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*J Immunol* 2014; 192:5074-5082; Prepublished online 23 April 2014; doi: 10.4049/jimmunol.1302916

http://www.jimmunol.org/content/192/11/5074

**Supplementary Material**

http://www.jimmunol.org/content/suppl/2014/04/23/jimmunol.1302916.DCSupplemental

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Impact of Asymptomatic Herpes Simplex Virus Type 2 Infection on Mucosal Homing and Immune Cell Subsets in the Blood and Female Genital Tract

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HSV-2 infection is common and generally asymptomatic, but it is associated with increased HIV susceptibility and disease progression. This may relate to herpes-mediated changes in genital and systemic immunity. Cervical cytobrushes and blood were collected from HIV-uninfected African/Caribbean women in Toronto, and immune cell subsets were enumerated blindly by flow cytometry. Immune differences between groups were assessed by univariate analysis and confirmed using a multivariate model. Study participants consisted of 46 women, of whom 54% were infected with HSV-2. T cell activation and expression of the mucosal homing integrin α4β7 (19.60 versus 8.76%; p < 0.001) were increased in the blood of HSV-2–infected women. Furthermore, expression of α4β7 on blood T cells correlated with increased numbers of activated (coexpressing CD38/HLA-DR; p = 0.004) and CCR5+ (p = 0.005) cervical CD4+ T cells. HSV-2–infected women exhibited an increase in the number of cervical CD4+ T cells (715 versus 262 cells/cytobrush; p = 0.016), as well as an increase in the number and proportion of cervical CD4+ T cells that expressed CCR5+ (406 versus 131 cells, p = 0.001; and 50.70 versus 34.90%, p = 0.004) and were activated (112 versus 13 cells, p < 0.001; and 9.84 versus 4.86%, p = 0.009). Mannose receptor expression also was increased on cervical dendritic cell subsets. In conclusion, asymptomatic HSV-2 infection was associated with significant systemic and genital immune changes, including increased immune activation and systemic α4β7 expression; correlation of the latter with highly HIV-susceptible CD4+ T cell subsets in the cervix may provide a mechanism for the increased HIV susceptibility observed in asymptomatic HSV-2–infected women. The Journal of Immunology, 2014, 192: 5074–5082.

Received for publication October 29, 2013. Accepted for publication March 25, 2014.

This work was supported by the Canadian Institutes of Health Research (Grant HET-85518 to R.K.), B.S. received a studentship from the Canadian Institutes of Health Research, and R.K. and T.J.Y. received salary awards from the Ontario HIV Treatment Network.

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The online version of this article contains supplemental material.

Abbreviations used in this article: DC, dendritic cell; LC, Langerhans cell; mDC, myeloid-derived dendritic cell; Treg, regulatory T cell.

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acyclovir did not reduce HIV incidence, despite lowering the frequency of clinical ulceration (2, 4), suggesting that increased HIV susceptibility is not simply due to compromised epithelial integrity.

There is a dramatic increase in the density of activated CD4+ T cells expressing CCR5 in the skin underlining a clinical HSV-2 ulcer that persists for >20 wk, despite healing and effective antiviral therapy (5). Asymptomatic HSV-2 infection is also associated with T cell alterations in the genital mucosa (3, 5, 6), an increased expression of CD69 and CCR5 by endocervical CD4+ T cells (3), and increased CD4+ T cell density (7) and CCR5 expression by CD4+ T cells (6) in the foreskin. Because CCR5+ T cells are the primary target for sexually transmitted R5-tropic strains of HIV (8), this increased density of target cells in the genital mucosa may be an important contributor to the increased HIV susceptibility observed in asymptomatic HSV-2 infection.

Whether asymptomatic HSV-2 induces additional mucosal or systemic immune alterations is not clear, and the mechanism by which activated T cells are targeted to the genital mucosa has not been described. α4β7 is a mucosal homing integrin expressed on CD4+ T cells that not only “targets” T cells to track from the blood to the gastrointestinal and genital mucosae (9), but may also directly enhance HIV infection through binding HIV gp120 (10, 11) and was shown to be enhanced in the blood and rectal mucosa of HSV-2–infected macaques (12). Dendritic cells (DCs) can also play a role in HIV acquisition through several mechanisms (13). Tissue-resident DCs are able to take up HIV through the interaction of their lectin-binding receptor DC-SIGN with HIV gp120 and infect CD4+ T cells in trans upon their subsequent migration to regional lymph nodes (14, 15). In addition to
DC-SIGN, several DC receptors may be capable of enhancing HIV transmission via a similar “Trojan horse” mechanism (16, 17), including langerin (CD207) (18, 19) and mannose receptor (MR/CD206) (20, 21). Furthermore, the association of HSV-2 infection with increased HIV disease progression in HIV-infected individuals (22, 23) suggests that this coinfection may also alter systemic immunology.

We hypothesized that HSV-2 infection would enhance HIV susceptibility in the genital mucosa through upregulation of the mucosal-homing integrin α4β7 in the blood, with enhanced mucosal recruitment of activated CD4+ T cells, as well as by increasing expression of HIV-binding lectins on genital DC populations. To address these questions, we conducted an observational clinical study in African/Caribbean women from Toronto, Canada; this community is disproportionately affected by HIV (24) and has an HSV-2 prevalence of 12.8% compared to 4.5% in all women in Canada (25). HSV-2–infected women had elevated T cell expression of α4β7 in the blood, corresponding with immune activation, and increased numbers of HIV-susceptible T cell subsets in the cervix; they also demonstrated increased expression of C-type lectins by cervical DC subsets.

Materials and Methods
Participant enrolment and inclusion criteria
Participants were recruited into this cross-sectional study from the Women’s Health in Women’s Hands Community Health Centre in Toronto, Canada. This is a community clinic that provides care for black women and women of color. Participants were either contacted directly by a clinic-based research coordinator or recruited through study fliers posted in the clinic waiting room. Informed written consent was obtained from all participants, and the study protocol was approved by the HIV Research Ethics Board at the University of Toronto. Participants completed a short demographic/behavioral questionnaire. Blood, urine, and genital secretions were collected by participants and processed as described below. Exclusion criteria included infection by HIV-1/2, Neisseria gonorrhoeae, Chlamydia trachomatis, Mycoplasma genitalium, Treponema pallidum, or Trichomonas vaginalis; previous menopause; or any symptomatic genital infection within 3 mo of study participation. All women were actively cycling and were sampled between 10 and 18 d after the last day of bleeding of their prior menstrual period. Participants completed a second demographic/behavioral questionnaire. Blood, urine, and a physician-collected vaginal swab were collected for sexually transmitted infection diagnostics. Participants self-collected undiluted cervicovaginal secretions using an InstaSoft cup (Evoform, San Diego, CA) for 1 min before vaginal secretions were collected. Additionally, two endocervical cytobrushes were collected for immune cell phenotyping. Each cytobrush was gently inserted into the cervical os, rotated through 360°, placed into R10 medium (RPMI 1640 with 10% heat-inactivated FBS [Sigma-Aldrich, Carlsbad, CA], 100 mg/ml streptomycin, 100 U/ml penicillin, and 1× GlutaMAX-1 [Life Technologies, Grand Island, NY] media) at 4°C and transported to the laboratory within 3 h. Cells from the two cytobrushes were combined, filtered through a 100-μm filter, washed, and divided into two equal aliquots for staining. PBMCs were isolated by Ficoll-Hypaque density centrifugation, counted, and washed twice in R10 medium. Two aliquots of 1 million PBMCs were used for staining.

Coinfection diagnostics
HSV-2 status was assessed using HepserSelect g-1 and g-2 ELISA (Focus Technologies, Cypress, CA), with an adjusted threshold value of 3.5 for defining seropositivity (26–28). A vaginal Gram stain was performed for the detection of yeast and bacterial vaginosis, with the latter diagnosed according to Nugent criteria (29). A first-void urine sample was tested for N. gonorrhoeae and C. trachomatis using a nucleic acid amplification test (ProbeTech Assay; BD, Sparks, MD). Genital secretions collected by instead cup were tested for HSV-2 shedding, using a previously validated in-house PCR assay (30). Briefly, DNA was extracted using Minirep DNA extraction kits (QIAGEN, Valencia, CA); SuperMix (Invitrogen, Burlington, ON, Canada) was used for master mix; and a Rotor-Gene 6000 (Corbett Life Science, Sydney, NSW, Australia) was used for real-time thermal cycling and data analysis. The following primers were used: 5’-CATC ACCGACCCGAGAGGGAC-3’ and 5’-GGGCCAGCGGCCTTGTGGT GTA-3’; and the TaqMan probe was 5’-FAM-CGCGGGACTGCGA CACCCGGCC-BHQ-3’ (Integrated DNA Technologies, Coralville, IA).

Immune cell phenotyping
PBMCs and cervical cells were stained with a T cell and DC mAb panel. The T cell panel consisted of α4–FITC (Miltenyi Biotec), CD3–ECD (Beckman Coulter, Marseille, France), CCR5–PE, β7–allophycocyanin, CD38–Alexa Fluor 700, HLA-DR–allophycocyanin–C7, CD69–eFluor 450 (BD Biosciences, Franklin Lakes, NJ), Live/Dead Aqua (Invitrogen), CD25–PE-Cy5.5, CD39–PE-Cy7, and DC-SIGN–PerCP–Cy5.5, CD206–allophycocyanin (BD Biosciences), CD3–fluorescein isothiocyanate (FITC)–PE, and Live/死者 Aqua (Invitrogen). Cells were enumerated using a BD LSR II flow cytometer (BD Systems) and analyzed with FlowJo 9.3.2 software (TreeStar, Ashland, OR) by a single researcher blinded to participants’ HSV-2 status. Cervical immune cell populations were reported as both proportion (%) and total number of cells/cytobrush, because the entire contents of the sample collected by the cytobrush (see above) were run through the flow cytometer. CD8+ T cells were defined as CD3+ CD4– lymphocytes. Regulatory T cells (Tregs) were defined as CD3+CD4+CD25+ CD39+ cells (31).

Statistical analysis
Baseline characteristics and outcomes were summarized with frequencies and proportions for categorical variables and median and range for continuous variables. Differences between HSV-2–infected and uninfected groups were assessed using the Fisher exact test for categorical variables and the Mann–Whitney U test for continuous variables. The number of cells/cytobrush was log transformed to normalize the data. Bivariate correlations were assessed using the Pearson correlation after ensuring that parameters were distributed normally using the Shapiro–
Wilk normality test. Univariate immune associations were confirmed in a multivariate model, controlling for demographic factors that varied with HSV-2 serostatus (see below). The Spearman rank correlation coefficient was used when parameters were not distributed normally. All statistical analyses were performed using SPSS version 19 (IBM, New York, NY).

**FIGURE 1.** Gating strategy and representative plots of cervical cells. (A) Overall gating strategy for CD4+ T cells. Cells were gated on lymphocytes, singlets, live cells, and CD3+CD4+ to isolate CD4+ T cells. Representative plots for CCR5 expression (lower middle panel) and CD38/HLA-DR expression (lower right panel) in the female genital tract. (B) Overall gating strategy for DCs. Cells were gated on DCs, singlets, live cells, and CD3− cells to isolate DCs. Within this population, cells were gated on CD14−CD11c+ cells to isolate mDCs (bottom row, middle) and CD14+ cells to isolate monocytes (middle row, middle). Representative plots for mannose receptor (MR; CD206) expression in monocytes (middle row, right) and mDCs (bottom row, right).
Results

Participant demographics

In total, 46 participants were enrolled; of these, 25 (54.35%) were HSV-2 infected. Three HSV-2–infected women (12.00%) had asymptomatic genital herpes reactivation detected by PCR and were excluded from subsequent analysis. The median age of HSV-2–infected participants was significantly higher than the uninfected group (38 versus 29 y, respectively, \( p = 0.001 \); Table I). All participants screened negative for HIV-1/2, \( N. \) gonorrhoeae, and \( C. \) trachomatis, and no other demographic or clinical differences were apparent between the HSV-2– and HSV-2+ groups, including self-reported number of sexual partners over the past year (median 1 in each group), hormonal contraceptive use (14.29 versus 13.64%, respectively), condom use during the last sex act (33.33 versus 36.36%), douching (9.52 versus 13.64%), bacterial vaginosis (14.29 versus 18.18%), altered vaginal flora (28.57 versus 31.81%), yeast (14.29 versus 9.09%), or HSV-1 seropositivity (85.71 versus 100%).

HSV-2 infection and T cell subsets in the blood

The association between asymptomatic HSV-2 infection and T cell subsets in blood was assessed (gating strategy, Fig. 1A). The peripheral blood CD4/CD8 T cell ratio was similar in HSV-2–infected and uninfected participants (median 1.579 versus 1.605, \( p = 0.782 \)). A higher proportion of CD4+ T cells in HSV-2–infected women expressed the early activation marker CD69 (0.76 versus 0.27%; \( p = 0.016 \)) and coexpressed the chronic activation markers CD38 and HLA-DR (0.65 versus 0.21%, \( p = 0.002 \); Fig. 2A), and there was also a trend toward increased expression of CCR5 by blood CD4+ T cells (4.68 versus 2.52%, \( p = 0.091 \); Fig. 2B). Similarly, an increased proportion of CD8+ T cells coexpressed the activation markers CD38 and HLA-DR (1.49 versus 0.62%, \( p = 0.038 \)) in HSV-2–infected individuals (Fig. 2C). HSV-2 infection was not associated with differences in Treg number or proportion (data not shown).

In addition, we observed a substantial increase in \( \alpha 4 \beta 7 \) expression by blood CD4+ T cells in HSV-2–infected women (19.60 versus 8.76%, \( p < 0.001 \); Fig. 2D). \( \alpha 4 \beta 7 \)-CD4+ T cells in the blood preferentially expressed increased levels of CCR5 (4.65 versus 2.88%, \( p = 0.005 \); Fig. 3A) and were more activated (coexpressed CD38/HLA-DR; 0.49 versus 0.28%, \( p = 0.014 \); Fig. 3B). \( \alpha 4 \beta 7 \) was not associated with CD69 expression (data not shown). Although HSV-2–infected women were older, age did not correlate with the frequency of \( \alpha 4 \beta 7 \)-CD4+ T cells (rs = 0.293, \( p = 0.086 \)), activated CD4+ T cells coexpressing CD38/HLA-DR (rs = 0.258, \( p = 0.094 \)), or other immune parameters (all \( p > 0.100 \), data not shown).

Association of blood \( \alpha 4 \beta 7 \) expression with genital T cell subsets

Blood \( \alpha 4 \beta 7 \)-CD4+ T cells expressed higher levels of CCR5 and the activation markers CD38/HLA-DR and would be expected to traffic to mucosal sites of possible HIV exposure. Therefore, we examined the association of blood \( \alpha 4 \beta 7 \) expression with genital T cell subsets. An increased level of \( \alpha 4 \beta 7 \) expression in blood CD4+ T cells was associated with increases in the number of...
cervical CCR5+ CD4+ T cells (r = +0.418, p = 0.005; Fig. 4B), cervical CD4+ T cells coexpressing CD38/HLA-DR (r = +0.426, p = 0.004; Fig. 4C), and cervical Tregs (r = +0.416, p = 0.005; Fig. 4D). These correlations remained significant after correcting for multiple comparisons (data not shown). In addition, there was a borderline correlation between blood α4β7 expression and an increased overall number of cervical CD4+ T cells/cytobrush (r = +0.292, p = 0.057; Fig. 4A).

**HSV-2 infection and T cell subsets in the endocervix**

Next, the association of asymptomatic HSV-2 infection with T cell subsets in the cervix was assessed using the same gating strategy as in blood (Fig. 1A). In HSV-2–infected women, there was an almost 3-fold increase in the total number of CD4+ T cells/cytobrush (median 715 versus 262 cells, p = 0.016; Fig. 5A). There was also an increase in both the number (406 versus 131 cells, p = 0.001) and percentage (50.70 versus 34.90%, p = 0.004) of CD4+ T cells expressing CCR5 in the HSV-2+ group (Fig. 5A).

The activation status of CD4+ T cells was then assessed in the cervix of HSV-2–infected women. Both the number (112 versus 13 cells, p < 0.001) and percentage (9.84 versus 4.86%, p = 0.009) of CD4+ T cells coexpressing the chronic activation markers CD38 and HLA-DR were increased in the HSV-2+ group (Fig. 5B). There was also a trend toward more cervical CD4+ T cells expressing the early activation marker CD69 (415 versus 120 cells, p = 0.072) but no significant difference in their proportion (56.95 versus 37.80%, p = 0.362) (Fig. 5B). Cervical Treg numbers (62 versus 10 cells, p = 0.001) and percentage (8.08 versus 4.17%, p = 0.002) were also increased in HSV-2–infected women (Fig. 5C). Again, although HSV-2–infected women were older, age did not correlate with the frequency of activated cervical CD4+ T cells expressing CCR5 (rs = 0.183, p = 0.239) or coexpressing CD38/HLA-DR (rs = 0.236, p = 0.127) or with other cervical T cell parameters (all p > 0.100, data not shown).

**HSV-2 infection and DC subsets in the female genital tract**

Cervical DC subsets assessed (gating strategy; Fig. 1B, Supplemental Fig. 1) included myeloid-derived DCs (mDCs; CD3−CD11c+), monocytes (CD3−CD14+), plasmacytoid DCs (CD3−CD11c−CD14−BDCA2+CD123+), and Langerhans cells (LCs; CD3−CD1a+langerin/CD207+). Insufficient numbers of plasmacytoid DCs were detected for analysis (data not shown).

There was no difference in the number of cells in each DC subset between the HSV-2+ and HSV-2− groups, including mDCs (5853 versus 2801 cells, p = 0.496), monocytes (6863 versus 5807 cells, p = 0.961), or LCs (380 versus 146 cells, p = 0.296; Fig. 6A). However, in HSV-2–infected women, a substantially higher proportion of cervical mDCs and monocytes expressed the mannose receptor (CD206) (31.00 versus 7.94%, p = 0.008 and 40.30 versus 11.60%, p = 0.012, respectively; Fig. 6B, 6C), and there was a nonsignificant trend toward increased numbers (CD206: 1162 versus 299 cells, p = 0.050; 860 versus 408 cells, p = 0.076, respectively). No difference was observed with regard to absolute numbers or percentage of DC-SIGN (CD209) expression on mDCs or monocytes (data not shown). No differences in mannose receptor or DC-SIGN expression on LCs were analyzed, because the expression of the C-type lectin receptor langerin (CD207) is included in the definition of these cells (see above). Participant age was not associated with cervical DC immune parameters (all p > 0.100, data not shown).

**Discussion**

HSV-2 infection prevalence is disproportionately high in sub-Saharan Africa (32) and within African/Caribbean communities in North America (25), and although infection is usually asymptomatic, it is associated with a 3-fold increase in HIV acquisition

![FIGURE 4](https://www.jimmunol.org/)

Blood α4β7 expression correlates with HIV-susceptible T cell subsets in the female genital tract. The proportion of blood CD4+ T cells expressing α4β7 was correlated with the number of CD3+CD4+ cells (A), CCR5+ cells (B), cells coexpressing CD38/HLA-DR (C), and cells coexpressing CD25/CD39 (D) in the female genital tract. Values were normalized through log transformation, and Pearson product-momentum correlation was performed.
risk (2). The current study confirms and substantially expands our previous finding that HSV-2 infection in Kenyan women was associated with more cervical cells expressing CCR5 and CD69 (3). These same cell populations were again increased, as were chronically activated cervical CD4+ T cells coexpressing CD38/HLA-DR. In addition, a higher proportion of cervical DCs and monocytes from HSV-2–infected women expressed mannose receptor (CD206), an HIV-binding lectin (although direct infection of monocytes by HIV would require expression of CCR5). Furthermore we show, for the first time to our knowledge, that asymptomatic HSV-2 infection was associated with systemic T cell immune activation and a dramatic increase in their expression of the mucosal homing integrin α4β7. Not only were α4β7+ T cells in the blood more activated and expressed more CCR5, α4β7 expression on blood T cells directly correlated with the number of activated and CCR5-expressing CD4+ T cells in the cervix.

HIV susceptibility at the site of sexual exposure may be enhanced by an increase in the overall number of mucosal CD4+ T cells, a relative enrichment of highly susceptible cells, or both (33). We find that asymptomatic HSV-2 infection was associated with an increase in the absolute number of cervical CD4+ T cells, as well as with increased levels of CD4+ T cell activation. Although HIV is capable of infecting “resting” memory CD4+ T cells, the efficiency of infection is much higher in activated cells, and these cells are thought to produce the majority of new virions (34, 35). Furthermore, the number and proportion of CD4+ T cells expressing CCR5 also were increased in the cervix, which

FIGURE 5. HSV-2 infection status and T cell subsets in the female genital tract. Association of HSV-2 with the absolute number of CD4+ T cells and the absolute number and proportion of CD4+ T cells expressing CCR5 (A), the absolute number and proportion of CD4+ T cells expressing CD69 and coexpressing CD38/HLA-DR (B), and the absolute number and proportion of CD4+ T cells coexpressing CD25/CD39 (C) in the female genital tract. Statistical comparisons were performed using the Mann–Whitney U test.
is important because R5-tropic HIV strains are responsible for the vast majority of sexual HIV transmission (8). However, this may reflect a host response to control recent herpes reactivation, because mice lacking CCR5 are unable to control herpes infection (36) and the T cells responsible for genital HSV-2 control in humans are predominantly CD4+ (37). Indeed, macaque studies showed elevated levels of αβ7+ CD4+ T cells in the blood and rectal mucosa of HSV-2+ macaques (12) and demonstrated that the expression of αβ7 by peripheral CD4+ T cells was correlated with both susceptibility to HIV infection and acute viral load (38). This suggests that the host’s natural response to control herpes reactivation may render individuals more susceptible to HIV. The observed Treg increases in HSV-2–infected participants were probably a secondary response and were less likely to be re-

FIGURE 6. HSV-2 infection status and DC subsets in the female genital tract. Association of HSV-2 with the absolute number of DC subsets, including LCs (CD3−CD1a+CD103+CD207+), mDCs (CD3−CD11c+), and monocytes (CD3−CD14+) (A), the absolute number and proportion of mDCs expressing mannose receptor (MR; CD206) (B), and the absolute number and proportion of monocytes expressing MR (CD206) (C) in the female genital tract. Statistical comparisons were performed using the Mann–Whitney U test.
sponsible for increased HIV susceptibility. Indeed, some groups showed that these cells may be associated with decreased HIV susceptibility (39). However, we believe that the increase in Treg number in the HSV-2 context is a host response to inflammation, because increases in Treg markers have been observed upon the activation of cells (40).

HSV-2–associated immune infiltration within the cervix, even in the absence of detectable HSV-2 shedding, provides a potential immune mechanism by which asymptomatic HSV-2 infection may enhance HIV susceptibility. Furthermore, this suggests that HSV-2–suppressive therapy might reverse this increase in susceptibility by inhibiting viral reactivation. However, two large clinical trials found that acyclovir had no impact on HIV acquisition in HSV-2–infected participants (41, 42), and valacyclovir therapy was unable to reverse the HSV-2–associated immune changes that we described in this study (43). This is likely because herpes-suppressive therapy is unable to completely prevent viral reactivation within the female genital tract. Indeed, a recent clinical trial showed that, even when a very high dose (3 g/d) of valacyclovir was used, there was an average of 16.5 HSV-2 shedding episodes/y; thus, asymptomatic infected participants (41, 42), and valacyclovir therapy was unable to enhance HIV susceptibility. Furthermore, this suggests that HSV-2–mediated mucosal immune changes are highly HIV–susceptible CD4+ T cell cervical subsets may have alterations. Our study was limited to asymptomatic HSV-2 infection by design, because most HSV-2–infected women are asymptomatic (1), and the local immune impact of HSV-2 ulceration was described previously (5).

In summary, asymptomatic HSV-2 infection was associated with increased systemic T cell immune activation and α4β7 expression, and the latter was closely correlated with highly HIV–susceptible CD4+ T cell subsets in the cervical mucosa. Although this may provide a mechanism for the increased HIV susceptibility that is observed in this population, the ability of targeted interventions to reduce these HSV-2–associated immune alterations or HIV transmission remains unclear.

Acknowledgments
We acknowledge the kind assistance of the staff at the Women’s Hands Clinic and the time and cooperation of all study participants.

Disclosures
The authors have no financial conflicts of interest.

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