

TLR4 sex dimorphism correlates with sex dimorphic phagocytosis in primary macrophages

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Summary. Men and women differ in their responses to viral infections and in autoimmune diseases. Isolated cultured cells are, in terms of sex, differentially sensitive to viruses. Here we pursue the question by evaluating the ability of primary macrophages collected from male and female mice to phagocytose fluorescent beads, and whether the cells differ in their expression of toll-like receptors (TLRs). In culture, cells isolated from female mice are substantially more aggressive than cells isolated from male mice: half as many female cells failed to phagocytose any beads; and, among those that succeeded, the modal number of beads phagocytosed was substantially higher. We evaluated the role of two TLRs for which specific inhibitors were available, TLR2 and TLR4. Under control conditions, male cells expressed more than twice as much TLR2, and the inhibitor suppressed production of the mRNA but did not affect either the number of cells that successfully phagocytosed beads or the distribution of the number of beads phagocytosed by an individual cell. Female cells produce approximately 30% more TLR4 mRNA; this differential is completely suppressed by the inhibitor, while male mRNA is unaffected. This inhibition affects phagocytosis, eliminating the differences in both the number of cells able to phagocytose and the higher number of beads that female cells are able to phagocytose. We conclude that TLR4 is an important component of the sex-differential response. Thus, one component of the sex differential in immune response, which is an important consideration in medicine, can be traced to a specific difference in the receptors that are responsible for mediating responses to PAMPs. Much more can be learned from pursuing such studies.

Key words. Macrophages, sex differential, TLR2, TLR4, phagocytosis.

Il dimorfismo sessuale del TLR4 è correlato alla fagocitosi dimorfa sessuale nei macrofagi primari

Riassunto. Uomini e donne differiscono nelle loro risposte alle infezioni virali e alle malattie autoimmuni. Anche le cellule isolate in coltura sono differenti a seconda del sesso di provenienza e sono sensibili ai virus in modo differente. In questo lavoro analizziamo la questione stabilendo la capacità dei macrofagi primari prelevati da topi maschi e femmine di fagocitare microsferine fluorescenti, e valutando altresì se le cellule differiscono o meno nell'espressione dei recettori toll-like (TLR, toll-like receptor). In coltura, le cellule isolate da topi femmine sono sostanzialmente più aggressive delle cellule isolate da topi maschi. Rispetto a quelle maschili, metà delle

cellule femminili non riusciva a fagocitare le microsferine; inoltre, tra quelle che vi riuscivano, il numero modale di microsferine fagocitate era significativamente più alto. Abbiamo valutato il ruolo di due TLR per i quali erano disponibili inibitori specifici, TLR2 e TLR4. In condizioni controllate, le cellule maschili hanno espresso più del doppio di TLR2, e l'inibitore specifico sopprimeva la produzione dell'mRNA ma non influenzava né il numero di cellule che erano riuscite a fagocitare le microsferine, né la distribuzione del numero di microsferine fagocitate da una singola cellula. Le cellule femminili producono circa il 30% in più di mRNA per il TLR4; questa differenza viene completamente soppressa dall'inibitore, mentre l'mRNA maschile non viene influenzato. Questa inibizione influisce sulla fagocitosi, eliminando le differenze sia nel numero di cellule in grado di fagocitare sia nel più elevato numero di microsferine che le cellule femminili sono in grado di fagocitare. In conclusione, possiamo affermare che il TLR4 è un componente importante della differente risposta osservata tra maschi e femmine. Pertanto, un elemento delle differenze tra i sessi nella risposta immunitaria – molto importante in medicina – può essere ricondotta a una differenza specifica nei recettori responsabili delle risposte ai PAMPs (*pathogen-associated molecular patterns*). Saranno necessari ulteriori studi per capire meglio questi meccanismi.

Parole chiave. Macrofagi, differenze tra i sessi, TLR2, TLR4, fagocitosi.

Introduction

Many diseases are more prevalent in one gender than the other, and the underlying causes for the sex dimorphism are not understood. These diseases include autoimmune disorders such as multiple sclerosis and systemic lupus erythematosus (higher prevalence in females) and Guillain Barré syndrome, fibrosis and psoriasis (higher in males).¹ In comparison to females, males are more susceptible to a broad range of fungal, bacterial, and viral systemic and respiratory infections.² Female mice also resist infection better than males, perhaps due to differing basal redox conditions.³ Sex hormones may potentially enhance immune response in females through cell activation and proliferation but the mechanisms by which sex hormones might affect the response remain unclear.⁴

We have previously documented sex differences at the cellular level,^{5,6} and shown that male primary murine macrophages are more easily killed by influenza virus.⁷ Here we examine the mechanisms behind the higher survival of female macrophages. Based on microscopic observations, we considered that female cells might more effectively phagocytose virus. Here we examine the ability of male and female cells to phagocytose fluorescent beads. We find that male and female macrophages differ in their handling of foreign material, even in the absence of sex hormones. If this is true as well for pathogens and cell debris, the difference might relate to the difference between male and female response to inflammatory disease. Dendritic cells, macrophages, B lymphocytes and T lymphocytes all participate in the immune response. Here we examine the readily available peritoneal derived primary macrophages, which engulf bacterial, fungal and viral components.⁸

Macrophages respond to specific proinflammatory cytokines⁹ by activation, and they present antigens to naïve T lymphocytes, enabling activation of the latter via expression of major histocompatibility complex II (MHC) molecules on the surface of macrophages. The expression of these molecules in macrophages is reported to be sex dimorphic.¹⁰

Macrophages express 12 toll-like receptors (TLR), pattern recognition receptors that recognize various microbial and viral molecules known as pathogen-associated molecular patterns (PAMPs). For example, TLR-4 expression on macrophages recognizes LPS expressed on gram (-) bacteria, TLR-2 gram (+) bacteria and yeast, and TLR-3 double-stranded viral RNA.¹⁰ TLR7 recognizes single-stranded RNA, TLR6 recognizes peptidoglycans and zymosan, and TLR9 recognizes unmethylated CpG DNA. TLRs1, 2, 4, 5, 6 are located on the surface where they recognize pathogenic components, whereas TLRs3, 7, 8, and 9 are found in endosomes where they recognize pathogenic nucleic acids.¹⁰ These toll-like receptors induce the release of cytokines such as TNF-alpha and IL-6, and chemokines such as CCL2. Female macrophages exhibit greater TLR expression in response to infection such as sepsis compared to their male counterparts.¹¹ High TLR8 in male monocytes may be related to high IL-10, while TLR9 may be modulated by sex hormones.¹¹ The mechanism is as follows: lipopolysaccharide binding protein forms a complex with lipopolysaccharide found on gram-negative bacteria. LBP:LPS complex transfers LPS to CD14 on the surface of macrophages. LPS-bound CD14 receptor interacts with TLR4 and induces downstream signaling.¹² Activation of TLR4 results in recruitment and activation of adaptor proteins such as MyD88 and TRIF. These adaptor proteins trigger downstream cell-signaling pathways resulting in production of cytokines and chemokines, which enable the initiation of an immune response.¹³ In this study we examine the ability

of macrophages derived from male and female mice to phagocytose fluorescent beads and how this ability is affected by inhibition of either TLR2 or TLR4.

Material and methods

Macrophage extraction and cell culture

Swiss Webster male and female mice were injected 1 ml of 5% thioglycolate media (Thomas Scientific, Cat. # 0190C81,) directly into the intraperitoneal cavity with a 1 cc BD insulin syringe outfitted with a 28G, 1/2 inch needle. The treated mice were sacrificed by CO₂ and cervical dislocation on the 4th day following injection. Mice were individually placed on clean sheet of aluminum foil and sprayed with 70% ethanol. The peritoneal cavity by a small slit with a scalpel. Ten ml of 1X sterile Phosphate Buffered Saline (PBS) was injected into the peritoneal cavity, in two injections separated by approximately 2 minutes. The peritoneal cavity was massaged several times to increase the yield of macrophages. Using a 22G, 1 inch needle, 5 ml of fluid was extracted from the peritoneal cavity and transferred to a 15 ml conical tube. This step was repeated 3 times to maximize extraction of cells, yielding 20 ml, which was collected and spun at 1500 g for 10 minutes. The supernatant was discarded, and the pellet was resuspended in RPMI medium 1640 (Gibco Cat. # 21870076,) supplemented with 20% FBS (Fetal Bovine Serum) 1% 100 U/ml penicillin, and 100 µg/ml streptomycin. The cells were seeded at 4 x 10⁵ into 35 mm plates with 22 x 22 mm sterile glass coverslips (Corning # 2870-22,). Cells were incubated at 37 °C in a humidified atmosphere with 5% CO₂ for a maximum of 24 hrs.

Treatments

Cells were allowed to adhere overnight after plating them on sterile glass coverslips. Twenty-four hrs after plating, media was aspirated and cells were washed twice with 1x PBS. The cells were then covered with either warm RPMI (Roswell Park Memorial Institute) media (control), RPMI media with 10 µm TLR4 (TLR4 inhibited cells) or RPMI with 5 µm TLR2 (TLR2 inhibited cells). The TLR4 inhibitor was diluted to 10 µm from 10 mm TLR4 inhibitor (Sigma, 40592-88-9) stock solution in RPMI. The TLR2 inhibitor was diluted to 5 µm from 5 mm TLR2 stock solution (Tocris, 1416324-85-0) in RPMI. The cells were covered with RPMI with treatments and incubated until ready for phagocytosis assay at 12 hrs.

Immunocytochemistry

For immunocytochemical analysis, 4 × 10⁵ cells were seeded onto heat-sterilized glass coverslips. After overnight incubation at 37 °C, attached cells were carefully

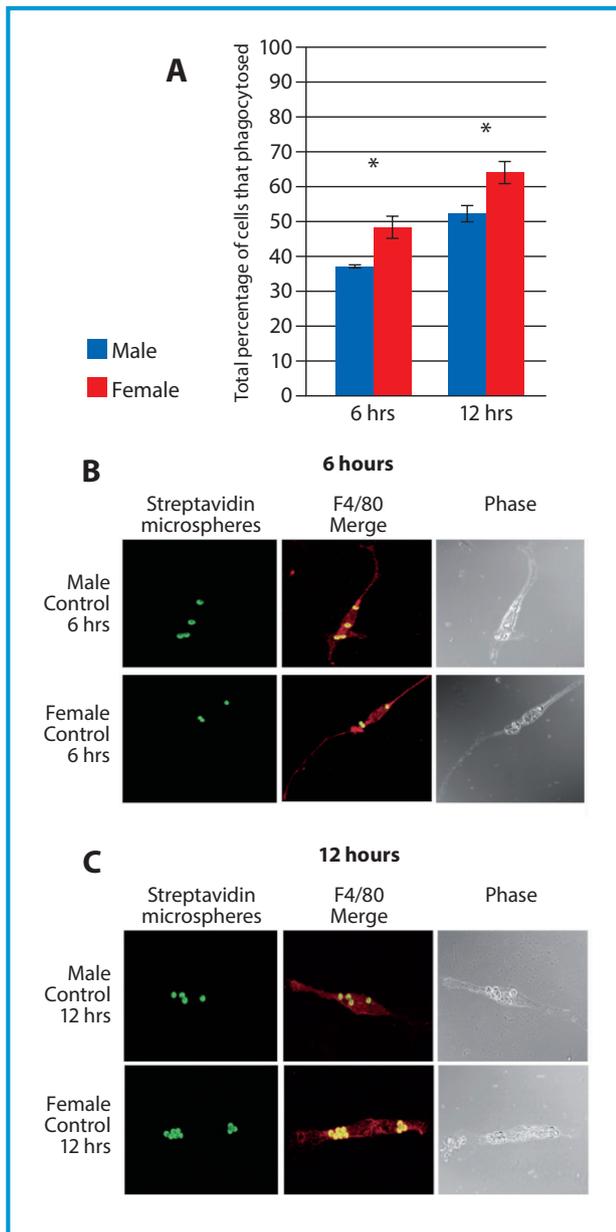


Figure 1. Phagocytosis of microspheres is sex dimorphic in primary macrophages at basal levels.

Primary macrophages were incubated in culture medium for 24 hrs, after which these cultures were exposed to virus diluting media for 1 h (to make the experiments fully compatible with other experiments, not reported here, involving infection with influenza virus) and then RPMI was added, and they were incubated in the presence of green streptavidin microspheres for 6 and 12 hrs. Microspheres phagocytosed (between 1 to 10) were determined by fluorescence in images in which macrophages were identified by F4/80 antibody (red) and engulfed beads were identified by the superimposition of their green fluorescence over the red of the macrophage, resulting in a yellow color in a z-stack of images from a confocal microscope. 1A. The number of cells that phagocytosed were counted and graphed in percentages. B and C. Cells were stained with 1:250 mouse anti-F4/80 antibody and 1:1000 anti-mouse IgG- AlexaFluor 555 as the secondary antibody. Cells were observed by confocal microscopy at 6 hrs (B) and 12 hrs (C). Male and female phagocytosis is sex dimorphic at 6 and 12 hrs. The graph represents experiments performed in triplicates. Asterisks (*) represent p-values of < 0.05.

rinsed with 1× PBS and treated as described above. The cells were then incubated with media and streptavidin YG (yellow-green) fluorescent latex beads 2 μm in size. For the rate of phagocytosis, cells were offered 5 latex beads per cell, directly added to cells with warm RPMI media and the preparation was incubated at 37 °C. Six or 12 hrs later, 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 immunoblotting with mouse F4/80 (C-7) (Santa Cruz Biotechnology # SC-377009). Cells were then rinsed three times in 1× PBS, followed by incubation with anti-mouse immunoglobulin G-Alexa Fluor 555 secondary antibody (Invitrogen # A11008,) solution at 1:1000 for 2 hrs. Cells were then rinsed twice with 1× PBS before staining nuclei with 4',6'-diamidino-2 phenylindole (DAPI) (Sigma #. D8417). Stained cells were then mounted using Fluoromount (Sigma # F4680) and visualized using a fluorescence microscope (Leica, Germany).

RNA extraction and qRT-PCR

The supernatant was aspirated and the cells were washed with 1X PBS. RNA was extracted from the cells using the GenElute Total RNA Purification Kit (Sigma # RNB100) according to the manufacturer's protocol. After RNA was collected, it was quantified using the NanoDrop2000 (Thermo Fisher Scientific, Waltham, MA, USA) and RNA concentration was adjusted to 1 μg/ml. The PCR was performed using Power SYBR™ Green RNA-to-Ct™ 1-Step Kit (for 1-step virus detection and gene expression).

Results

Phagocytosis is sex dimorphic at basal levels

Male and female cells were incubated with 5 microspheres per cell for 6 and 12 hrs. The number of the microspheres engulfed by these cells was assessed using fluorescence microscopy. We confirmed that microspheres were actually phagocytosed by male and female cells by observing cells by confocal microscopy. We utilized Z plane stacking to examine microspheres being phagocytosed. The streptavidin microspheres, which fluoresce green, appear yellow when they are inside red-fluorescing (F4/80) macrophages. The images acquired were developed by superimposing Z plane stacks (Figures 1A-C). The varying numbers of microspheres phagocytosed were grouped as 1, 2-5, 6-10, and >10 per cell as counted using fluorescence microscopy. Under control conditions by both 6 and 12 hrs, approximately 25% more female cells had ingested one or more beads than had male cells (Figure 2). At 6 hrs, there were sex differences in phagocytosis under control conditions with male macrophages engulfing fewer

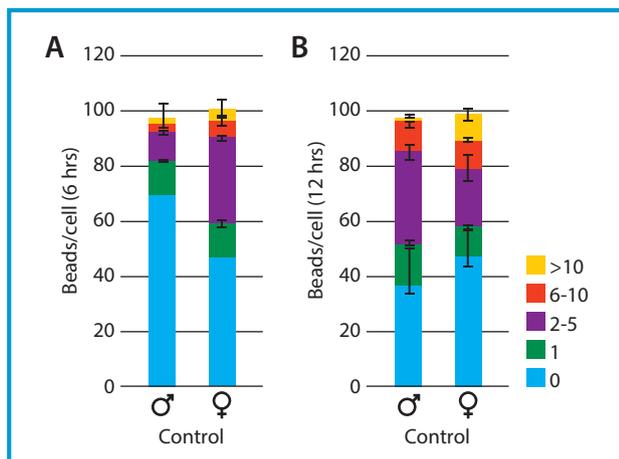


Figure 2. Sex dimorphism in phagocytosis of varying numbers of microspheres at 6 and 12 hrs.

Primary macrophages were incubated in culture medium for 24 hrs, following which they were exposed to VDM for 1 hrs and then RPMI was added. The cultures were incubated in streptavidin microspheres for 6 (A) and 12 (B) hrs. Cells were stained with 1:250 mouse anti-F4/80 antibody and 1:1000 anti-mouse IgG- AlexaFluor 555 as the secondary antibody. The varying number of microspheres phagocytosed was determined by fluorescence microscopy. Male and female cells phagocytosis is sex dimorphic under normal conditions. Males phagocytose fewer beads per cell (modal class 1 microsphere/cell) compared to the female cells, which phagocytosed more beads per cell (modal 2-5 beads/cell). The graph represents experiments performed in triplicates.

microspheres per cell than females, with the difference predominantly in cells phagocytosing 2-5 beads (Figure 2A). By 12 hrs the difference persisted, with substantial numbers of female but not male cells having engulfed >10 beads (Figure 2B).

Under control conditions, males express more TLR2 than females

We examined whether this difference could be explained by toll-like receptors. Since the difference was consistent but increased by 12 hrs, we focused on 12 hrs exposure to the beads. We estimated the production of TLR receptors by using qRT-PCR for cells 12 hrs post treatment, using cycle number to calculate the amount of TLR2 mRNA relative to β -tubulin as a housekeeping control (Figure 3A). The inhibitor suppresses expression of TLR2 by 80-90% on a linear scale relative to tubulin. In the inhibited state, the relatively higher expression in males (almost 3:1) is reduced to approximately 10% more than in females.

Inhibiting TLR2 does not affect phagocytosis of beads

Inhibition of TLR2 increases the variability of the number of female cells actively phagocytosing but otherwise has little effect on this parameter (Figure 3B). Likewise, the sex difference disappears, primarily because of the

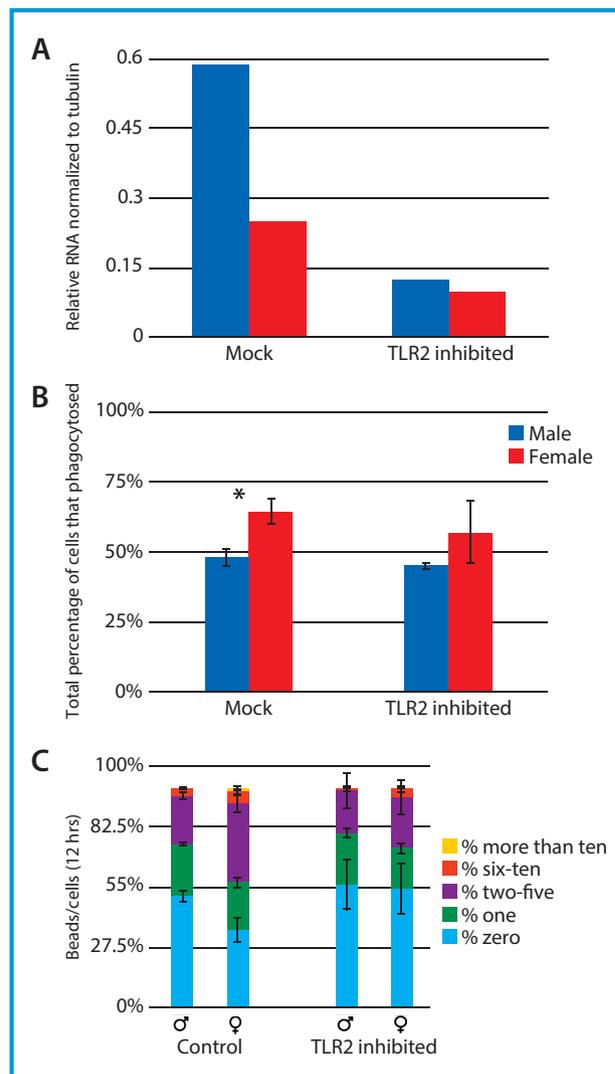


Figure 3. At baseline culture values, male cells express more TLR2 than female cells.

Total RNA was collected from primary macrophages treated as described in Material and Methods and qRT-PCR was performed for TLR2. The amount of RNA message, calculated from CT values, was expressed relative to β -tubulin, also calculated from CT. TLR2 can be suppressed 60-80%, with the sex differential nearly disappearing (A). Primary macrophages were incubated in cultured medium for 24 hrs, following which control cells exposed to virus diluting media for 1 hrs and then RPMI was added. Experimental cells were exposed to 5 mM solution of TLR2 inhibitor dissolved in RPMI for 12 hrs and then incubated in the presence of streptavidin microspheres for a further 12 hrs. The number of microspheres phagocytosed by fluorescence-positive macrophages was determined as above. Male and female phagocytosis is sex dimorphic at 12 hrs. After TLR2 was inhibited total percent phagocytosis did not change in either sex although the increased variability among female cells eliminated the statistical difference (B). The varying number of microspheres phagocytosed was determined by fluorescence microscopy (C). Males cells phagocytose fewer beads per cell (modal 1 bead/cell among active cells) compared to the female cells, which phagocytosed modal 2-5 beads per cell. After inhibition of TLR2 this pattern did not change. The graph represents experiments performed in triplicates. Asterisks (*) represent p-values of < 0.05.

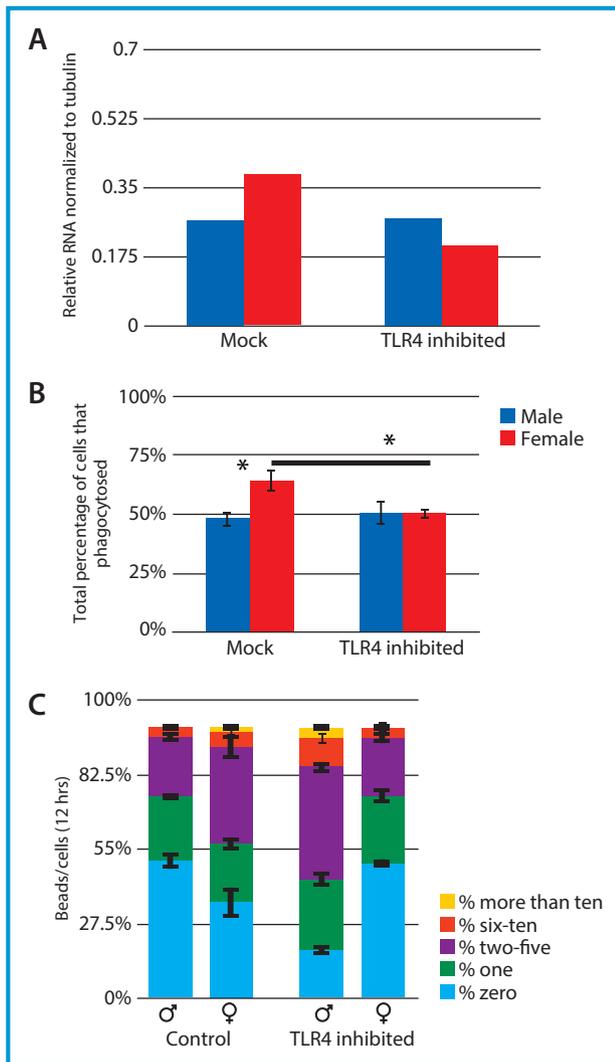


Figure 4. At basal levels, female cells express more TLR4 than male cells and its inhibition abolishes sex dimorphic phagocytosis.

Total RNA was collected from primary macrophages treated as described in Material and Methods and qRT-PCR was performed for TLR4. Female cells produced more TLR4 relative to tubulin than did male cells (A). The inhibitor did not affect the TLR4 mRNA in male cells, but in female cells reduced it nearly 50%, lower than in male cells. Primary macrophages were incubated in culture medium for 24 hrs, following which the control cells were exposed to virus diluting media for 1 hr and then RPMI was added. Experimental cells were exposed for 12 hrs to 10 mM solution of TLR4 inhibitor dissolved in RPMI. These cultures were incubated in the presence of streptavidin microspheres for 12 hrs. Microspheres phagocytosed were determined by z-stack of confocal images in which macrophages were confirmed by antibody. The number of cells that phagocytosed, and the number of beads per cell, were determined. Male and female phagocytosis is sex dimorphic at 12 hrs, with almost 20% more female cells ingesting beads than male cells. When TLR4 was inhibited, this differential disappeared by loss of the female advantage (B). The number of microspheres phagocytosed per cell was determined by fluorescence microscopy (C). Male cells phagocytose fewer beads per cell (modal 1 microsphere/cell) compared to the female cells, for which the modal number was 2-5 beads/cell. In the presence of TLR4 inhibitor, males phagocytose more beads per cell (2-5 beads/cell) whereas females phagocytose fewer beads per cell (1 bead/cell). The graph represents experiments performed in triplicates. Asterisks (*) represent p-values of < 0.05.

decrease in number of female cells phagocytosing, but the number of beads ingested per active cell is relatively unaffected by inhibition of TLR2 (Figure 3C).

Females have higher TLR4 levels compared to males in mock

In control conditions (mock) female cells produce more TLR4 mRNA (Figure 4A, left). When we add TLR4 inhibitor, this difference is eliminated, primarily by suppression of the higher female production (Figure 4A, right), which is reduced approximately 50%, while male production of TLR4 mRNA is barely changed (Figure 4A).

Inhibiting TLR4 abolishes sex dimorphic phagocytosis

The TLR4 inhibitor abolishes the sex differential in number of cells actively phagocytosing, exclusively by reducing the number of female cells actively phagocytosing (Figure 4B). This difference reduces the number of female cells ingesting 2-5 beads. The number of male cells ingesting beads becomes more variable (Figure 4C).

Discussion

There exists no clear explanation why, on a statistical basis, men and women differ in their responses to seemingly similar viral infections and in their manifestations of diseases presumed to be autoimmune in origin. We have attempted to trace these differences to the fundamental differences between male and female cells, their chromosomal differences. We have previously shown that isolated cultured cells are, in terms of sex, differentially sensitive to viruses, and that this difference depends on more than the hormonal milieu from which they came. Here we pursue the question by evaluating the ability of primary macrophages collected from male and female mice to phagocytose fluorescent beads, and we examine whether the cells differ in their expression of toll-like receptors (TLRs), which are important receptors for phagocytosis.

Macrophages isolated by peritoneal lavage and placed in culture are undoubtedly stimulated but represent our closest approach to a control condition. In this type of culture, cells isolated from female mice are substantially more aggressive than cells isolated from male mice. Half as many female cells failed to phagocytose any beads; and, among those that succeeded, the modal number of beads phagocytosed was substantially higher (because of the limitations of resolving beads, we could not establish a precise count when cells had engulfed more than 5 beads.)

We pursued this difference by evaluating two TLRs for which specific inhibitors were available, TLR2 and TLR4. For both of these receptors, we applied the in-

hibitor and measured the response both by measuring production of the appropriate mRNA and by evaluating how well the inhibited cells phagocytosed beads. Under our control conditions, male cells expressed more than twice as much TLR2, and the inhibitor suppressed production of the mRNA to approximately 20% of the male level, and to nearly equal amounts for both sexes. The TLR4 inhibitor blocks a coreceptor, MD2, by binding to the hydrophobic pocket. The TLR2 inhibitor specifically blocks the dimerization of TLR 1/2. Other than currently-unexplained feedback, we have no explanation for the suppression of mRNA expression. Likewise, we found no reports concerning sex hormone dependence of TLR 2 or 4. In Coxsackie-B virus-infected cardiac tissue, TLR2 expression is higher in females while TLR4 is higher in females,¹⁴ but the relevance of these findings to ours in macrophages is unclear. However, the inhibitor failed to affect the number of cells of either sex that successfully phagocytosed beads and likewise failed to affect the distribution among the cells of the number of beads phagocytosed by an individual cell. We conclude that TLR2, though more expressed in male cells, does not contribute to the sex differential.

The situation was different for TLR4. Female cells produce approximately 30% more mRNA for this receptor, and the inhibitor differentially suppresses female production so that, in the presence of the inhibitor, male cells are barely affected while female mRNA decreases to approximately 75% of the male level, equivalent to 50% of the level of mock-infected cells. This inhibition affects phagocytosis, eliminating the 33% greater number of female beads able to phagocytose at all and likewise eliminating the higher number of beads (estimated as modal number) that female cells are able to phago-

cytose. In both means of counting, the differentially greater ability of female cells is eliminated with the female responses being reduced to the level of the male cells. We conclude that TLR4 is an important component of the sex-differential response.

This of course is far from a complete story, but this study documents that one component of the sex differential in immune response, which is an important consideration in medicine, can be traced to a specific difference in receptors that are responsible for mediating responses to PAMPs. Much more can be learned from pursuing such studies.

Conclusions

1. Female macrophages can phagocytose more beads than their male counterparts for control conditions.
2. Female macrophages also phagocytose higher number of beads per cell than male macrophages.
3. At basal levels female macrophages express higher TLR4 levels than males, leading us to conclude that the higher TLR4 can explain the higher levels of phagocytosis since TLRs are important for recognizing foreign materials.
4. Inhibition of TLR4 removes the sex dimorphic phagocytosis by lowering the levels of phagocytosis in females, leading us to conclude that TLR4 is necessary for the sex dimorphic phagocytosis pattern.
5. At basal levels males express more TLR2 but inhibition of TLR2 did not result in change in phagocytosis, leading us to conclude that since inhibition of TLR2 doesn't affect sex dimorphic phagocytosis TLR2 does not contribute to differential phagocytosis.
6. Here we demonstrate a difference in behavior of primary murine macrophages to the same stimuli based on their gender.

Key messages

- More freshly isolated peritoneal macrophages from females can phagocytose beads and each cell can also phagocytose more beads than can macrophages from male mice.
- Cells from males express more TLR2, known mostly for recognizing gram positive bacteria, but inhibition of TLR2 alters neither the number of active phagocytosing cells nor the number of beads phagocytoses, indicating TLR2 does not contribute to the sex difference in phagocytosis.
- Macrophages from females express more TLR4, known mostly for recognizing gram negative bacteria, than males.
- Inhibition of TLR4 decreases phagocytosis in cells taken from females, eliminating the sex differential.
- We conclude that TLR4 is necessary for the sex dimorphic phagocytosis pattern.

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Author contribution statement: all the Authors have contributed to the manuscript, and all have reviewed and approved the final copy.

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

Ethic statement: the research has been approved by all relevant animal care and safety committees.

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