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CUTTING EDGE

Cutting Edge: Cross-Regulation by TLR4 and T cell Ig Mucin-3 Determines Sex Differences in Inflammatory Heart Disease[1]

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***Recent clinical studies have reinforced the importance of sex-related differences in the pathogenesis of cardiovascular diseases, with an increased incidence and mortality in men. Similar to humans, male BALB/c mice infected with coxsackievirus B3 (CVB3) develop more severe inflammation in the heart even though viral replication is no greater than in females. We show that TLR4 and IFN-γ levels are significantly elevated and regulatory T cell (Treg) populations significantly reduced in the heart of males following CVB3 infection, whereas females have significantly increased T cell Ig mucin (Tim)-3, IL-4 and Treg. Blocking Tim-3 in males significantly increases inflammation and TLR4 expression while reducing Treg. In contrast, defective TLR4 signaling significantly reduces inflammation while increasing Tim-3 expression. Cross-regulation of TLR4 and Tim-3 occurs during the innate and adaptive immune response. This novel mechanism may help explain why inflammatory heart disease is more severe in males. The Journal of Immunology,* 2007, 178: 6710–6714.**

The incidence and severity of heart disease, including myocarditis, is higher among men (1, 2). Coxsackievirus B (CVB)[3] infection is commonly associated with myocarditis (2). Although myocarditis is increased in men, rates of infection with CVB are similar between men and women worldwide (3–5). The lower incidence of heart disease in women has been attributed to the cardioprotective effects of estrogen (6). Previously, we reported that gonadectomy of male mice reduces myocarditis, indicating that testosterone increases inflammation in the heart (7). In contrast, STAT4-induced IFN-γ was not found to increase inflammation in males (8). In this study, we further examine mechanisms that increase inflammation in the heart of male mice.

In the present investigation, an isolate of coxsackievirus B3 (CVB3) originally obtained from a patient was passaged through the heart of BALB/c mice to produce a viral stock that contains cardiac myosin and virus (9). Acute myocarditis develops from days 7 to 14 postinfection (p.i.) and progresses to chronic myocarditis and dilated cardiomyopathy from day 28 p.i. (9). Previously we showed that TLR4 signaling increases myocarditis and IL-1β/IL-18 levels in the heart following CVB3 infection in male BALB/c mice (10), whereas inflammation is controlled by T cell Ig mucin (Tim)-3 signaling and regulatory T cell populations (Treg) (11). However, it was unknown whether communication occurred between these two receptors.

Materials and Methods

Mice

Male and female BALB/cJ (BALB/c), male TLR4 defective (C.C3-Tlr4$^{Lps\text{-}d}$/J) or male IFN-γ deficient (C.129S7(B6)-Ifng^{tm1Ts}/J) mice (6 to 8 wk old) (The Jackson Laboratory) were inoculated i.p. with 10^3 PFU of a heart-passaged stock of CVB3 (Nancy strain; American Type Culture Collection) and examined at 6 h, 12 h, or day 2 p.i. (innate response) or days 8 or 12 p.i. for acute myocarditis. Male BALB/c mice received one i.p. injection of 100 μg of anti-Fc (clone 93; eBioscience) to reduce nonspecific binding and either 100 μg of anti-Tim-3 (clone 8B.2C12; eBioscience) or control IgG1 (catalog no. 16-4301; eBioscience) on day 0 of infection, as described previously (11). Mice inoculated i.p. with heart homogenate from uninfected mice did not develop myocarditis (data not shown). Mice were maintained under pathogen-free conditions in the animal facility at Johns Hopkins University School of Medicine, and approval was obtained from the Animal Care and Use Committee of the Johns Hopkins University for all procedures.

Plaque assay and cytokine measurement

Hearts were homogenized at 10% w/v in 2% MEM and individual supernatants used in plaque assays to determine the level of infectious virus (10). IL-4 and IFN-γ levels were examined using Quantikine ELISA kits (R&D Systems) according to manufacturer's instructions, as previously (10).

FACS analysis

Mast cells (MC), macrophages, and lymphocytes were separated from heart cells using anti-CD117, anti-FITC, or anti-CD45 paramagnetic beads on a

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[3] Abbreviations used in this paper: CVB3, coxsackievirus B3; DC, dendritic cells; Foxp3, forkhead box p3; MC, mast cells; p.i., postinfection; Tim-3, T cell Ig mucin-3; Treg, regulatory T cells.

magnetic column (Miltenyi Biotec) (11). In innate studies, pooled cells were FACS sorted using a FACS Aria cell sorter (BD Biosciences) obtaining approximately 85% purity (data not shown). Gates were set on pure populations. Cells were stained with the following mAbs (eBioscience) diluted in 1% FBS in PBS: F4/80 (clone BM8), CD11b (clone M1/70), GR-1 (clone RB6–8C5), CD3 (clone 17A2), CD4 (GK1.5), CD8 (clone 53–6.7), B220 (clone RA3–6B2), CD117 (clone ACK2), NK (CD49b, clone DX5), CD11c (clone N418), TLR4 (clone MTS5510; isotype control rat IgG2a), Tim-3 (clone 8B.2C12; isotype control rat IgG1), CD80 (B7–1, clone 16–10A1), CTLA-4 (CD152, clone UC10–4B9), and forkhead box p3 (Foxp3, clone FJK-165). For intracellular staining, cells were fixed and permeabilized using BD Cytofix/Cytoperm or an anti-mouse Foxp3 staining kit (BD Pharmingen). Cell fluorescence was measured using a FACSCalibur flow cytometer and data analyzed using Cell Quest software (BD Biosciences).

Statistical analysis

Normally distributed data were analyzed by Student's *t* test. The Mann-Whitney *U* test was used to evaluate nonparametric data. A value of $p < 0.05$ was considered significant.

Results and Discussion

Increased inflammation in males not due to virus

To better understand the mechanisms responsible for increased heart disease in males, we examined the cellular infiltrate of male and female BALB/c mice during acute CVB3-induced myocarditis. Males developed significantly increased acute myocarditis compared with females by histologic assessment (Fig. 1*A*), which was confirmed by FACS analysis of $CD45^+$ immune cells (Fig. 1*B*) (8). Males had significantly increased numbers of monocyte/macrophages, granulocytes, MC, and dendritic cells (DC), whereas females had significantly increased numbers of B cells in the heart (Fig. 1*C*) consistent with the robust Ab response known to occur in female mice and humans (12).

Since increased inflammation in males may be due to increased viral replication in the heart, we examined the level of CVB3 by plaque assay at days 2, 8, and 12 p.i. We found no significant difference in the level of replicating virus in the heart at day 2 (females 702 ± 551 vs males 746 ± 499 PFU/g heart), day 8 (Fig. 1*D*) or day 12 p.i. (females 229 ± 137 vs males 657 ± 329 PFU/g heart), indicating that factors other than viral replication increase inflammation in males (8, 13). We found previously that increased IL-1β and IL-18 levels correlate with more severe myocarditis in male BALB/c mice (10, 13). In this study, females had significantly increased IL-4 levels in the heart (Fig. 1*E*) and B cell numbers (Fig. 1*C*). Males produced significantly more IL-1β, IL-18 (8), and IFN-γ (Fig. 1*E*). IL-1 and IL-18 increase IFN-γ via MyD88 signaling (14) and may be responsible for the dominant Th1 response in males.

Males and females express different levels of TLR4 and Tim-3

Because TLR4 signaling increases inflammation and IL-1β/IL-18 levels in males (10), we compared TLR4 expression on APC isolated from the heart of males and females during acute myocarditis (Fig. 2*A*). We found TLR4 expression was significantly increased on $F4/80^+$ macrophages from males (Fig. 2*A*). In several experiments TLR4 expression was also increased on male MC (data not shown), but was not increased on B cells or DC (Fig. 2*A*). Thus, males have greater numbers of macrophages (and MC) in the heart (Fig. 1*C*) with increased expression of TLR4 (Fig. 2*A*). We next asked whether elevated IFN-γ levels in males (Fig. 1*E*) increase TLR4 expression on MC and macrophages from the heart during acute myocarditis (Fig. 2*A*). We found that TLR4 levels were not decreased on APC in IFN-γ-deficient males (data not shown), indicating that IFN-γ does not increase TLR4 expression in males. These data suggest that elevated TLR4 expression in males leads to a Th1 response via increased IL-18 (IFN-γ-inducing factor) (8, 10).

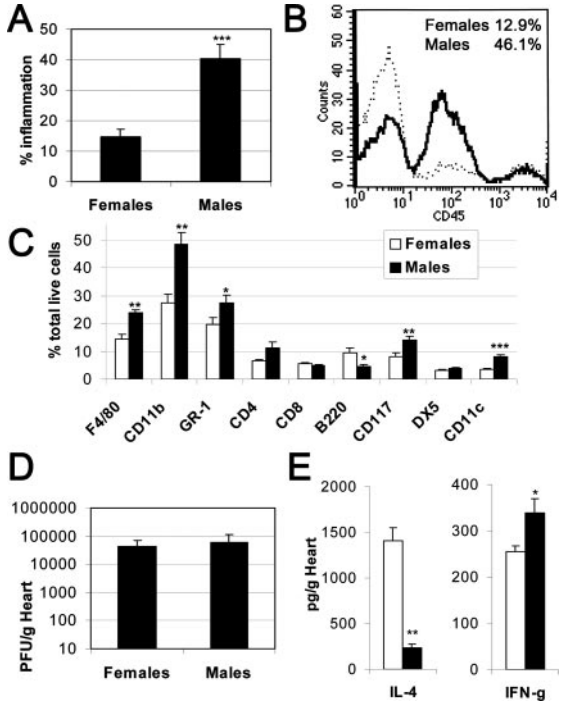

FIGURE 1. Males develop increased inflammation in the heart. Male and female BALB/c mice were compared for acute myocarditis by histological (*A*) or FACS analysis of total lymphocytes (*B*) or individual immune cells (*C*), infectious virus by plaque assay (*D*) or cytokines (*E*) in the heart. Mice received 10^3 PFU of CVB3 i.p. on day 0 and hearts were collected on day 8 (*B, C, D*) or day 12 (*A, E*) p.i. Sections were stained with H&E and myocarditis assessed as the percentage of the heart section with inflammation compared with the overall size of the heart section (*A*). Males (solid line) and females (dotted line) were compared by FACS for the percentage of $CD45^+$ cells (*B*). The following cell types were evaluated by FACS (*C*): macrophages (F4/80), macrophages/granulocytes/MC (CD11b), granulocytes (GR-1), $CD4^+$ T cells (CD4), $CD8^+$ T cells (CD8), B cells (B220), MC (CD117), NK cells (DX5), and DC (CD11c). Similar results were obtained in four separate experiments using 7 to 10 mice per group. Data show the mean ± SEM. *, $p < 0.05$; **, $p < 0.01$, ***, $p < 0.001$.

We recently showed that signaling by Tim-3 significantly decreases $CD11b^+$ inflammation in the heart (which include macrophages, granulocytes, and MC) during acute CVB3-induced myocarditis, and increases $CD4^+Tim\text{-}3^+CTLA\text{-}4^+$ and $CD4^+CD25^+Foxp3^+$ Treg populations (11). We therefore compared the level of Tim-3 on immune cells isolated from the heart of males and females during acute myocarditis (Fig. 2*B*). Tim-3 expression was significantly increased on MC, macrophages, and $CD4^+$ T cells from females on day 8 p.i. (Fig. 2*B*). At day 12 p.i., Tim-3 was increased by 122% on MC from females (Fig. 2*C*) compared with males (Fig. 2*D*). $CD4^+Tim\text{-}3^+CTLA\text{-}4^+$ (T&CTLA4) and conventional $CD4^+Foxp3^+$ Treg populations were also significantly increased in the hearts of females compared with males (Fig. 2*E*). Previously, estrogen treatment was shown to reduce inflammation in experimental autoimmune encephalomyelitis by increasing Treg (15). Our findings suggest that estrogen may increase Treg numbers by increasing Tim-3 expression.

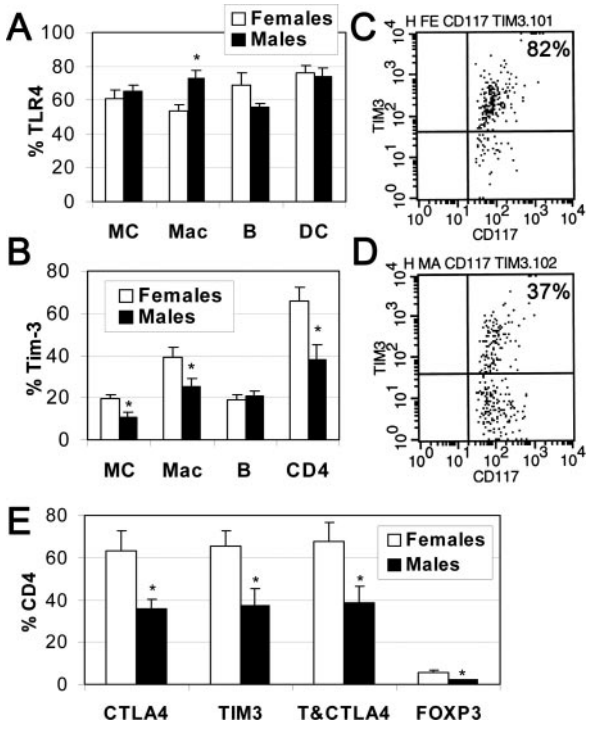

FIGURE 2. TLR4 and Tim-3 expression in males and females. Mice received 10^3 PFU of CVB3 i.p. on day 0 and $CD45^+$ immune cells were isolated from the heart of female and male BALB/c mice on day 8 (*A, B, E*) or day 12 (*C, D*) p.i. FACS analysis depicts the percentage of MC, macrophages (Mac), B cells (B), DC, and $CD4^+$ T cells (CD4) expressing TLR4 (*A*) or Tim-3 (*B-D*). Expression levels of CTLA-4, Tim-3, Tim-3 and CTLA-4 (T&CTLA4), or Foxp3 were assessed on/in $CD4^+$ T cells (*E*). Similar results were obtained in 4 separate experiments using 5 to 7 mice per group. Data show the mean ± SEM. *, $p < 0.05$.

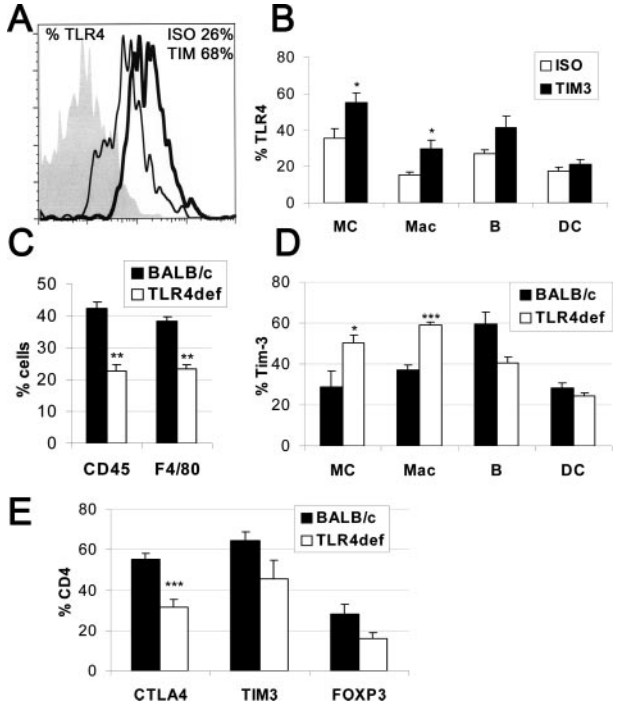

FIGURE 3. Cross-regulation by TLR4 and Tim-3 during acute myocarditis. Male BALB/c mice were inoculated with anti-Tim-3 (TIM3) or isotype control (ISO) Ab at day 0 of CVB3 infection and $CD45^+$ immune cells were isolated from the heart at day 8 p.i. (*A, B*). FACS analysis depicts the percentage of MC (*A, B*), macrophages (Mac), B cells (B) and DC (*B*) expressing TLR4 in anti-Tim-3-treated males (heavy line) compared with isotype control-treated males (thin line) (*A*). FACS isotype control is filled (*A*). TLR4-deficient (TLR4def) and wild-type BALB/c males were infected with 10^3 PFU CVB3 i.p. on day 0 and analyzed similarly (*C-E*). FACS analysis depicts the percentage of $CD45^+$ cells from the heart and $CD45^+$ cells expressing F4/80 (*C*) as well as the percentage of MC, Mac, B cells, DC or $CD4^+$ T cells (CD4) expressing Tim-3 (*D, E*). Expression levels of CTLA-4, Tim-3 and Foxp3 were assessed on/in $CD4^+$ T cells from TLR4 deficient mice (*E*). Similar results were obtained in at least four separate experiments using 5 to 7 mice per group. Data show the mean ± SEM. *, $p < 0.05$; **, $p < 0.01$, ***, $p < 0.001$.

Cross-regulation by TLR4 and Tim-3

We then examined TLR4 levels on immune cells isolated from the heart of male mice treated with mAb to block Tim-3, as before (11). Mice received only one i.p. treatment with anti-Tim-3 at the same time as CVB3 inoculation, thereby influencing the innate immune response. We reported previously that anti-Tim-3-treated males have significantly increased inflammation and reduced Treg cell populations in the heart indicating that Tim-3 expression increases Treg during acute myocarditis (11). In this study, TLR4 expression was significantly increased on MC (Fig. 3, *A* and *B*) and macrophages (Fig. 3*B*) in anti-Tim-3-treated males (TIM3) during acute myocarditis compared with isotype control-treated males (ISO). We report here for the first time, that in addition to reducing inflammation by increasing Treg, Tim-3 also reduces TLR4 expression on MC and macrophages present in the heart during acute myocarditis.

$CD45^+$ inflammation and $F4/80^+$ macrophages were significantly reduced in the heart of mice with defective TLR4 signaling (TLR4def) (Fig. 3*C*) (10). Tim-3 expression was significantly increased on MC and macrophages in TLR4-deficient mice, but not on B cells or DC (Fig. 3*D*). Tim-3 expression was similarly increased on MC and macrophages from females (Fig. 2*B*). However, $CTLA\text{-}4^+$, $Tim\text{-}3^+$ and conventional $Foxp3^+$ $CD4^+$Treg were not increased in the hearts of TLR4-deficient males (Fig. 3*E*) as they were in wild-type (WT) females (Fig. 2*E*). In fact, CTLA-4 was significantly decreased in TLR4-defective $CD4^+$ T cells isolated from the heart (Fig. 3*E*), indicating that TLR4 signaling increases CTLA-4 expression in T cells. These results suggest that TLR4 and Tim-3 signaling are both required to increase Treg populations during acute myocarditis (11). Recently, TLR2 signaling on $CD4^+$ T cells was shown to be necessary for Treg development following bacterial infection (16). Our findings suggest that both TLR4 and Tim-3 expression influence the number of Treg in the heart during acute myocarditis.

Innate cross-regulation

Infection with CVB3 increases TLR4 and Tim-3 expression on APC compared with uninfected controls immediately after infection (11, 13). Examining the innate immune response in males and females, TLR4 expression was increased on male MC (Fig. 4*A*) and macrophages (Fig. 4*B*) from the peritoneum at 6 to 12 h p.i., but was not increased on other APC (data not shown). Increased TLR4 expression in males occurs immediately following infection, because males and females have similar constitutive expression of TLR4 on APC before infection (data not shown). In contrast, Tim-3 and CD80 levels (CD80 binds CTLA-4 increasing Treg numbers, 11) were increased on MC and macrophages from females at 6 h p.i. (Fig. 4, *A* and *B*).

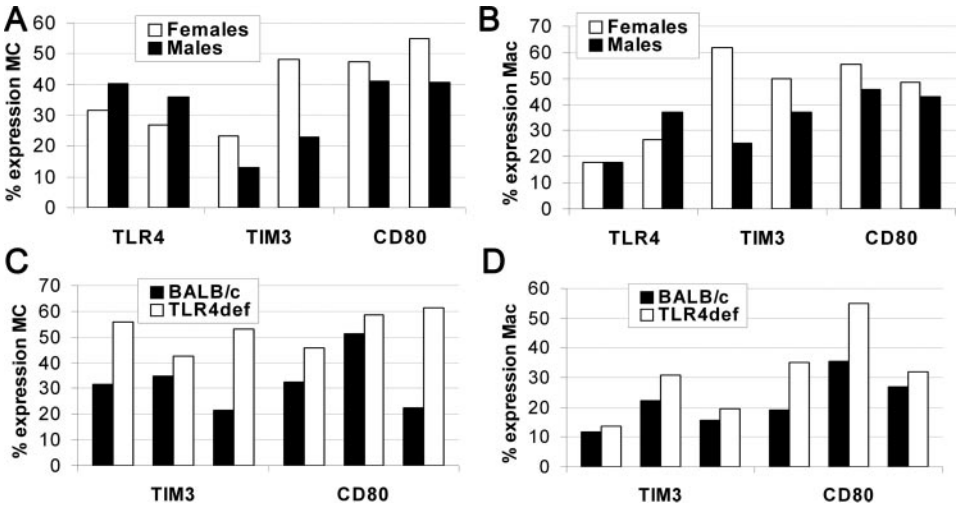

FIGURE 4. Cross-regulation by TLR4 and Tim-3 during innate immunity. Male and female BALB/c mice or wild-type BALB/c and TLR4-deficient (TLR4def) males received 10^3 PFU of CVB3 i.p. on day 0 and $CD45^+$ immune cells were isolated from the peritoneum at 6 to 12 h p.i. FACS analysis depicts the percentage of MC (*A, C*) or macrophages (*B, D*) expressing TLR4, Tim-3 or CD80 in two (*A, B*) or three (*C, D*) separate experiments. Data show results from 5 to 7 pooled mice per group per experiment.

Intracellular CTLA-4 increased by 200% in $CD4^+$ splenocytes (females 39% vs males 13%) from females at 12 h p.i. (data not shown), similar to acute myocarditis (Fig. 2*E*). Similar results were obtained with cells from the peritoneum, spleen or heart, but changes in expression of TLR4 and Tim-3 occurred earlier in the peritoneum and heart (6 h p.i.) than in the spleen and lymph node (12–24 h p.i.).

We showed recently that Tim-3 expression during innate immunity determines the severity of acute inflammatory heart disease by altering CD80 expression on MC and macrophages and intracellular CTLA-4 levels in $CD4^+$ T cells (11). In this study, blocking Tim-3 increased TLR4 expression during innate immunity by 20% on MC and 79.2% on $F4/80^+$ macrophages at 6 h p.i. in males (data not shown), similar to acute myocarditis (Fig. 3, *A* and *B*). In mice with defective TLR4 signaling, Tim-3 and CD80 expression was increased on MC (Fig. 4*C*) and macrophages (Fig. 4*D*) in males at 6 h p.i., but was not increased on B cells or DC (data not shown). Although CD80 levels were increased on MC and macrophages (Fig. 4, *C* and *D*), intracellular CTLA-4 levels were not increased in $CD4^+$ T cells from TLR4-deficient males (WT vs TLR4 expt. 1: 17.4% vs 7.7%; expt. 2: 10.4% vs 9.9%; expt. 3: 7% vs 4%), similar to acute myocarditis (Fig. 3*E*). These findings suggest that both TLR4 and Tim-3 are needed during innate immunity to increase Treg numbers later during the adaptive immune response.

We demonstrate in these studies that Tim-3 inhibits TLR4 expression while TLR4 signaling reduces Tim-3 expression on MC and macrophages, indicating that cross-regulation occurs between these two receptors. Our findings indicate that reduced inflammation in the heart of females following CVB3 infection is due to increased Tim-3 expression on APC, resulting in increased CD80 and CTLA-4 expression and increased Treg populations. MC and macrophages are known to express estrogen and androgen receptors (17–19). Estrogen has been shown to decrease the proliferation of MC and macrophages, to decrease the release of histamine and to reduce proinflammatory cytokines in humans, rats, and other species (20, 21), whereas testosterone increases the number of MC and macrophages, histamine, and cytokine release (22). Thus, increased numbers of MC and macrophages and TLR4 expression in males inhibits regulation of the inflammatory response by reducing Treg populations and Tim-3-mediated apoptosis (11, 23). In support of these findings, it was recently shown that patients with multiple sclerosis have increased IFN-γ levels but lower TIM-3 expression on T cells (24). We suggest that cross-regulation by TLR4 and Tim-3 during the innate and adaptive immune response to infection determines the severity of inflammatory heart disease between the sexes.

Disclosures

The authors have no financial conflict of interest.

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