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Sexual dimorphism in cell behaviour and sensitivity to death of primary macrophages

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Summary. *Introduction.* Although males differ from females in their responses to viral and bacterial infections, whether cells of the immune system manifest intrinsic sex differences has not been examined. We therefore investigated the sex differences in viability of peritoneal macrophages when they were exposed to influenza A virus (WSN/33) or lipopolysaccharide (LPS) and evaluated the impact of androgens and estrogens on their responses. *Methods.* We extracted mouse peritoneal macrophages from male and female mice, exposed them to influenza A virus or bacterial lipopolysaccharides, measured cell death by trypan blue exclusion, and also assessed the impact of sex steroids on the ability of the macrophages to resist the virus. *Results.* Peritoneal macrophages differed by sex in their response to both influenza A virus and to LPS, with influenza killing 100% of male cells within 24 h but leaving approximately 15% of the female cells viable. Pretreatment with sex hormones, 17β-estradiol and testosterone, reduced cell death in both genders but did not alter the dimorphism. *Discussion.* We conclude that the sex-dependent response of macrophages to viral and bacterial infections derives from sex chromosomes rather than sex hormones. This sexual dimorphism should be taken into account for treatment of both viral and bacterial infections.

Key words: macrophages, sex dimorphism, influenza, cell death.

Dimorfismo sessuale nel comportamento cellulare e sensibilità alla morte dei macrofagi primari

Riassunto. *Introduzione.* Sebbene i maschi differiscano dalle femmine nelle risposte alle infezioni virali e batteriche, non è stato esaminato se le cellule del sistema immunitario manifestano differenze intrinseche tra i sessi. Abbiamo quindi studiato le differenze di sesso nella vitalità dei macrofagi peritoneali esposti al virus dell'influenza A (WSN / 33) o al lipopolisaccaride (LPS) e abbiamo valutato l'impatto degli androgeni e degli estrogeni sulle loro risposte. *Metodi.* Abbiamo estratto macrofagi peritoneali da topi maschi e femmine, li abbiamo esposti al virus dell'influenza A o a lipopolisaccaridi batterici, abbiamo misurato la morte cellulare con il test di esclusione del trypan blue e anche valutato l'impatto degli steroidi sessuali sulla capacità dei macrofagi di resistere al virus. *Risultati.* I macrofagi peritoneali differivano per sesso nella loro risposta sia al virus dell'influenza A che all'LPS, e l'influenza uccideva il 100% delle cellule maschili entro 24 ore, lasciando però circa il 15% delle cellule femminili vitali. Il pretrattamento con ormoni sessuali, 17β-estradiolo e testosterone, ha ridotto la morte cellulare in entrambi i sessi ma non ha alterato il dimorfismo. *Discussione.* Concludiamo che la risposta sesso-dipendente dei macrofagi alle infezioni virali e batteriche deriva dai cromosomi sessuali piuttosto che dagli ormoni sessuali. Questo dimorfismo sessuale dovrebbe essere preso in considerazione per il trattamento sia delle infezioni virali sia di quelle batteriche.

Parole chiave: macrofagi, dimorfismo sessuale, influenza, morte cellulare.

Introduction

Viral-bacterial coinfections in humans are well documented, with secondary bacterial pneumonia an important cause of influenza-associated death[1]. In both human and animals the sexes differ significantly in immune responses to several viral and bacterial infections[2-7]. Usually, females mount an enhanced immune response to viral infection, allowing them to better resist infections[8] but in female mice influenza A infection causes greater morbidity and mortality, suggesting that heightened response may also increase female susceptibility to immunopathology[3]. Also, males are more susceptible to several bacterial illnesses[5,7,9].

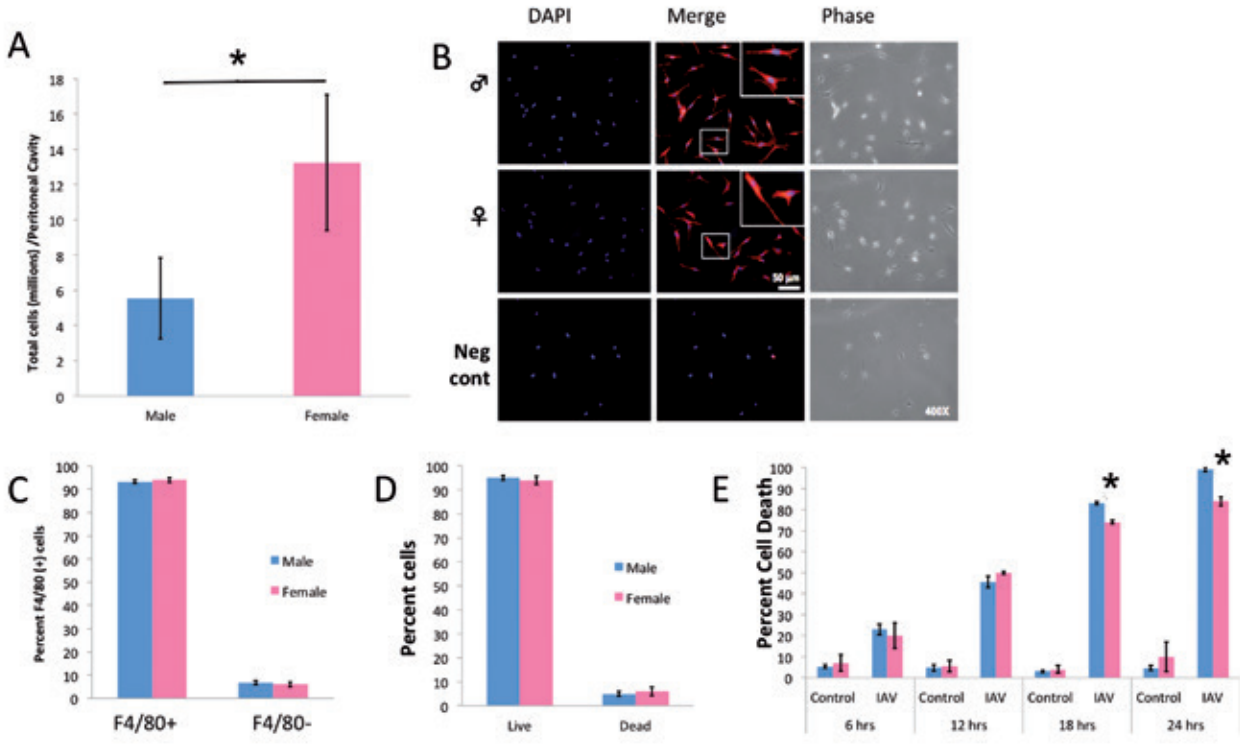
Some authors argue that the difference in susceptibility and response to viral and bacterial infections in males and females is related to both the effects of sex hormones and to gene expression on the X and Y chromosomes[4,10]. The importance of other involved factors is still obscure[3,4,11]. For instance, male and female cells *in vitro* respond differently to several stresses even in the absence of sex hormones. This difference possibly derives from different levels of DNA methylation[12,13]. Since males and females respond differently to viral and bacterial infections, further studies of cell-specific responses to specific viruses or bacteria are needed to better understand the sex dimorphism under these conditions.

We therefore examined how male and female mouse peritoneal macrophages survive in the presence of influenza virus and *E. coli* lipopolysaccharides (LPS). We further applied exogenous sex hormones to determine their impact on the responses of these cells to viral and bacterial infections (the latter mimicked by LPS). In both cases the sex difference persists, even in the presence of sex hormones.

Materials and methods

Macrophage extraction and cell culture

To attract macrophages to the peritoneum, we injected 1.0 ml of 5% thioglycollate media (catalog no. 0190C81, Thomas Scientific) into the intraperitoneal cavity of Swiss Webster male and female mice using a 1ml BD insulin syringe outfitted with a 28G, 1/2 inch (1.27 cm) needle. The mice were sacrificed by CO_2 and cervical dislocation on the 4th day following injection. One mouse at a time was placed on a clean sheet of aluminum foil and sprayed with 70% ethanol. A small incision was made in the center of the abdomen to expose the peritoneal cavity. Using a 5 ml syringe, we washed the peritoneal cavity with 5 ml of 1X sterile phosphate buffered saline (PBS), followed by additional 5 ml of PBS. The peritoneal cavity was massaged several times to increase the yield of macrophages. Using a 22 G 1 inch (2.54 cm) needle, we extracted 5 ml of fluid from the peritoneal cavity and transferred it to a 15 ml conical tube. This step was repeated 3 times for a total of 20 ml, which was then spun at 1500 x g for 10 minutes. The supernatant was discarded, and the pellet was resuspended in ice cold sterile distilled water to lyse red blood cells. The cells were spun again at 1500 x g for 10 minutes. The supernatant was discarded and the pellet was resuspended in RPMI medium 1640 (catalog no. 21870076, Gibco) supplemented with 10% FBS, and 1% penicillin-streptomycin. The cells were seeded at 4 x 10^5 per well into 35 mm plates with 22 x 22 mm sterile glass coverslips (catalog no. 2870-22, Corning). Cells were incubated at 37 °C in a humidified atmosphere with 5% CO_2 for 24 h prior to treatment.

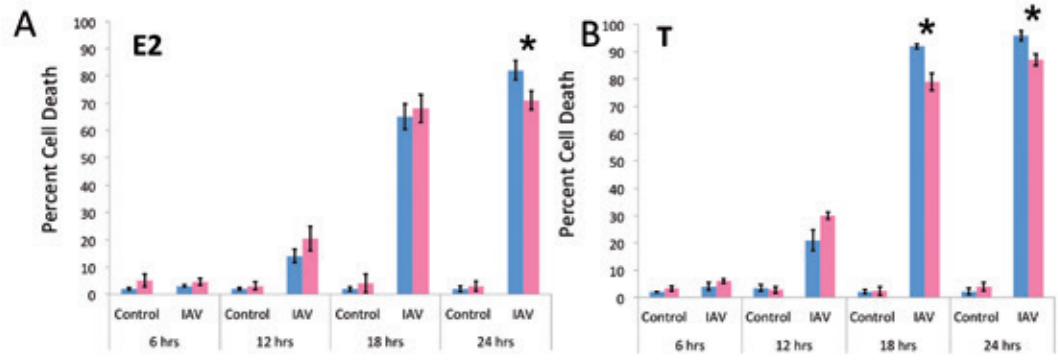


A. Differing yield of peritoneal extract cells. Male and female mice were matched in both age and weight. The amount of 1X PBS injected into the cavity to isolate cells was consistent between the sexes. Data represents 12 independent experiments for both male and female. Error bars represent standard error and significance is represented as asterisk (*) indicating P <0.05.
B. For immunocytochemistry, cells were stained with DAPI (blue) nuclear stain, and F4/80 (red). Insets show a 2-fold increase of magnification. Negative control illustrates that the red fluorescence is specific to the antibody.
C. Quantification of F4/80 positive cells from Figure 1B. Total nuclei were counted, followed by cells positive for F4/80 as indicated by an abundant red signal. Graphs represent data from three separate mice per sex. Asterisks (*) indicate (P <0.05). Over 90% of the cells collected are macrophages.
D. After harvest, cells were incubated in culture medium for 24 h, then incubated in trypan blue for 30 seconds. Graphs represent data from five or more experiments, each including three separate mice per sex. Asterisks (*) indicate (P <0.05). In extracted but otherwise undisturbed macrophages, mortality did not differ between sexes.
E. Primary macrophages were incubated in culture medium for 24 h, after which cultures were exposed to influenza virus for 6-24 h (WSN/33 MOI: 5). Male cells are more sensitive to influenza at 18 and 24 hours. Graphs represent data from five or more experiments, each representing three separate mice per sex. Asterisks (*) indicate (P <0.05).

Figure 1. Influenza virus induces sex dimorphic cell death.

Virus growth and determination of titer

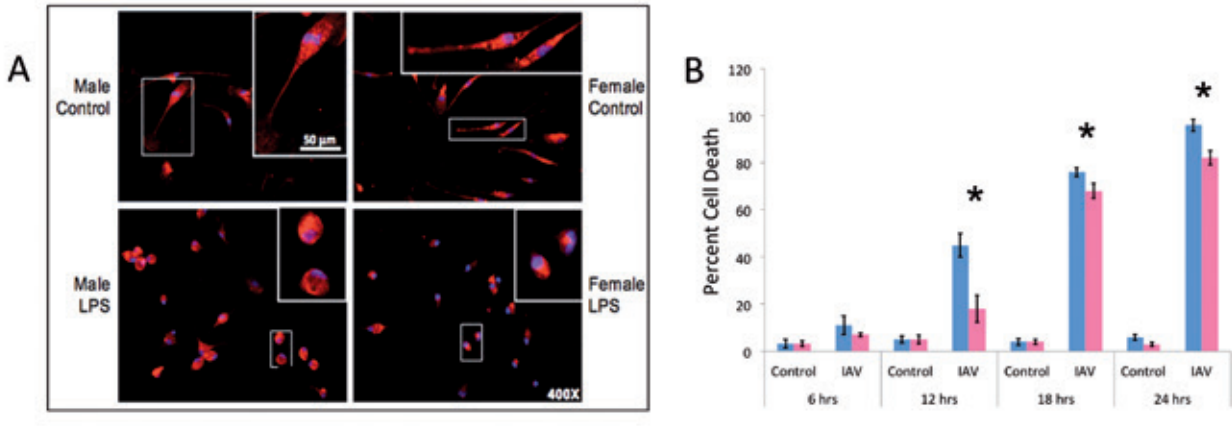
Influenza A/WSN/33 virus (gift of Dr Adolfo Garcia-Sastre, Mt. Sinai Medical School, New York) was cultured in 10-day-old specific-pathogen-free embryonated chicken eggs (Charles River SPAFAS, North Franklin, CT) for 2 days at 35 °C. Infected egg albumen was collected and cleared by centrifugation at 1,000 rpm for 10 min. The supernatant was collected and stored at −80 °C. Virus titer was determined by plaque assay, which was conducted as follows: MDCK cells were incubated overnight in DMEM containing 10% FBS and 1% penicillin-streptomycin. This incubation was followed by infection with 10-fold serial dilutions of virus suspension for 1 hour at room temperature. Cells were then covered with warmed Eagle's minimum essential medium (catalog no. 12-668E, Thermo Fischer, Waltham, Massachusetts) containing 0.1% DEAE-dextran (catalog no. D9885, Sigma-Aldrich) and 1% purified agar (catalog no. LP0028, Oxoid, Thermo Fischer). This agar medium was allowed to solidify at room temperature and incubated for 2 days at 37 °C to promote plaque development.



A. The same procedure as Figure 1E was used, except that a 24 h pre-treatment with 20nM E_2 was administered prior to exposure to influenza virus. Graphs represent data from five or more experiments, each including three separate mice per sex. Asterisks (*) indicate (P <0.05) when sexes are compared. E_2 provides a little protection, but the sex difference persists.

B. The same procedure as Figure 2A was used, except a 24 h pre-treatment with 20nM testosterone was administered prior to influenza virus. Compared to controls without hormone (Figure 1E), T does not offer protection, and neither hormone overcomes the sex differential. Graphs represent data from five or more experiments, each including three separate mice per sex. Asterisks (*) indicate (P <0.05) difference between sexes.

Figure 2. Sex hormone pretreatment prior to influenza exposure reveals sex dimorphism.



A. Following 24 h in culture media, cells were exposed to 2 µg/ml LPS for 24 h. For morphological analysis, cell cultures were stained with DAPI (blue) nuclear stain, F4/80 (red). Image acquisition performed using fluorescence microscopy using 40x objectives. Activated macrophages are more rounded than quiescent macrophages, and there is no difference in morphology between male and female cells.

B. Cells were exposed to pre-treatment with 2 µg/mL LPS 24 h prior to influenza virus. Controls are cells exposed to LPS but no virus; they manifest no difference in cell death from samples without LPS. Graphs represent data from five or more separate experiments involving three different animals for each sex. Compared to no activation (Figure 1), activation transiently improves survival, more effectively for females than males, so that the sex differential persists. Asterisks (*) indicate (P <0.05).

Figure 3. Effect of LPS on lethality of influenza virus.

Immediately prior to plaque analysis, the solidified agar was removed, and cells were fixed and stained with a methanol-crystal violet solution. Plaques were counted, and the virus titer was expressed as PFU/ml.

For experimental determinations of virus replication, cells were infected with influenza A/WSN/33 virus at an MOI (multiplicity of infection) of 0.01, and at 24-h intervals, cell supernatant samples were collected and plaques were assayed.

Culture and treatment of cells

Cells were allowed to adhere overnight on sterile glass coverslips. 24 h after plating, media was aspirated and cells were washed twice with 1x PBS. For the treatments, LPS (catalog no. L8274, Sigma) were dissolved in 1x PBS, and 17β-estradiol (E_2, catalog no. 250155, Sigma) or testosterone (T, catalog no. 58220, Sigma) was dissolved in DMSO. LPS and hormone solution were then added to warm RPMI media directly, resulting in a final concentration of 2 μg/mL LPS in RPMI and 20 nM for the hormones in RPMI when added to cells. The cells were then covered with warm RPMI media, or RPMI media with additives and then incubated for a further 24 h. After 24 h, media was aspirated and cells were washed twice with 1x PBS. The cells seeded at 4×10^5 cells per plate were exposed to influenza A virus at an MOI of 5 for 1 h at room temperature. Control cells were incubated in virus diluting media (VDM) for 1 h at room temperature. The cells were covered with RPMI containing the appropriate additives and incubated until ready for estimation of cell death by exclusion assay (below) at 6, 12, 18 and 24 h.

Assessment of cell death by trypan blue exclusion

The ability of influenza A to kill primary macrophages was assessed using a trypan blue exclusion assay. At the end of treatment, medium with floating cells was pelleted. The pellet was resuspended in 1x PBS and samples were mixed 1:1 with 0.4% trypan blue (catalog no. T8154, Sigma-Aldrich) and incubated at room temperature for 30 s. Cells were counted by use of a hemocytometer. Adherent cells on coverslips were incubated in 0.4% trypan blue for 30 s at room temperature. Cells were washed twice with 1x PBS and fixed with 4% paraformaldehyde for 10 min on ice. Fixed cells were mounted on slides with Fluoromount (catalog no. F4680, Sigma). The floating cells were counted separately and added to the total counts. Floating cells were encountered only at longer times. At least 200 cells (dead or alive) were counted under a microscope, and each treatment was conducted at least three times on three different samples. Blue cells were categorized as nonviable. Using appropriate multipliers, the total number of cells (floating and adhering) from each well was calculated. The percentage of cell death was calculated as 100 × the number of dead cells, divided by the total cell number. Statistical significance of the results was calculated by standard t test; values of $P < 0.05$ were considered significant.

Immunocytochemistry

For immuno-cytochemical analysis, 4×10^5 cells were seeded onto heat-sterilized glass coverslips. After overnight incubation at 37 °C, attached cells were carefully rinsed with 1× PBS. At 6 or 12 h, cells were fixed with ice-cold 4% PFA for 10 min on ice and rinsed with ice-cold 1× PBS. Nonspecific binding sites were blocked using 1× PBS containing 18 μg/ml bovine serum albumin before exposure to mouse F4/80 (C-7) (catalog no. sc-377009, Santa Cruz Biotechnology, Inc., Dallas, Texas). Cells were then rinsed three times in 1× PBS, followed by incubation with anti-mouse immunoglobulin G-Alexa Fluor 555 secondary antibody (catalog no. A11008, Invitrogen Waltham, Massachusetts) solution for 2 h. Cells were then rinsed twice with 1× PBS before staining nuclei with 4', 6'-diamidino-2-phenylindole (DAPI) (catalog no. D8417, Sigma). Stained cells were then mounted using Fluoromount (catalog no. F4680, Sigma) and visualized using a fluorescence microscope (Leica, Buffalo Grove, IL) and a confocal microscope (Leica, Buffalo Grove, IL).

Results

Influenza A virus induces sex dimorphic cell death

Peritoneal macrophages were extracted from male and female mice following thioglycollate stimulation as explained in Materials and Methods. As shown in Figure 1A (see p. 137), female mice yielded more than twice as many peritoneal cells compared to male mice. The morphology of male and female macrophages was the same and all were elongated, and approximately 90% of harvested and cultured cells were positive for the murine macrophage marker, F4/80 (Figures 1B, C). The percentage of macrophage survival over 48 h was similar for both genders (Figure 1D).

Male and female macrophages were challenged with 5 MOI, which is considered to be a lethal infection for macrophages exposed to influenza A virus, for 6, 12, 18 and 24 h. Cell death was assessed by the modified Trypan Blue Exclusion assay described in Material and Methods. Approximately 5% of male and female mock-treated cells were dead at any assessment time. For both sexes, the number of dead virus-exposed cells increases with time, but by 18 and 24 h the male cells are 10 to 15% more vulnerable. Most importantly, by 24 h, none of the male cells, but up to 15% of the female cells, had

survived (Figure 1E). We find no significant difference in the production of virus between male and female as determined by plaque assay (data not shown).

We tested whether sex hormones modulated this death by exposing the cells to sex hormones prior to the administration of virus. Macrophages from males and females were exposed to 20nM 17-β estradiol (E_2) or 20nM testosterone (T) for 24 h and then to influenza A virus at different indicated times before assessing cell viability. Neither E_2 nor T alone affected spontaneous cell death, compared to control without hormone, at any times. Pretreatment with E_2 for 24 h offers slight protection against influenza virus compared to the cells that were treated with influenza virus alone (killing reduced from 99% to 82% in male macrophages and from 84% to 71% in female macrophages). Nevertheless, the difference between male and female susceptibility persisted (Figure 2A, see p. 138). Pretreatment of male and female macrophages with testosterone for 24 hours transiently decreased cell death in both male and female cells compared to the cells that were treated by influenza virus alone, but after 24 h cell death was similar to the cells that were exposed to influenza virus alone (Figure 2B), again with the difference between male and female susceptibility persisting.

LPS activated macrophages show sex dimorphic response to influenza

Once they encounter and identify invading pathogens by virtue of cell-surface receptors, macrophages are activated, like other cells of the immune system[14]. They can also be activated by lipopolysaccharides from bacteria. We tested whether activation of macrophage through bacterial infection contributed to the macrophage sex dimorphism in response to influenza virus. Twenty-four hours after isolation, male and female macrophages were challenged with 2 μg/mL LPS for 24 h. The cell morphology was assessed by using immunocytochemistry. After LPS stimulation, the cells were fixed and stained with DAPI (blue, nuclear stain) and anti-F4/80 (red). LPS stimulated macrophages differed in morphology from untreated control cells. LPS stimulated cells are less elongated and dendritic, and are more rounded (Figure 3A, see p. 138). Male and female cells are similar in appearance.

We treated extracted cells with LPS and then exposed them to influenza virus. Male and female LPS stimulated macrophages were exposed to influenza A (MOI 5) and observed at 6, 12, 18 and 24 h following the same protocol as above. As in the other experiments, control cells exposed to only LPS exhibit less than 10% cell death throughout the experiment. Compared to cells without LPS activation, LPS activated female cells initially do better when infected with IAV, manifesting only 25% cell death at 12 hpi, whereas up to 50% of infected but originally quiescent female cells are dead by this time (Figure 1). In the presence of LPS, cell death provoked by influenza virus was similar in quantity at later times to that seen in the absence of LPS, and the sex differences persisted at all times. However, at 18 and 24 hours there was no significant difference between unactivated cells (Figure 1E) and LPS-activated cells (Figure 3B).

Discussion

Female macrophages are more resistant to cell death induced by influenza virus. Hormone pretreatment, and LPS pretreatment modestly affect, but do not completely abolish, this difference. As we present elsewhere (submitted), female macrophages more readily engage in phagocytosis than male macrophages, and female cells that have engaged in phagocytosis are more likely to contain more beads per cell than male cells under control and LPS conditions.

The difference in survival is only transient, reflecting the fact that the virus continues to replicate and ultimately kills the macrophages. Nevertheless, the difference presumably reflects an underlying difference in vulnerability that creates substantial differences under more modest stresses and may affect the dynamics of infection and recovery in men and women.

In an *in vivo* mouse model of influenza pathogenesis, E_2, which is anti-inflammatory through an interruption of nuclear factor kappa B (NF-kB) activity, increased female survival and decreased TNF-α and chemokine ligand 2 (CCL2) in lung homogenates after influenza exposure[4]. Our findings suggest that E_2 offers macrophages modest protection *in vivo* against influenza.

Macrophage activation with LPS modestly and transiently protects both sexes against influenza at 6 h in male and female cells, and a large dimorphism in male and female death is seen at 12 h with LPS pretreatment (Figure 3B). Female macrophage death is significantly lower at 12 h when LPS is presented to macrophages in addition to influenza. The reasons for this are unclear. The pathway from LPS to activation is known and, based on other lines of evidence, it is likely that the sex-based difference in response derives at least in part from differences in the cell surface receptors for LPS, most notably the TLR receptors. Very preliminary data from our laboratory suggests that the macrophages from males and females differ in the expression of the protein receptors. Even this observation, though, leaves open the question as to whether transcription, translation, post-translation modification, turnover, or binding or other modifications of receptors are responsible for the differences. This is a larger topic that our laboratory is currently assessing. Nevertheless, LPS activation prior to exposure to influenza does not affect the sex differential at 18 and 24 h.

This sex dimorphic cell sensitivity in activated macrophages is likely to be biologically important to the severity of disease caused by viral pathogens.

The sex dimorphisms of primary macrophages are important to recognize for both biological and clinical reasons. The differing biology by sex of the cells should be taken into account for all research purposes. Furthermore, our findings demonstrate that primary macrophages are a valuable model for the study of sex dimorphism. As an intimation of greater impact, our findings underscore the importance for clinicians to look for and monitor differences in disease progression and response to treatment between men and women.

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Key messages

- Although males differ from females in their responses to viral and bacterial infections, whether cells of the immune system manifest intrinsic sex differences has not been examined.
- Therefore the sex differences in viability of peritoneal macrophages when they were exposed to influenza A virus (WSN/33) or lipopolysaccharide (LPS) were investigated and the the impact of androgens and estrogens on their responses was further evaluated.
- Peritoneal macrophages differed by sex in their response to both influenza A virus (WSN/33) and to LPS, with influenza killing 100% of male cells within 24 h but leaving approximately 15% of the female cells viable.
- Pretreatment with sex hormones, 17β-estradiol and testosterone reduced cell death in both genders but did not alter the dimorphism.
- We conclude that the sex-dependent response of macrophages to viral and bacterial infections derives from sex chromosomes rather than sex hormones. This sexual dimorphism should be taken into account for treatment of both viral and bacterial infections.

Conflict of interest statement: the Authors declare no conflicts of interest.

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