphorylation, decreased PI3K/Akt activation may lead to TSC activation and shifts macrophage polarization towards a M2 phenotype.

Another important question raised by our study is how does DUSP3 deletion lead to decreased activation of the ERK1/2 and Akt signalling molecules under the control of oestrogen. Decreased phosphorylation of these kinases clearly suggests that they are not directly targeted by DUSP3. The observed decreased phosphorylation could be due to reduced activation of specific ERK1/2 and PI3K/Akt yet unknown phosphatase. Indeed, preliminary data from our laboratory show that pervanadate (non-specific protein tyrosine phosphatases inhibitor) treatment of LPS-stimulated peritoneal macrophages restores ERK1/2 phosphorylation while okadaic acid (inhibitor of Ser/Thr PP1/PP2A), at low and high concentrations, did not (data not shown). Further investigations using, among others, phosphoproteomic approaches are required to confirm this hypothesis and identify the specific substrate(s) for DUSP3 and assess the exact role of this phosphatase in TLR4 signalling under the influence of female sex hormones.

In summary, we identified DUSP3 dual-specificity phosphatase as a new key signalling molecule playing an important role in macrophage alternative activation and sexual dimorphism in innate immune response to infection. Our data suggest that DUSP3 inhibition, combined to oestrogen administration, may lead to protection from sepsis and septic shock.

Consent for publication: not applicable

Availability of data and materials: not applicable

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Author Contributions:

S.R designed the research. M.V., C.W., P.S., M.A., L.M. M.S and L.D., performed the experiments. S.R. and C.L. analyzed data. S.R. and M.V. wrote the manuscript.

Competing Financial Interests statement: The authors declare that they have no competing financial interests.

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Figure legends

Figure 1: *Female sex hormones and myeloid cells are required for DUSP3 deletion-induced resistance to endotoxemia and septic shock*. (**A**) $DUSP3^{+/+}$ male (n = 12) and female (n = 17), $DUSP3^{-/-}$ male (n = 13) and female (n = 19) mice were i.p injected with 6 mg/kg of LPS. Percent survival was assessed twice a day for 10 days. (**B**) Body temperature of $DUSP3^{+/+}$ and $DUSP3^{-/-}$ mice before, 6 h, and 24 h after LPS injection. (**C**) $DUSP3^{+/+}$ male (n = 10) and female (n = 11) and $DUSP3^{-/-}$ male (n = 9) and female (n = 11) mice were subjected to CLP (one puncture with 21-gauge needle). Survival was documented twice a day for 7 days. (**E-G**) $DUSP3^{+/+}$ and $DUSP3^{-/-}$ were sham operated (n= 9 for $DUSP3^{+/+}$ and n=8 for $DUSP3^{-/-}$) or OVX (n=9 for $DUSP3^{+/+}$ and n=11 for $DUSP3^{-/-}$) 4 weeks after birth. (**E**) Representative macroscopic view of uterus after sham surgery or OVX is shown in. (**F**) 6 weeks after surgery, mice were i.p injected with 6 mg/kg LPS. Percent survival was assessed twice a day for 5 days. (**G**) Body temperature of $DUSP3^{+/+}$ and $DUSP3^{-/-}$ mice before, 8 h, and 24 h after LPS injection. (**H-K**) $10x10^6$ bone marrow cells (BM) from $DUSP3^{-/-}$ C57BL/6-CD45.2 female mice were intravenously injected into lethally irradiated $DUSP3^{+/+}$ C57BL/6-CD45.1 recipient male and female mice ($DUSP3^{-/-}$ > M-$DUSP3^{+/+}$ and $DUSP3^{-/-}$ > F-$DUSP^{+/+}$, respectively). As control, $DUSP3^{+/+}$ females BMs were transplanted into lethally irradiated $DUSP3^{+/+}$ male or female mice ($DUSP3^{+/+}$ > M-$DUSP3^{+/+}$ and $DUSP3^{+/+}$ > F-$DUSP3^{+/+}$, respectively). (**H**) Representative dot plot of CD45.1 and CD45.2 immune cells in BM transplanted mice. (**I**) Percentage of CD45.1 and CD45.2 immune cells in all transplanted mice. (**J**) Western blot was performed on peritoneal cells from transplanted mice using anti-DUSP3 antibody. Anti-GAPDH was used as a loading control. Each line corresponds to one mouse. Line 1: lysate from peritoneal cavity cells of $DUSP3^{+/+}$ mouse. Lines 2-8: ♀ $DUSP3^{-/-}$ into ♂-$DUSP3^{+/+}$. Lines 9-14: ♀ $DUSP3^{-/-}$ into ♀-$DUSP3^{+/+}$ (**K**). Transplanted mice survival

after LPS i.p. injection (6mg/mL). Data are presented as mean ± SEM. Survival data were compared using Kaplan–Meir with log-rank test. *p < 0.05, ***p <0,001, ***p <0,001.

Figure 2. *DUSP3-deletion-induced LPS shock resistance in female mice, but not in male, OVX and wild type mice, is associated with increased M2-like macrophages in the peritoneal cavity*. (**A**) Peritoneal cells harvested from PBS and 24h LPS-challenged $DUSP3^{+/+}$ and $DUSP3^{-/-}$ mice were analyzed by flow cytometry to evaluate the percentage of T, B, NK, NKT, neutrophil and macrophage cell populations. For lymphocyte and NK cell phenotyping, cells were stained using PE-anti-CD3, FITC-anti-CD4, PE-Cy7-anti-B220 and PerCp-Cy5-anti-NK1.1. FSC and SSC were used for gating on live cells and lymphocyte populations. CD4 T cells were $B220^{neg}/NK1.1^{neg}/CD3^{pos}/CD4^{pos}$. CD8 T cells were $B220^{neg}/NK1.1^{neg}/CD3^{pos}/CD8^{pos}$. B cells were $B220^{pos}/NK1.1^{neg}/CD3^{neg}$. NK cells were $B220^{neg}/CD3^{neg}NK1.1^{pos}$ and NK-T cells were $B220^{neg}/CD3^{pos}NK1.1^{pos}$. For neutrophils and macrophages, phenotyping was performed using PerCP-Cy5.5-anti-CD11b, APC-Cy7-anti-Ly6G and APC–anti-F4/80. Neutrophils were $F4/80^{neg}/CD11b^{pos}/Ly6G^{pos}$ while macrophages were considered as $Ly6G^{neg}/F4/80^{pos}/CD11b^{pos}$. Percentage of the indicated cell population out of live cells (total live cells for macrophages and neutrophils analysis and leucocytes gate for the analysis of lymphocytes and neutrophils) are presented as histogram of means (n=3 in each group) ± SEM. (**B**) Peritoneal cells from PBS or LPS (24h) injected $DUSP3^{+/+}$ and $DUSP3^{-/-}$ male mice, $DUSP3^{+/+}$ and $DUSP3^{-/-}$ sham operated or OVX female mice were analysed to discriminate between M1-like macrophages ($F4/80^{int}CD11b^{int}$) and M2-like macrophages ($F4/80^{hi}CD11b^{hi}$). Analysis was performed on $Ly6G^{neg}$ live cell gate. Representative dot plot from each group of mice is shown. (**C**) Quantification of M1-like and M2-like macrophages out of total live $Ly6G^{neg}$ cells. Results are presented as means ± SEM. N=6-10 mice per group. (**D**) Quantitative RT-PCR analysis for the expression of *Arg1* and

Nos2 transcripts in harvested peritoneal macrophages of the indicated groups of mice at basal levels and 24h after LPS injection. The expression of genes of interest was relative to β2M. n=4 mice in each group. Results are presented as mean ± SEM. *p<0.5, **p<0.01.

Figure 3. *DUSP3-KO female mice survival to LPS is not due to a modification in pro-inflammatory cytokines production*. Plasma levels of TNF (**A**), IL-6 (**B**), IFNγ (**C**) and IL-10 (**D**) in DUSP3$^{+/+}$ and DUSP3$^{-/-}$ male and sham operated or OVX female mice before, at 2h and at 24h after LPS challenge (6 mg/mL). Cytokine levels were determined using MSD assays. Results are presented as mean ± SEM. n = 5 mice per group. The same mice were used for all time points. *p < 0.05, **p < 0.01.

Figure 4. *DUSP3-deficiency affects ERK 1/2 phosphorylation in female mice macrophages but not in males*. Peritoneal macrophages isolated from 12-weeks-old DUSP3$^{+/+}$ and DUSP3$^{-/-}$ female, male and OVX mice were stimulated *ex vivo* with 1 mg/ml LPS for the indicated time points. (A) Western blots were performed using anti–phospho-ERK1/2 (Thr202/Tyr204) and anti-ERK1/2 as a loading control. Representative blots are shown for each detected (phospho) protein. Densitometry quantifications of phosphor-ERK1/2 and ERK1/2 were performed. (B) Anti–phospho- MEK1/2 (Ser217/221) and anti-MEK1/2, as loading control and densitometry quantifications of phospho-MEK and MEK. Results are presented as a ratio of phospho-ERK/ERK and phospho-MEK/MEK from four independent experiments. For each experiment, peritoneal cells from 2-3 individual mice were pooled prior to stimulation with LPS and lysis. Data are shown as mean ± SEM. *p < 0.05.

Figure 5. *DUSP3-deficiency affects PI3K/Akt pathway in female, but not in male mice macrophages*. Peritoneal macrophages isolated from 12-weeks-old DUSP3$^{+/+}$ and DUSP3$^{-/-}$

784 female, male and OVX mice were stimulated *ex vivo* with 1 mg/ml LPS for the indicated time
785 points. (A) Western blots were performed using anti–phospho-PI3K (p85 Tyr 458/ p55
786 Tyr199), anti-phospho-Akt (Ser473), anti-phospho-GSK3α/β (Ser21/9) and anti-PI3K, anti-
787 Akt and anti-GSK3α/β as loading controls. (B) Densitometry quantifications of phospho-
788 PI3K, phospho-Akt, phospho-GSK3α/β, PI3K, Akt and GSK3α/β. Results are presented as a
789 ratio of phospho-PI3K/PI3K, phospho-Akt/Akt and phospho-GSK3α/β/GSK3α/β from four
790 independent experiments. For each experiment, peritoneal cells from 2-3 individual mice were
791 pooled prior to stimulation with LPS and lysis. Data are shown as mean ± SEM. *p, 0.05.
792
793 **Figure 6.** *Alteration of ERK1/2 and Akt phosphorylation in DUSP3$^{-/-}$ female macrophages is*
794 *oestrogen-depend*. Peritoneal macrophages isolated from OVX DUSP3$^{+/+}$and DUSP3$^{-/-}$ and
795 from OVX DUSP3$^{+/+}$and DUSP3$^{-/-}$ under estrogens complementation?(3 weeks, 1.5µg/day)
796 were stimulated *ex vivo* with 1 µg/ml LPS for the indicated time points. (**A**) Western blots
797 were performed using anti–phospho-ERK1/2 (Thr202/Tyr204), anti–phospho-PI3K (p85 Tyr
798 458/ p55 Tyr199), anti-phospho-Akt (Ser473), anti-PI3K, anti-ERK1/2 and anti-Akt as
799 loading controls. (**B**) Densitometry quantifications of phospho-ERK, phospho-PI3K,
800 phospho-Akt, ERK1/2, PI3K and Akt. Results are presented as a ratio of phospho-
801 ERK1/1/ERK1/2, phospho-PI3K/PI3K and phospho-Akt/Akt from 3 independent
802 experiments. For each experiment, peritoneal cells from 3 individual mice were pooled prior
803 to stimulation with LPS and lysis. Data are shown as mean ± SEM. *p < 0.05. **p < 0.01.
804

Figure 1

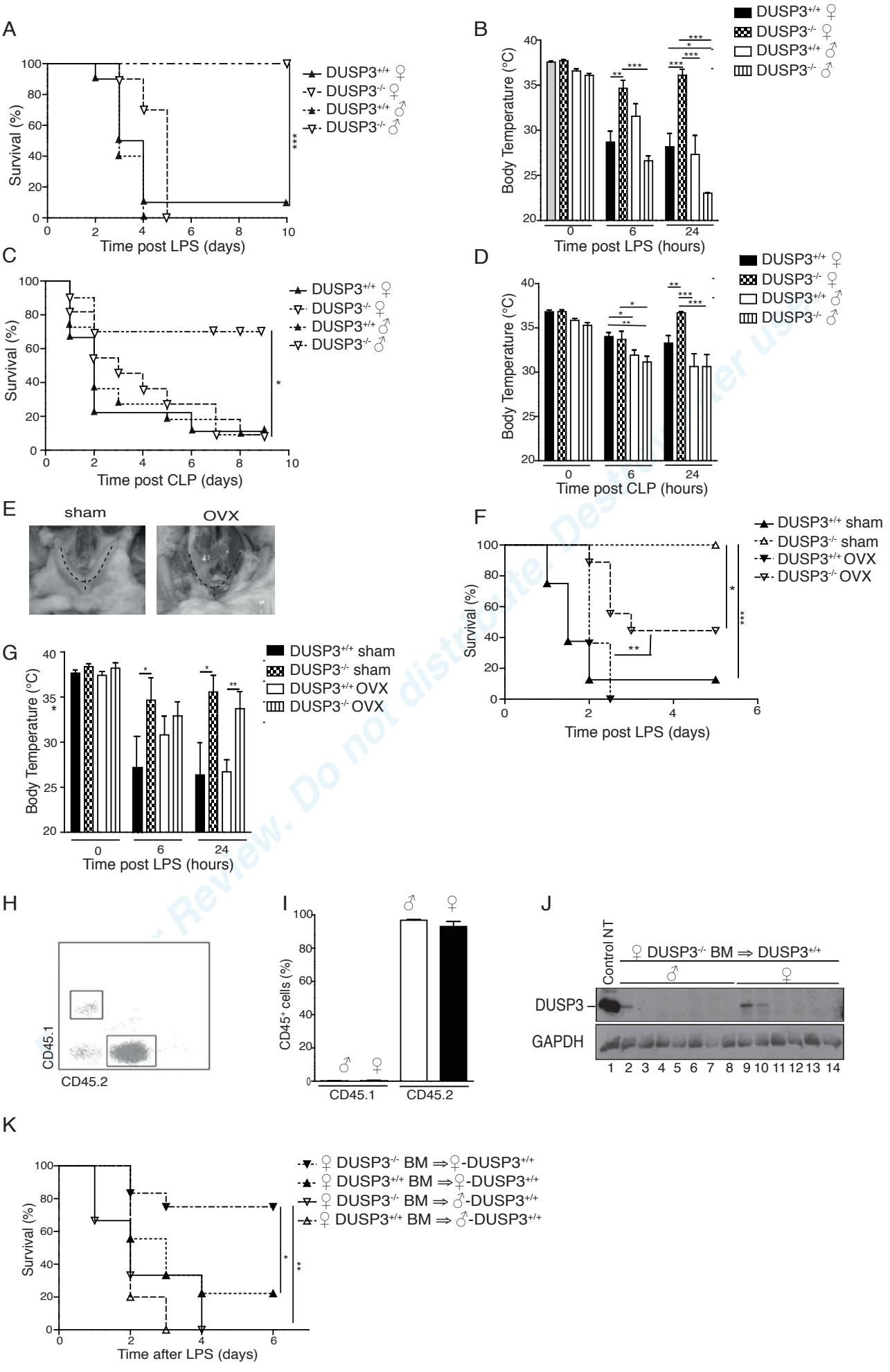

Figure 2

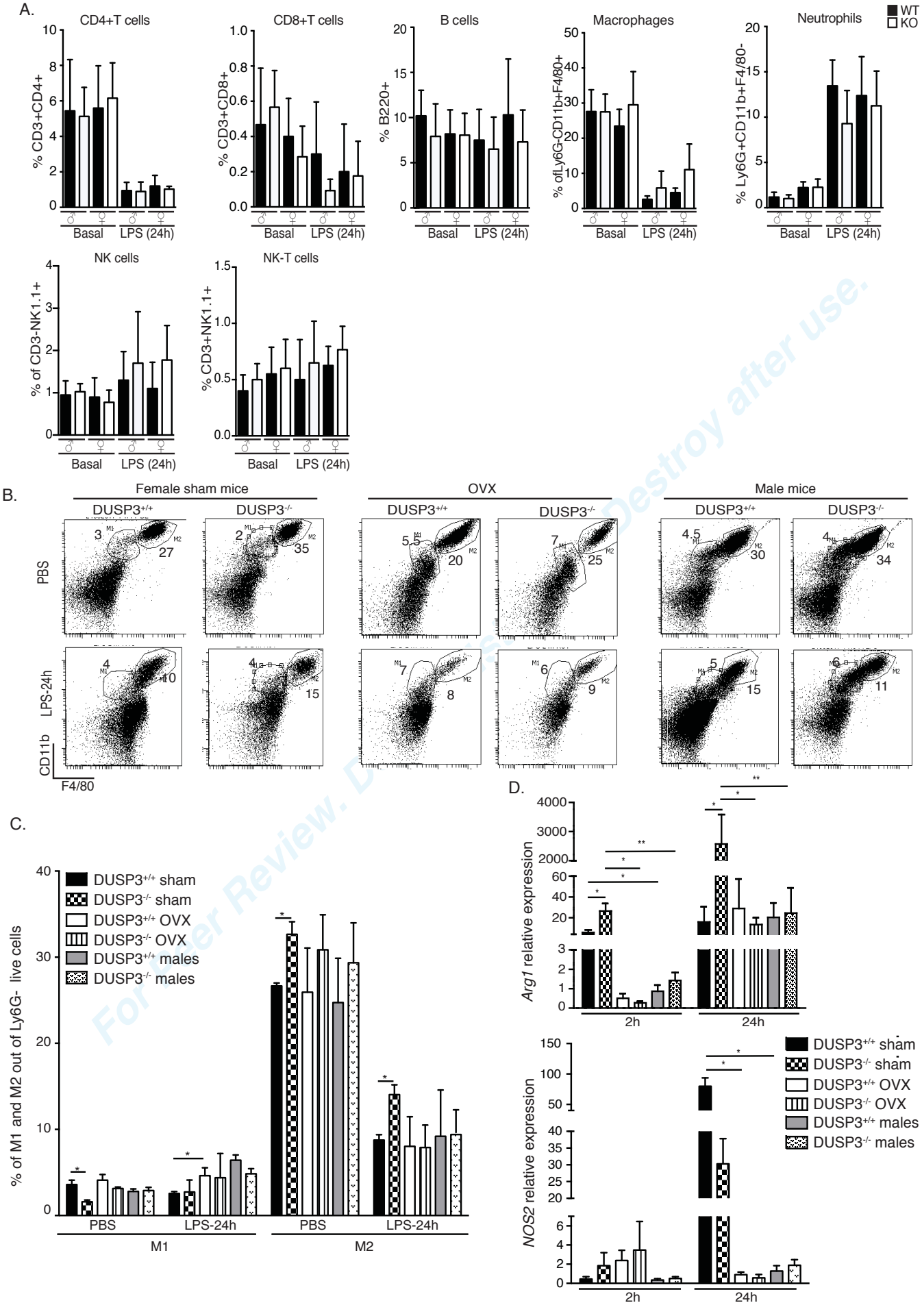

Figure 3

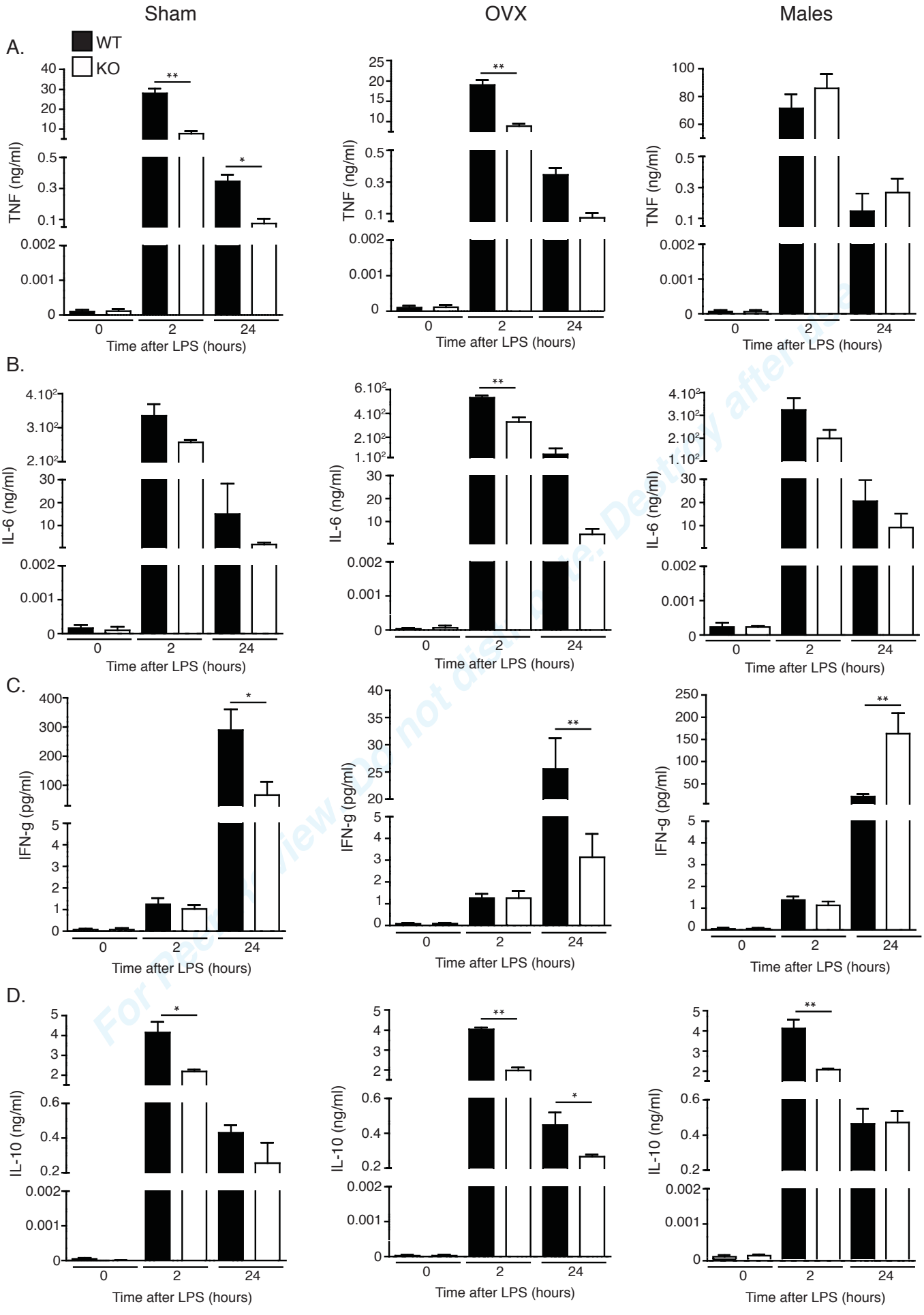
Sham
OVX
Males
A.
WT
KO
TNF (ng/ml)
B.
IL-6 (ng/ml)
C.
IFN-g (pg/ml)
D.
IL-10 (ng/ml)
Time after LPS (hours)
0
2
24
*
**

Figure 4

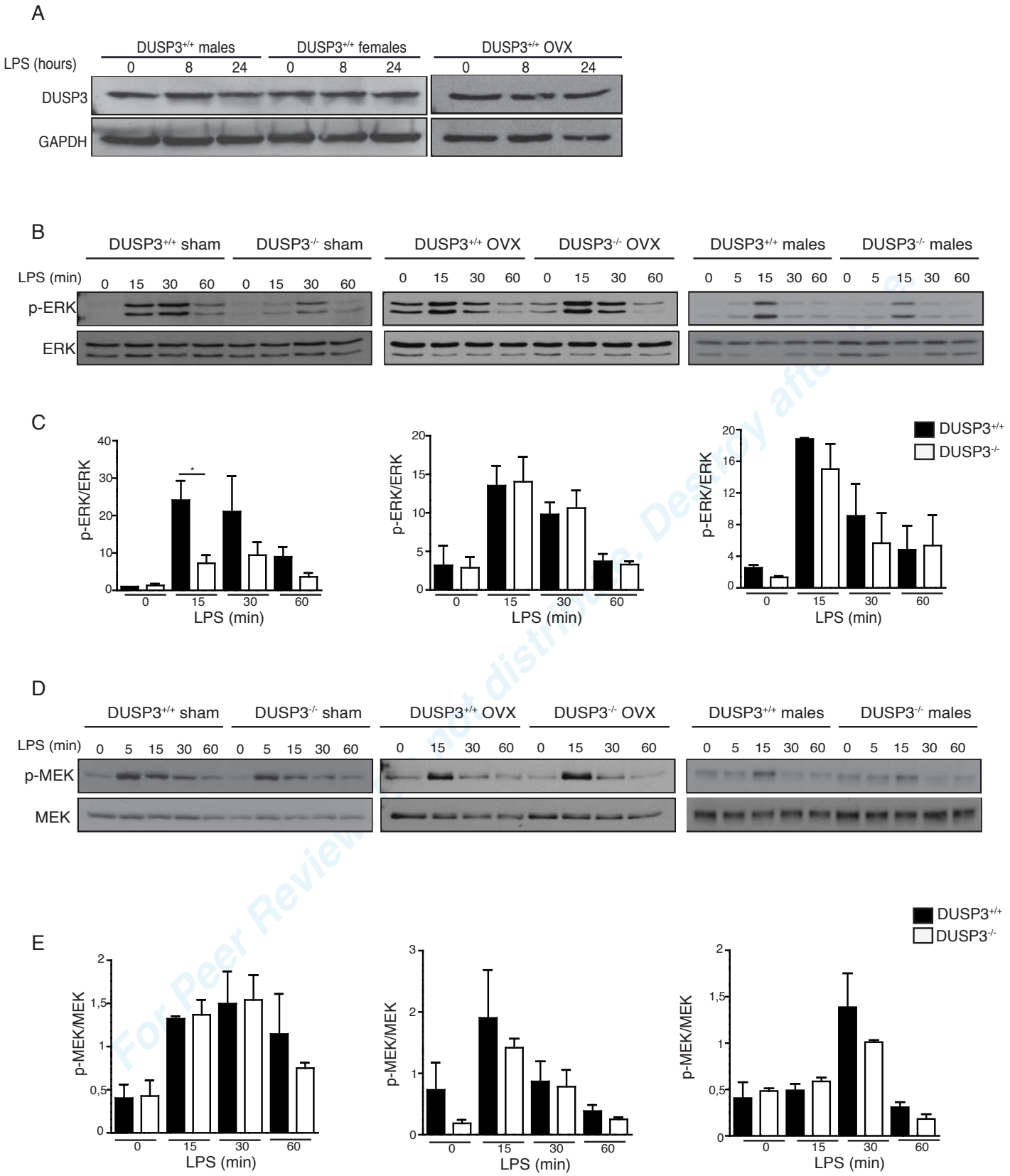

Figure 5

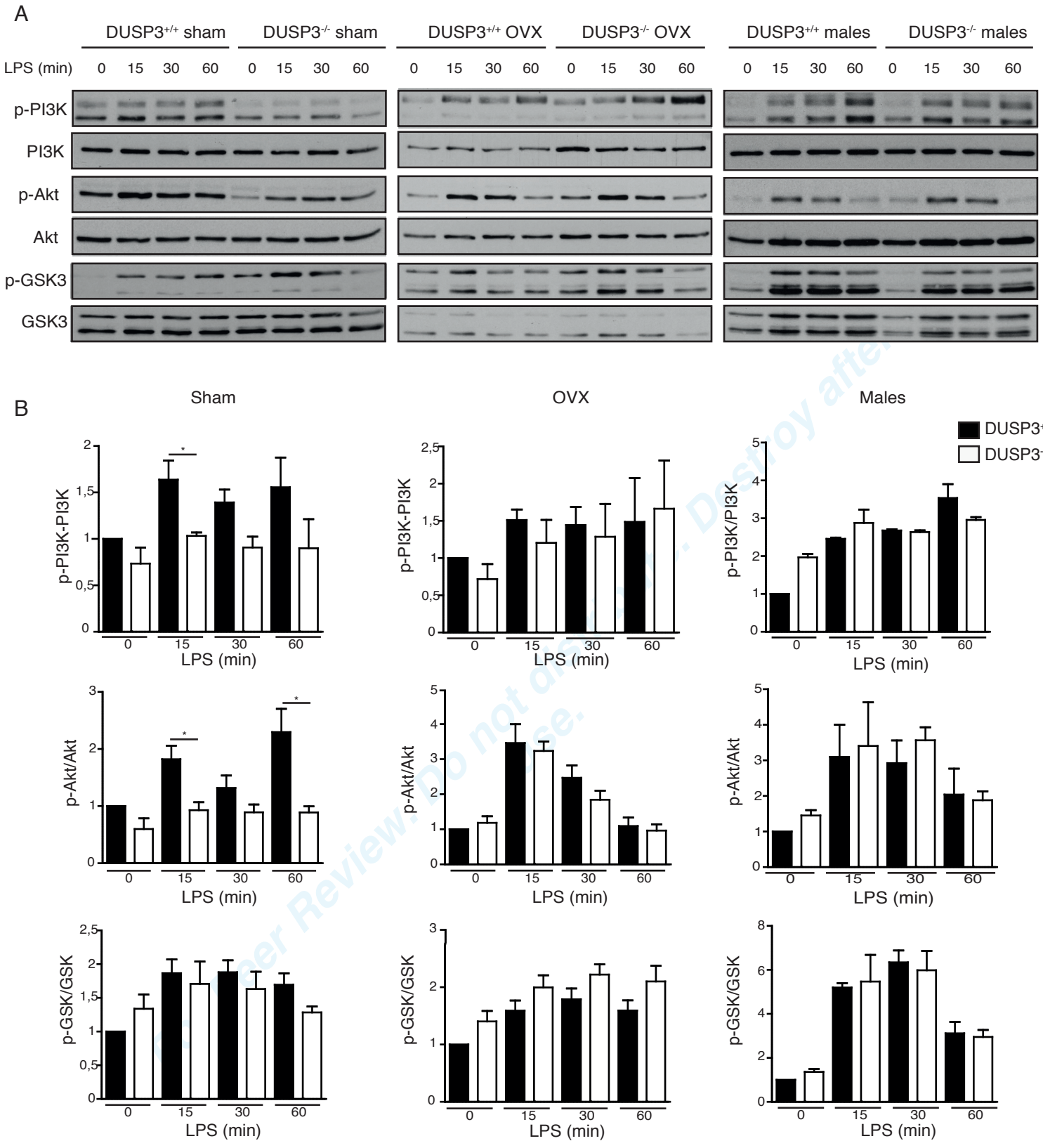

Figure 6