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L-Selectin and P-Selectin Are Novel Biomarkers of Cervicovaginal Inflammation for Preclinical Mucosal Safety Assessment of Anti-HIV-1 Microbicide

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A major obstacle thwarting preclinical development of microbicides is the lack of a validated biomarker of cervicovaginal inflammation. Therefore, the present study aims to identify novel noninvasive soluble markers in a murine model for assessment of microbicide mucosal safety. By performing cytokine antibody array analysis, we identified two adhesion molecules, L-selectin and P-selectin, which significantly increased when mucosal inflammation was triggered by nonoxynol-9 (N9), an anti-HIV-1 microbicide candidate that failed clinical trials, in a refined murine model of agent-induced cervicovaginal inflammation. We found that patterns of detection of L-selectin and P-selectin were obviously different from those of the two previously defined biomarkers of cervicovaginal inflammation, monocyte chemotactic protein 1 (MCP-1) and interleukin 6 (IL-6). The levels of these two soluble selectins correlated better than those of MCP-1 and IL-6 with the duration and severity of mucosal inflammation triggered by N9 and two approved proinflammatory compounds, benzalkonium chloride (BZK) and sodium dodecyl sulfate (SDS), but not by two nonproinflammatory compounds, carboxymethyl celluose (CMC; microbicide excipients) and tenofovir (TFV; microbicide candidate). These data indicated that L-selectin and P-selectin can serve as additional novel cervicovaginal inflammation biomarkers for preclinical mucosal safety evaluation of candidate microbicides for the prevention of infection with HIV and other sexually transmitted pathogens.

The epidemic of human immunodeficiency virus type 1 (HIV-1), when combined with the lack of an effective vaccine, has created an urgent need for a female-controlled method to reduce the risk of HIV-1 transmission (35). Microbicides are defined as products that can be applied topically for the prevention of HIV-1 and other sexually transmitted infections by creating chemical, biological, and/or physical barriers. As such, they may offer one of the most promising preventive interventions by their accessible cost, ready availability, wide acceptance, and female-controlled use (35). To date, seven candidate microbicides have been tested in clinical trials. These include nonoxynol-9 (N9), Savvy, cellulose sulfate, Carraguard, PRO 2000, BufferGel, and tenofovir (TFV). While tenofovir showed some degree of preventive effects, the other products were shown to have no effect on HIV-1. Among them, cellulose sulfate and N9 were even shown to have a trend toward an increased risk of HIV-1 infection (1, 35).

N9, a nonionic surfactant, was the first candidate microbicide to be tested in efficacy clinical trials. Early studies indicated that N9 was active against a range of bacteria and enveloped viruses *in vitro*, including HIV-1 and herpes simplex virus (HSV), and various N9 products, including COL-1492, were defined to be generally safe by colposcopic examination in phase I and phase II clinical trials (30, 40, 41). However, clinical trials to evaluate the efficacy of COL-1492 (containing 52.5 mg N9) in protecting against HIV-1 infection have generated disappointing outcomes. Specifically, N9 did not prevent HIV-1 infection when used at a low frequency, and it even increased the risk of HIV-1 infection by frequent use (42). A series of studies that followed found that genital inflammation and the disruption of the cervicovaginal epithelium induced by microbicides are critical risk factors associated with the incidence of HIV-1 infection (9, 13, 15, 16, 18).

In constructing a safety profile, much can be learned from the N9 story. First, microbicidal efficacy depends on striking a balance between specific activity and safety, and the absence of inflammatory events is a key issue for microbicidal safety. Second, colposcopic evaluation is an unreliable predictor of local inflammation. Finally, inflammation resulting from exposure to N9 has been detected by different methods, including histology and determination of neutrophil counts and cytokine-chemokine release (18). Although histology analysis could enable investigators to identify inflammation, it would also require biopsy specimens of vaginal or cervical tissue to be taken shortly before and after exposure to the test agent. In addition, this method is qualitative and not sensitive enough to determine mucosal inflammation without obvious tissue damage. Thus, a noninvasive, more sensitive, and quantifiable method to describe the alterations caused by microbicidal agents with proinflammatory potential would be preferable (12).

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Many studies have used cervicovaginal fluid samples for the analysis of proteins, and correlations between different expression profiles and specific pathologies were found, resulting in the identification of biomarkers of these specific conditions and diseases (3, 28, 45). Use of a biomarker in drug development can reduce the costs and time required to get a drug from discovery to market. Relevant research in the area of microbicide safety is yielding new assays and biomarkers which, if validated, will be essential to the rational selection of microbicide candidates for efficacy trials (10, 29).

In fact, a number of animal and human studies have produced evidence that the levels of proinflammatory cytokine interleukin 1 (IL-1), IL-6, and chemokines IL-8, monocyte chemotactic protein 1 (MCP-1), and macrophage inflammatory protein 3α (MIP-3α) in cervicovaginal lavage (CVL) fluid positively correlate with histopathological and clinical signs of vaginal inflammation (2, 9, 13–16). Accordingly, these cytokines/chemokines have been defined as biomarkers of cervicovaginal inflammation. Similar to systemic or other local inflammation, cervicovaginal inflammation is characterized by infiltration of leukocytes. However, these molecules are not leukocyte specific and may be blocked by endogenous antagonists (39). These properties complicate the interpretation of cytokine profiles and, hence, their use as biomarkers of cervicovaginal inflammation. Thus, additional biomarkers are needed to facilitate the assessment of HIV-1 transmission risk and evaluate the mucosal safety of microbicides.

In as early as 2004, Fichorova and her colleagues found that IL-1, IL-6, and IL-8 could predict the mucosal toxicity of vaginal microbicidal compounds by using an improved rabbit irritation model (13). In 2007, they reported two soluble adhesion molecules, E-selectin and vascular cell adhesion molecule 1 (VCAM-1), to be biomarkers of leukocyte traffic and activation in the vaginal mucosa (39). These studies demonstrated that a single dose or three consecutive doses of detergents such as N9, sodium dodecyl sulfate (SDS), and benzalkonium chloride (BZK) could induce obvious cervicovaginal inflammation and that soluble adhesion molecules besides proinflammatory cytokines and chemokines might be used as biomarkers of mucosal inflammation. This is understandable because of the pivotal role that adhesion molecules play in the onset and progress of inflammation and inflammatory diseases (5, 23, 25), in which a well-defined and regulated multistep cascade and initial capture of leukocytes are primarily mediated by the selectin family, which is composed of three members: L-, P-, and E-selectins (25). In the present study, we tried to identify novel biomarkers of cervicovaginal inflammation and focused on the selectin family for use in preclinical assessment of microbicides in a murine model. By using a cytokine antibody array, which can analyze multiple proteins simultaneously, we identified two adhesion molecules, L-selectin and P-selectin, which significantly increased when mucosal inflammation was triggered by N9. Further studies suggested that L-selectin and P-selectin correlated closely with the severity of cervicovaginal inflammation induced by different proinflammatory agents. The early response and long duration of increased levels of soluble L- and P-selectins detected after mucosal inflammation occurred suggest that L- and P-selectins may be recruited as additional cervicovaginal inflammation biomarkers and used for preclinical safety evaluation of candidate microbicides for the prevention of HIV-1 infection and other sexually transmitted diseases.

MATERIALS AND METHODS

Reagents. A panel of compounds was selected for the study. While N9, BZK, and SDS were selected as positive controls in models of vaginal irritation and inflammation, as reported in previous works (2, 9, 13, 15, 16), carboxymethyl cellulose (CMC; which is widely used as a microbicide excipient and control) and TFV (a microbicide candidate which is proven to be efficacious and safe [1]) were chosen as negative controls. N9 and TFV were purchased from LKT Laboratories, Inc., and Molekula Limited (United Kingdom), respectively. BZK, SDS, and CMC were purchased from Sigma-Aldrich. All agents were diluted in phosphate-buffered saline (PBS) to avoid the confounding effects of different formulations.

Murine model. Female C57BL/6 mice (6 to 8 weeks old) were obtained from the Beijing Laboratory Animal Research Center and housed under specific-pathogen-free (SPF) conditions in the Animal Center of the Wuhan Institute of Virology, Chinese Academy of Sciences. Animal studies were performed according to Regulations for the Administration of Affairs Concerning Experimental Animals in China (1988), and protocols were reviewed and approved by the Laboratory Animal Care and Use Committee of the Wuhan Institute of Virology, Chinese Academy of Sciences. Seven days before the experiment, the reproductive cycle was synchronized through subcutaneous injection of 0.1 ml of medroxyprogesterone acetate injectable suspension (Depo-Provera; Pfizer Pharmacia & Upjohn Company) diluted in lactated Ringer's saline solution for a final concentration of 30 mg/ml per animal (9).

Medroxyprogesterone-treated mice were anesthetized with a formulation of pentobarbital (80 to 85 mg/kg of body weight) and received an intravaginal inoculation (10 μl) of the compounds tested. The mice were treated with the following compounds: 40 mg/ml or other indicated concentrations, when dose-dependent effects were considered, of N9 in PBS, 40 mg/ml SDS in PBS, 20 mg/ml BZK in PBS, 10 mg/ml TFV, and 40 mg/ml CMC in PBS. Untreated mice and mice treated with the PBS alone were used as controls to evaluate normal tissue morphology and inflammation status in the cervicovaginal mucosal surface.

In this study, two different protocols were designed for parallel assessment of proinflammatory cytokine and soluble selectin release. In the first protocol, mice received a single dose of 10 μl compound in PBS or PBS alone, and CVL fluid was collected 4 h after dose administration or at the indicated time points when time course effects were considered. In the second protocol, mice were dosed on three consecutive days, and CVL fluid was collected at 24-h intervals, 4 h after each treatment, and on two consecutive days after the last application. CVL fluid was collected from groups of 5 to 10 mice at the indicated time points by three consecutive washes with 40 μl of PBS per wash delivered and recovered five consecutive times; a total volume of 100 to 110 μl was usually recovered. CVL fluid was then centrifuged (400 × *g* at 4°C for 10 min in a Thermo Scientific Sorvall Legend Micro 17R centrifuge with a 75003424 rotor) to separate the soluble supernatant from cell debris and stored at −80°C until analyzed. After lavage fluid collection, mice from each treatment group were then immediately sacrificed, and the cervix and vagina were excised for histological analysis.

Cytokine antibody array. CVL fluid was analyzed with a cytokine antibody array by using a RayBio mouse cytokine antibody array III (RayBiotech, Inc., Norcross, GA), according to the manufacturer's instructions. Signal intensities were quantified directly with a chemiluminescence imaging system (FluorChem HD2; Alpha Innotech) and analyzed with its software. Spots were digitized into pixel densities (PDs), which were exported into the Excel program for analysis. For the control group (PBS treatment), the PD of each protein was determined as the mean of duplicate spots, and then the fold change of each protein was calculated as $PD_{N9}/PD_{control}$.

Flow cytometry assay. Cells obtained from CVL fluid were resuspended at a concentration of 1×10^6 to 2×10^6 cells/100 μl and blocked with Fc block (anti-CD16/32, 0.5 μg per test; eBioscience) for 15 min at 4°C and then incubated with fluorochrome-conjugated antibodies against mouse CD45 (0.03 μg per test), F4/80 (0.25 μg per test), and Gr1 (0.125

μg per test) (all from eBioscience) for 30 min at 4°C. Dead cells were excluded by 7-amino-actinomycin D (0.5 μg per test; BD Pharmingen) staining. Samples were washed three times with 1% fetal bovine serum in PBS and were resuspended in 300 μl of PBS containing 2% paraformaldehyde. Samples were assessed with a FACSCalibur apparatus (Becton Dickinson, Heidelberg, Germany) and gated according to side scatter (SSC), forward scatter (FSC), and excitation spectra. Leukocytes, neutrophils, and macrophages were identified as $CD45^+$, $CD45^+$ $Gr1^+$, and $CD45^+$ $F4/80^+$ cells, respectively (16). The counts were derived from the total viable cell count by the following formula: total viable cell count × percentage of leukocytes (or macrophages or neutrophils [from differential staining]) = total number of leukocytes (or macrophages or neutrophils).

Histological analysis. Groups of 5 to 10 mice were treated intravaginally with the tested compounds (or PBS for controls) and sacrificed at the indicated times immediately before dissecting out the vagina. After macroscopic observations were recorded, each vagina was fixed in 5 ml 10% neutral buffered formalin. The vaginas were embedded, sectioned horizontally, stained with hematoxylin and eosin (H&E), examined by bright-field microscopy, and evaluated blindly for epithelial cell disruption, leukocyte infiltration, edema, and congestion. The histopathological scores were assigned by a semiquantitative system which was based on Eckstein's scoring system (11) and employed in our previous work (27). The detail of the scoring system is shown in Table S1 in the supplemental material.

Determination of soluble factors by ELISA. Soluble factors in mouse CVL fluid were measured by enzyme-linked immunosorbent assays (ELISAs), which were run on diluted mouse CVL fluid: 1:3 for MCP-1, IL-6, E-selectin, and P-selectin and 1:20 for L-selectin. Mouse MCP-1 and IL-6 kits were obtained from BD Biosciences, and soluble E/L/P-selectin ELISA kits were bought from Boster Biological Technology, Ltd. (China). Cytokine concentrations were determined using the calibration procedure and cytokine reference standards supplied with the ELISA kits. There was no cross-reactivity between the different cytokines.

Statistical analysis. Time course effects (a single dose and three consecutive doses) were analyzed by two-way analysis of variance (ANOVA); all other data analyses were performed with one-way ANOVA. When the *P* value was significant at the 5% level, further pairwise comparisons between the experimental group and control conditions were made using Dunnett's test. Statistical analyses were carried out with Instat GraphPad software, version 5.0. Data are represented as mean ± standard deviation (SD) for individual mice of one representative experiment or as mean ± standard error (SE) of several experiments. The Pearson *r* (linear regression) analysis was used to assess the correlation between the soluble factors and histopathology scores. A *P* value of less than 0.05 was considered significant.

RESULTS

Identification of novel protein molecules related to cervicovaginal inflammation by antibody array. To identify novel biomarkers of cervicovaginal inflammation by antibody array, we first needed to determine the time point of lavage fluid collection when proinflammatory molecules peaked. As Alt et al. (2) and Galen et al. (16) reported previously, MCP-1 was obviously increased in CVL fluid by N9 treatment. Therefore, we first analyzed the kinetics of MCP-1. MCP-1 peaked at 4 h ($P < 0.001$) and then declined sharply at later time points (Fig. 1). Therefore, we collected CVL fluid 4 h after treatment with N9 and PBS (as a negative control) to determine cytokine induction in the cervicovaginal environment by using a cytokine antibody array. In the N9-treated mice, 13 proteins were significantly increased compared with the levels in PBS-treated control mice (Fig. 2): one binding protein, insulin-like growth factor binding protein 6 (IGFBP-6; 1.58-fold); two cytokines, including IL-6 (6.46-fold) and IL-12 p40/70 (1.61-fold); three adhesion molecules, L-selectin (1.54-fold), P-selectin

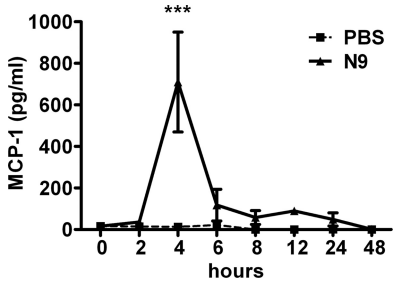

FIG 1 MCP-1 in CVL fluid peaks at the 4-h time point after a single application of N9. CVL fluid was collected from the mice, and the mice were sacrificed at the indicated time points following a single application of 40 mg/ml N9 in PBS or PBS alone. The untreated mice were regarded as the baseline, i.e., the 0-h time point. Five mice were used for each time point of each treatment. MCP-1 in the supernatant of CVL fluid collected at the indicated time point after a single 10-μl application of 40 mg/ml N9 was quantified by ELISA. MCP-1 in CVL fluid peaked at the 4-h time point, followed by a sharp decline and a return to baseline values at later time points. Experiments were repeated two times and showed similar results. Data are represented as mean ± SD for individual mice of one representative experiment. ***, $P < 0.001$.

(1.49-fold), and VCAM-1 (1.98-fold); five chemokines, MCP-1 (3.4-fold), platelet factor 4 (PF4 1.47-fold), regulated upon activation, normal T-cell expressed and secreted (RANTES; 1.58-fold), thymus and activation regulated chemokine (TARC; 1.29-fold), and T-cell activation-3 (TCA-3; 1.45-fold); and two receptors, soluble tumor necrosis factor receptor I (sTNFR I; 1.25-fold) and sTNFR II (1.68-fold). Among these proteins, the ones with the most strikingly increased levels were cytokine IL-6 (6.46-fold) and chemokine MCP-1 (3.4-fold), both proinflammatory cytokines. Moreover, most of these proteins have been previously studied and showed increases with the introduction of proinflammatory compounds. Most interestingly, we also found that three adhesion molecules, L-selectin, P-selectin, and VCAM-1, exhibited significant increases after N9 treatment. In previous studies of soluble biomarkers for microbicidal safety, most attention was focused on proinflammatory cytokines and chemokines, while little attention was given to soluble adhesion molecules (2, 9, 13, 15, 16, 39). Actually, it is well documented that adhesion molecules play a pivotal role in the onset and progress of inflammation and inflammatory diseases (5, 23, 25). Inflammation usually consists of a well-defined and regulated multistep cascade; in fact, the initial capture of leukocytes is primarily mediated by the selectin family, which is composed of three members: the L-, P-, and E-selectins. Our results also suggest that L- and P-selectins may be involved in the cervicovaginal inflammation process. Thus, we focused our subsequent studies on the potential association of these soluble selectins and proinflammatory compound-induced cervicovaginal inflammation.

The time course and duration of soluble L- and P-selectin levels correlated better than those of MCP-1 and IL-6 levels with time course and duration of toxic effects after a single application of N9. We next analyzed the time course of histopathology after vaginal application of a single dose of 40 mg/ml N9. At all time points, the morphological observations of the stained vaginal tissue sections revealed that the vaginal epithelium was a single-column layer, which is consistent with medroxyprogesterone treatment. The vaginal mucosa was intact in the PBS-treated mice, with some leukocytes randomly spread throughout the tissue. However, N9 caused rapid exfoliation of epithelial cells and infil-

A

	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1	POS	POS	NEG	NEG	Blank	AxL	BLC	CD30L	CD30T	CD40	CRG-2	CTACK	CXCL 16	Eotaxin
2	POS	POS	NEG	NEG	Blank	AxL	BLC	CD30L	CD30T	CD40	CRG-2	CTACK	CXCL 16	Eotaxin
3	Eotaxin-2	Fas Ligand	Fractalkine	GCSF	GM-CSF	IFN-γ	IGFBP-3	IGFBP-5	IGFBP-6	IL-1α	IL-1 β	IL-2	IL-3	IL-3Rb
4	Eotaxin-2	Fas Ligand	Fractalkine	GCSF	GM-CSF	IFN-γ	IGFBP-3	IGFBP-5	IGFBP-6	IL-1α	IL-1 β	IL-2	IL-3	IL-3Rb
5	IL-4	IL-5	IL-6	IL-9	IL-10	IL-12 p40/70	IL-12 p70	IL-13	IL-17	KC	Leptin R	Leptin	LIX	L-Selectin
6	IL-4	IL-5	IL-6	IL-9	IL-10	IL-12 p40/70	IL-12 p70	IL-13	IL-17	KC	Leptin R	Leptin	LIX	L-Selectin
7	Lymphotactin	MCP-1	MCP-5	M-CSF	MIG	MIP-1α	MIP-1γ	MIP-2	MIP-3β	MIP-3α	PF-4	P-Selectin	RANTES	SCF
8	Lymphotactin	MCP-1	MCP-5	M-CSF	MIG	MIP-1α	MIP-1γ	MIP-2	MIP-3β	MIP-3α	PF-4	P-Selectin	RANTES	SCF
9	SDF-1α	TARC	TCA-3	TECK	TIMP-1	TNF-α	sTNF RI	sTNF RII	TPO	VCAM-1	VEGF	Blank	Blank	POS
10	SDF-1α	TARC	TCA-3	TECK	TIMP-1	TNF-α	sTNF RI	sTNF RII	TPO	VCAM-1	VEGF	Blank	Blank	POS

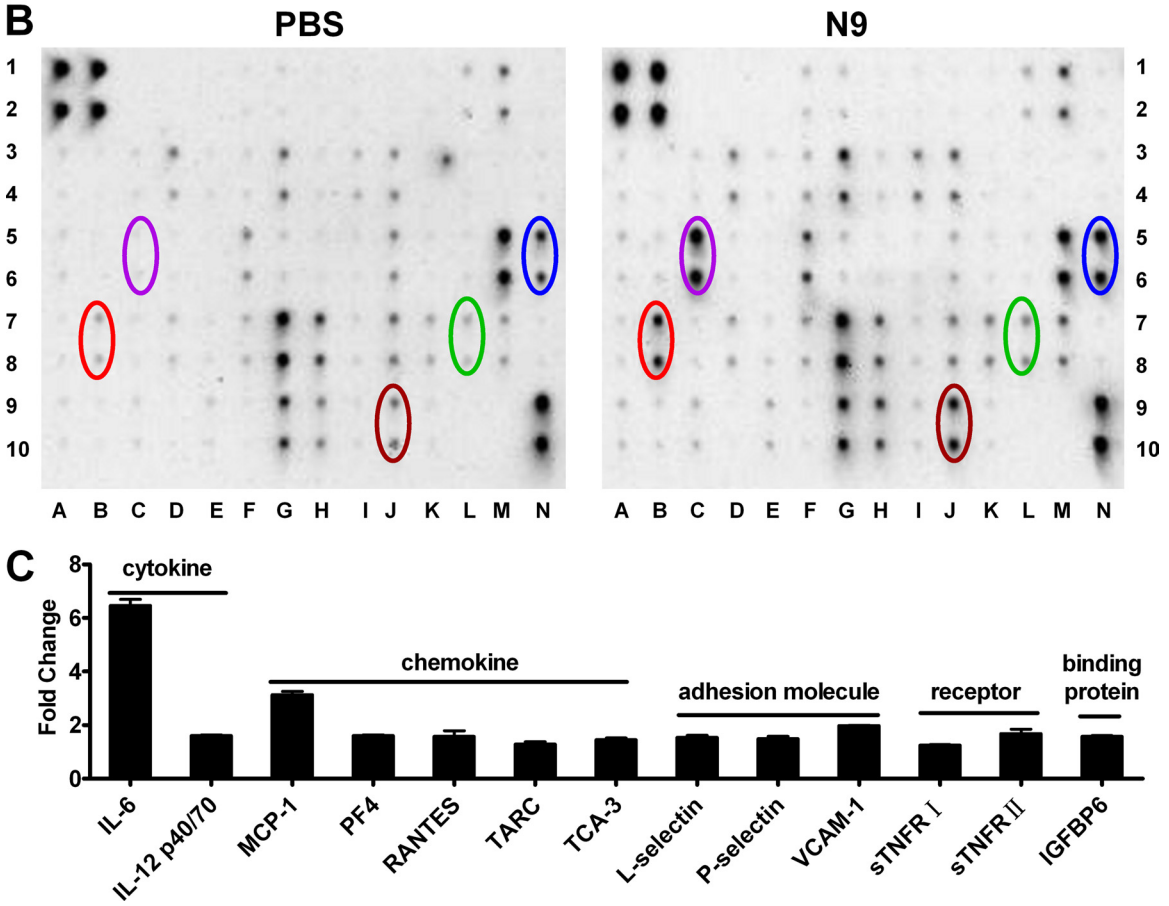

FIG 2 Cytokine profile of murine CVL fluid by N9 treatment. Mice were treated intravaginally with a single 10-μl dose of 40 mg/ml N9 in PBS or PBS alone for 4 h, and CVL fluid was harvested as described in Materials and Methods. The supernatant of CVL fluid was subjected to analysis with a RayBio mouse cytokine antibody array III, as described in Materials and Methods. Reactive protein spots were visualized by enhanced chemiluminescence detection. (A) Template showing the location of cytokine antibodies spotted in duplicate onto the RayBio mouse cytokine antibody array III. SDF-1α, stromal cell-derived factor 1α; GCSF, granulocyte colony-stimulating factor; M-CSF, macrophage colony-stimulating factor; TECK, thymus-expressed chemokine; GM-CSF, granulocyte-macrophage colony-stimulating factor; MIG, monokine induced by gamma interferon; TIMP-1, tissue inhibitors of metalloproteinases 1; IFN-γ, gamma interferon; BLC, B-lymphocyte chemoattractant; TPO, thrombopoietin; CRG-2, cytokine responsive gene-2; VEGF, vascular endothelial growth factor; CTACK, cutaneous T-cell-attracting chemokine; LIX, lipopolysaccharide-induced CXC chemokine; POS, positive; NEG, negative. (B) Representative photographs of cytokine arrays using CVL fluid from N9- or PBS (as a control)-treated mice. Colored circles mark differentially expressed cytokines: purple, IL-6; red, MCP-1; blue, L-selectin; green, P-selectin; and brown, VCAM-1. CVL fluid samples from 13 mice per group were pooled for each experiment, and the cytokine array was repeated three times and showed similar results each time. (C) Fold change in levels of proteins significantly increased by N9 treatment compared with PBS control treatment. Data are represented as mean ± SD for duplicate spots of one experiment.

TABLE 1 Time points of composite mouse histopathology scores obtained after a single administration of 40 mg/ml N9 in PBS or PBS alone (as control)[d]

	Histopathology score									
	PBS					N9				
Time (h)	Total	Epithelial cell disruption	Leukocyte infiltration	Edema	Congestion	Total	Epithelial cell disruption	Leukocyte infiltration	Edema	Congestion
0	2.0 ± 0.0	0.0 ± 0.0	1.0 ± 0.0	0.0 ± 0.0	1.0 ± 0.0	2.0 ± 0.0	0.0 ± 0.0	1.0 ± 0.0	0.0 ± 0.0	1.0 ± 0.0
2	2.2 ± 0.45	0.0 ± 0.0	1.2 ± 0.45	0.0 ± 0.0	1.0 ± 0.0	7.8 ± 1.48[c]	3.2 ± 0.45[c]	2.8 ± 0.45[c]	0.6 ± 0.55[c]	1.2 ± 0.45
4	2.8 ± 0.45	0.0 ± 0.0	1.8 ± 0.45	0.0 ± 0.0	1.0 ± 0.0	9.6 ± 0.89[c]	4.0 ± 0.0[c]	4.0 ± 0.0[c]	0.2 ± 0.45	1.4 ± 0.55
6	2.8 ± 0.45	0.0 ± 0.0	1.8 ± 0.45	0.0 ± 0.0	1.0 ± 0.0	8.4 ± 0.89[c]	4.0 ± 0.0[c]	2.8 ± 0.45[b]	0.0 ± 0.0	1.6 ± 0.55[a]
8	2.8 ± 0.84	0.0 ± 0.0	1.8 ± 0.84	0.0 ± 0.0	1.0 ± 0.0	7.4 ± 0.55[c]	4.0 ± 0.0[c]	2.2 ± 0.45	0.0 ± 0.0	1.2 ± 0.45
12	2.2 ± 0.45	0.0 ± 0.0	1.2 ± 0.45	0.0 ± 0.0	1.0 ± 0.0	6.4 ± 0.89[c]	3.4 ± 0.55[c]	1.8 ± 0.45	0.0 ± 0.0	1.2 ± 0.45
24	2.2 ± 0.45	0.0 ± 0.0	1.2 ± 0.45	0.0 ± 0.0	1.0 ± 0.0	5.0 ± 1.23[c]	2.4 ± 0.55[c]	1.4 ± 0.55	0.0 ± 0.0	1.2 ± 0.45
48	2.4 ± 0.55	0.0 ± 0.0	1.4 ± 0.55	0.0 ± 0.0	1.0 ± 0.0	3.0 ± 0.71	0.4 ± 0.55	1.6 ± 0.55	0.0 ± 0.0	1.0 ± 0.0

[a] $P < 0.05$ compared with that of corresponding control group (two-way ANOVA, followed by Bonferroni posttests).
[b] $P < 0.01$ compared with that of corresponding control group (two-way ANOVA, followed by Bonferroni posttests).
[c] $P < 0.001$ compared with that of corresponding control group (two-way ANOVA, followed by Bonferroni posttests).
[d] Mice were sacrificed at the indicated time points following a single application of 40 mg/ml N9 in PBS or PBS alone. The vaginal tissues were immediately taken and processed for histopathological examination, which was performed blindly by evaluation for epithelial cell disruption, leukocyte infiltration, edema, and congestion. Untreated mice were utilized as baseline controls, i.e., the 0-h time point, to document the normal tissue architecture and inflammation status in the vaginal mucosa. The histopathological scores were assigned by a semiquantitative system which was based on the scoring system of Eckstein et al. (11) and employed in our previous work (27). The detail of the scoring system is shown in Table S1 in the supplemental material. Data are represented as mean ± SD obtained from five mice per group of one experiment.

tration of leukocytes. Changes were detectable at as early as 2 h, and cumulative scores are statistically significant during 24 h. The epithelial damage rose to a peak at about 4 h, and the epithelium began to slowly regenerate after 12 h of treatment, recovering almost to the control level by 48 h. Leukocyte infiltration exhibited a similar increase but resolved much earlier (at 8 h). Edema was not seen in PBS-treated mice and was seen in only some tissues at 4 and 6 h in N9-treated animals. Conversely, dilation could be seen in all tissues, but it was more obvious in tissues from N9-treated mice (Table 1).

To verify the histological changes and further elucidate the quality and quantity of infiltrated leukocytes, leukocytes entering the vaginal tract in CVL fluid were collected and analyzed by flow cytometric analysis. At all time points shown in Fig. 3A, more than 95% of the leukocytes present in CVL fluid of PBS-treated mice were neutrophils. In contrast, the percentage of neutrophils of N9-treated mice was approximately 96% at 2 h and then decreased following influx of macrophages. Leukocyte and neutrophil counts were variable among individual mice, and mean counts were not significantly different between N9-treated and control mice at any time point during this experiment (Fig. 3A). The macrophage count exhibited a significant increase at the 12-h time point after N9 treatment. The percentage of macrophages started to rise at 2 h post-N9 treatment, reached a peak at 12 h, and then returned to the control baseline by 48 h. Because the percentage of macrophages displayed a clear trend during the cervicovaginal inflammation process, we next used the macrophage percentage as an index of leukocyte infiltration.

As shown in Fig. 3B and Fig. 1, proinflammatory cytokine IL-6 and chemokine MCP-1 in CVL fluid increased only transiently, with peaks at the 2- and 4-h time points, respectively, followed by a sharp decline and an immediate return to baseline values at the later time points. There was no significant increase of soluble E-selectin detected at any time point. Strikingly, L-selectin and P-selectin were rapidly increased at the 2- and 4-h time points, respectively, peaked at the corresponding later time point, and persisted at significantly increased levels over 12 h. It is noted that the significant release of soluble L-selectin and P-selectin was contemporaneous with tissue damage (total score), which started at 2 h, peaked at 4 h, and persisted for 12 h with the most severe damage (Table 1). Therefore, the time course and duration of the soluble L-selectin and P-selectin levels correlated better than those of MCP-1 and IL-6 levels with the time course and duration of tissue damage.

Soluble L-selectin and P-selectin were more sensitive than MCP-1 and IL-6 in determining the mild degree of inflammation induced by treatment with a low concentration of N9. To compare the sensitivity of soluble selectins with that of proinflammatory cytokine IL-6 and chemokine MCP-1 in determining cervicovaginal inflammation, we tested different degrees of inflammation by intravaginal application of various concentrations of N9 (40, 8, 1.6, and 0.32 mg/ml in PBS) or PBS only (0 mg/ml). At 4 h after the treatment, significant tissue damage was found in mice treated with N9 at a concentration of 1.6 mg/ml or higher (Fig. 4A). Flow cytometric analysis demonstrated that significant macrophage infiltration occurred in mice treated with N9 at a concentration of 8 mg/ml or higher (Fig. 4B).

Compared to the 0 dose (PBS only), a dose of 40 mg/ml N9 was sufficient to induce significant increases of MCP-1 and IL-6, while 8 mg/ml N9 could induce mild, but not significant, expression of MCP-1 and IL-6 (Fig. 4C and D). However, the 8-mg/ml N9 dose induced significant increases of L- and P-selectins (Fig. 4E and F) which correlated closely with the obvious tissue damage induced by that dosage (Fig. 4A). These data demonstrated that L- and P-selectins can predict cervicovaginal inflammation and tissue damage more sensitively than MCP-1 or IL-6, especially for determining a mild degree of cervicovaginal inflammation. Exposure to none of the concentrations of N9 tested induced any significant increase of E-selectin (Fig. 4G).

L- and P-selectins were increased in animals treated with several detergent-based proinflammatory compounds. We wondered whether the increase of selectins in cervicovaginal inflammation is unique to N9 only or if it could be generalized to other proinflammatory compounds. Furthermore, we were intrigued to

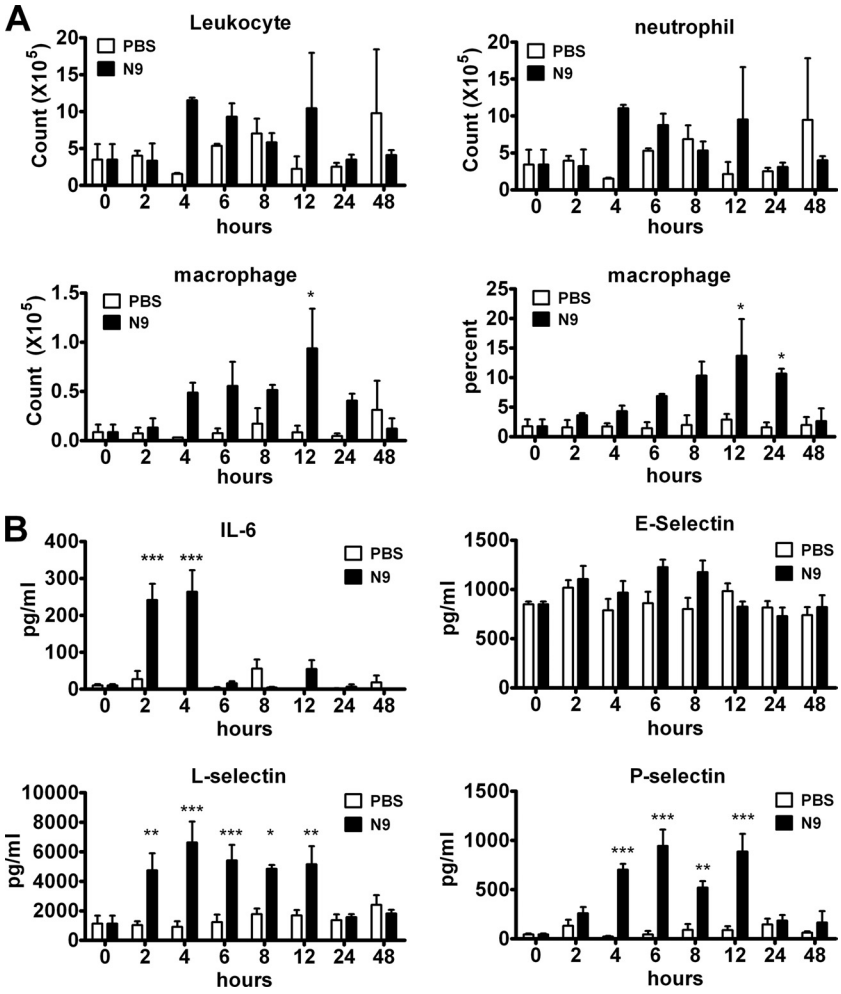

FIG 3 Soluble selectins show distinct time course compared with proinflammatory cytokines by application of N9. Mouse CVL fluid was collected, and the mice were sacrificed at the indicated time points following a single application of 40 mg/ml N9 in PBS or PBS alone. The untreated mice were regarded as the baseline, i.e., the 0-h time point. Five mice were set for each time point of each treatment. Supernatant was separated from cell debris by centrifugation. (A) Recruitment of inflammatory cells into the vaginal lumen determined by flow cytometric analysis. Leukocytes of each group were harvested by lavage, pooled, and stained for expression of CD45, Gr1, and F4/80. Gates for leukocytes, neutrophils, or macrophages were set on CD45/FSC, CD45/Gr1, or CD45/F4/80 dot plots to identify the desired subsets. Leukocytes, neutrophils, and macrophages were identified as $CD45^+$, $CD45^+$ $Gr1^+$, and $CD45^+F4/80^+$ cells, respectively. Data are represented as the mean ± SE of two experiments. (B) Soluble factors in supernatant of CVL fluid were quantified by ELISA. The proinflammatory cytokine IL-6 increased only transiently in CVL fluid. For E-selectin, there was no difference between the N9 treatment group and controls at all time points. Notable exceptions were soluble L-selectin and P-selectin, which rapidly increased and persisted at significantly increased levels over 12 h. Experiments were repeated two times and showed similar results. Data are represented as mean ± SD for individual mice of one representative experiment. *P* values are for the N9 treatment group versus the corresponding PBS control: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

know the correlation of selectins and TFV, the only proved efficacious and safe microbicide candidate at present. To address these questions, we tested two additional proinflammatory compounds (SDS and BZK) together with an excipient (CMC) and a microbicide candidate (TFV), which were expected to be noninflammatory, besides the PBS blank control. TFV and BZK were tested at concentrations of 10 mg/ml and 20 mg/ml diluted in PBS, respectively; N9, SDS, and CMC were tested at a 40-mg/ml concentration, since this concentration has inflammatory consequences and increased susceptibility to HSV in a mouse model (9).

The histopathological examination of cervicovaginal tissue performed 4 h after a single compound application revealed that the vaginal mucosa was intact in the PBS-, CMC-, and TFV-treated mice, with some leukocytes and dilated blood vessels randomly spread throughout the tissue. The tissue from N9-, SDS-, and BZK-treated mice, however, showed various degrees of epithelial damage, leukocyte infiltration, edema, and dilation (Fig. 5A). BZK ranked first in epithelial damage with edema, while N9 ranked first in leukocyte infiltration. All three detergents completely disrupted the epithelial lining, while BZK damage extended to the lamina propria. Multiple intra- and submucosal leukocyte infiltrates were found in the N9-treated mice; however, leukocyte infiltration was also seen, albeit in a low degree, in BZK- and SDS-treated mice. Dilated blood vessels were more obvious in tissues from N9-, SDS-, and BZK-treated animals than in those from controls (Fig. 5A). All histopathology findings were in accordance with previous reports (13, 39).

The results of flow cytometric analysis of cells collected from the mouse vaginal lumen by lavage demonstrated that the percentage of macrophages remained relatively unchanged after ap-

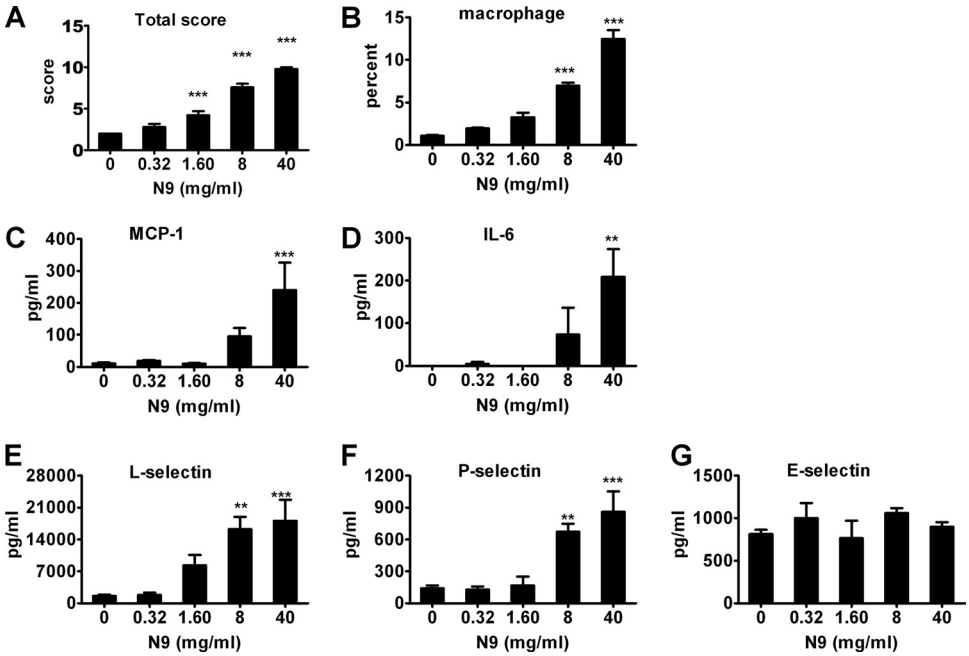

FIG 4 L- and P-selectins are more sensitive than IL-6 and MCP-1 as an indicator of inflammation induced by N9. In three independent experiments, a group of 10 mice was treated intravaginally with a single dose of a serial 5-fold dilution of N9 from 40 mg/ml to 0.32 mg/ml. CVL fluid was collected at 4 h after application. Supernatant was separated from cell debris by centrifugation and was evaluated by ELISA for detection of soluble factors. Cells of each group were pooled and analyzed for macrophages by flow cytometric analysis. Mice were sacrificed immediately after lavage fluid collection, and vaginas were dissected for histology analysis. (A) Cumulative (total) scores are shown for one representative experiment with 5 animals per group; (B) macrophages as a percentage of leukocytes of three independent experiments determined by flow cytometric analysis; (C to G) soluble factors of CVL fluid: MCP-1 (C), IL-6 (D), L-selectin (E), P-selectin (F), and E-selectin (G). *P* values are for various concentrations (except the 0 concentration) of N9 treatment versus the 0 concentration (PBS control): *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (A and C to G) Data are represented as mean ± SD for individual mice of one experiment; (B) data are represented as mean ± SE value of three independent experiments.

plication of CMC and TFV but increased significantly after treatment with N9, SDS, and BZK (Fig. 5B) compared with the PBS control.

At 4 h after a single application, the excipient CMC and the microbicide candidate TFV did not significantly increase any soluble factor compared with PBS alone. On the contrary, all detergents tested here produced a significant increase in two or more soluble factors. MCP-1 and IL-6 were increased for only one or two detergents, and E-selectin remained unchanged for all detergents. However, L-selectin and P-selectin were significantly increased for all detergents tested (Fig. 5C). As Fichorova and her associates found that IL-1 and IL-8 could predict the mucosal toxicity of a vaginal microbicidal (13), we also checked the level of IL-1β and keratinocyte-derived cytokine (KC) and found that IL-1β was increased only by N9 treatment and KC remained unchanged for all compounds tested in our murine model (Fig. S1 in the supplemental material).

Furthermore, as shown in Fig. 5D, Pearson's correlation analysis between these five soluble factors and the total scores obtained in 30 test mice 4 h after a single-dose administration found (i) a significant correlation between CVL fluid L-selectin, P-selectin, and IL-6 levels and the total score ($r = 0.664$, $P < 0.0001$; $r = 0.665$, $P < 0.0001$; and $r = 0.523$, $P = 0.003$, respectively) but (ii) no significant correlation between MCP-1 ($r = 0.257$, $P = 0.171$) or E-selectin ($r = 0.092$, $P = 0.627$) and the total score. Hence, soluble L-selectin and P-selectin correlated better with the tissue damage.

In parallel, three consecutive compound applications with a 48-h posttreatment follow-up period showed a similar pattern of proinflammatory cytokines and soluble selectins (Fig. 6). All detergents tested here induced a significant increase of L-selectin and P-selectin at 4 h after each application, and BZK also induced a delayed increase of these two molecules. N9, SDS, and BZK also induced a significant increase of E-selectin at some time points. On the other hand, MCP-1 and IL-6 were significantly induced only by N9 and BZK at a few time points and not triggered by SDS at all.

DISCUSSION

This study is the first to systematically characterize the soluble selectin levels in vaginal fluids during cervicovaginal inflammation induced by irritating compounds in a murine model. In addition, this is the first report to compare the levels of soluble selectins and those of proinflammatory cytokine IL-6 and chemokine MCP-1 in the prediction of cervicovaginal inflammation.

In progestin-treated C57BL/6 mice, we observed that the baseline CVL fluid level of P-selectin was low and that the levels of L-selectin and E-selectin were moderate. A single dose or three consecutive daily doses of a proinflammatory agent (N9, SDS, or BZK) promoted the significant increase of L-selectin and P-selectin but that of E-selectin only on some occasions. Furthermore, compared with MCP-1 and IL-6, the previously defined biomarkers of cervicovaginal inflammation, L-selectin and P-selectin, had prolonged significant increases. They were also more sensitive, broad-spectrum indications of mucosal inflammation induced by

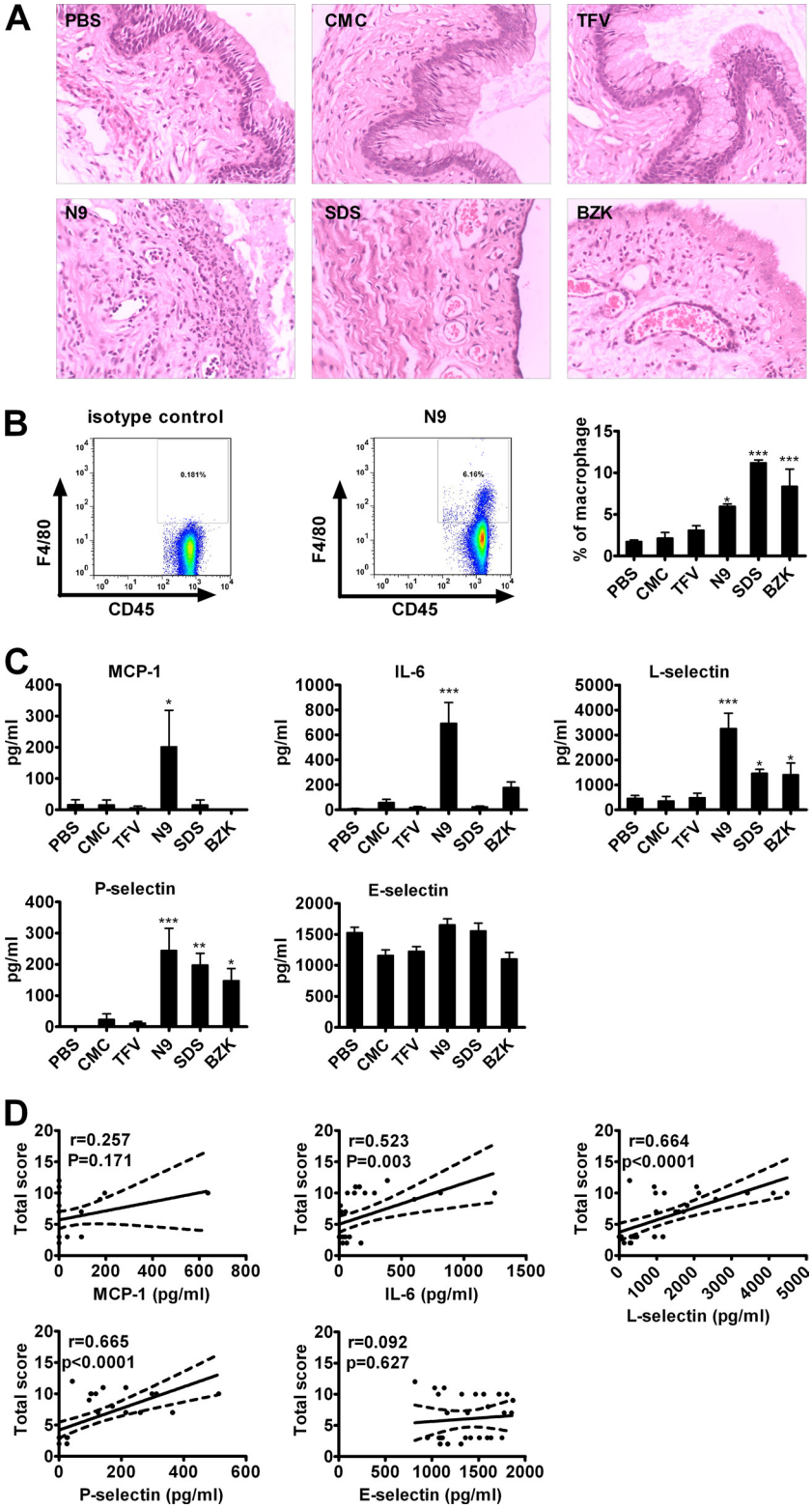

FIG 5 L- and P-selectin correlate closely with the severity of mucosal inflammation induced by N9, SDS, and BZK. A group of 10 mice was treated intravaginally with a single dose of 40 mg/ml CMC, 10 mg/ml TFV, 40 mg/ml N9, 40 mg/ml SDS, and 20 mg/ml BZK in PBS or PBS alone. CVL fluid was collected at 4 h after application. Supernatant was separated from cell debris by centrifugation and was evaluated for detection of soluble factors by ELISA. Cells of 3 to 5 mice from each group were pooled and analyzed for macrophage by flow cytometric analysis. Mice were sacrificed immediately after lavage fluid collection, and vaginas were dissected for histology analysis. (A) Representative photographs comparing histological findings in full-thickness paraffin-embedded abdominal vaginal sections from mice treated with a single application of CMC, TFV, N9, SDS, and BZK in PBS or PBS alone. H&E staining; magnification, ×400. (B) The percentage of macrophages present in leukocytes was determined by flow cytometric analysis of macrophages prepared from CVL fluid. (Left and middle) Density plots of

different compounds, and their levels showed a better positive correlation to the time course and severity of inflammatory tissue damage. Thus, our study suggests that L-selectin and P-selectin, in addition to the proinflammatory chemokine MCP-1 and cytokine IL-6, should be novel biomarkers of cervicovaginal inflammation, improving prediction of the proinflammatory side effects of other microbicide candidates for vaginal application.

The human cervicovaginal mucosa is the primary target of HIV-1 infection during male-to-female transmission. Mounting evidence indicates that cervicovaginal inflammation caused by sexually transmitted infections, bacterial vaginosis (BV), and some vaginal products, including microbicides, might increase HIV-1 infection risk (19, 22, 35). However, the mechanism underlying such a phenomenon is not well-known, and a validated biomarker of cervicovaginal inflammation is lacking. It has been suggested that epithelial damage or activation in the mucosa leads to IL-1β secretion and transactivation of NF-κB, resulting in release of cytokines (IL-1β, IL-6, and tumor necrosis factor alpha [TNF-α]) and chemokines (IL-8, IL-10, and MIP-3α). The resulting influx of neutrophils and HIV-1 target cells can lead to subsequent HIV-1 infection and virus shedding (10). Because the concentrations of these proinflammatory cytokines and chemokines in vaginal secretions correlate with epithelial damage, these cytokines/chemokines have been defined to be biomarkers of cervicovaginal inflammation. However, these molecules reflect early events in the inflammatory cascade that may be blocked by endogenous antagonists that have pleiotropic or opposing functions. These properties complicate the interpretation of cytokine profiles and their use as biomarkers of vaginal product safety. Therefore, markers specific for leukocyte infiltration, the focus of inflammation, would be a useful addition to current tools for monitoring cervicovaginal mucosal inflammation (10, 39).

Selectins mediate rolling, the first step of leukocyte infiltration, which is essential for both the development of an appropriate inflammatory response to injury or infection and the debilitating sequence of events leading to inflammatory diseases (20). The selectins are a calcium-dependent, type I transmembrane glycoprotein family of adhesion molecules. Three selectins exist, including E-selectin, P-selectin, and L-selectin, named for the cell type in which they were originally identified (endothelium, platelet, and leukocyte). All three selectins share a similar structure containing an N-terminal lectin-like domain, an epidermal growth factor-like domain, a variable number of consensus repeats (CRs), a single transmembrane domain, and a short cytoplasmic tail (20). The most important ligand for selectins is the glycoprotein P-selectin glycoprotein ligand-1 (PSGL-1), which is present as a homodimer on leukocytes and can bind to the three selectins (25). Selectins are involved in leukocyte recruitment and in acute and chronic inflammation processes, including postischemic inflammation in muscle; kidney, heart, and skin inflammation; atherosclerosis; glomerulonephritis; and lupus erythematosus (24). Soluble forms of selectins, which most likely arise by shedding or cleavage from the cell membrane, have been identified in serum and inflamed synovial fluid (4, 33, 43). Although considerable evidence has indicated that elevated serum concentrations of soluble selectins are present in patients with different inflammatory diseases and correlate with disease duration and severity (5, 17, 36), only one previous study has evaluated the association between soluble E-selectin in cervicovaginal secretions and mucosal inflammation (39).

In 2007, Trifonova and coworkers (39) reported that soluble E-selectin was expressed at low, but detectable, levels in blank rabbits and was raised by BZK application. Similarly, our results showed that soluble E-selectin is also raised by three daily doses of detergents at some time points. However, some differences also existed: the baseline level of E-selectin in mice was moderate. The most rational explanation for this discrepancy is the difference in animal models studied: a rabbit model was used in the study of Trifonova et al. (39), and a murine model was used in ours. The progestin treatment may also be a reason. In our study, to minimize the influence of the reproductive cycle, the mice were pretreated 7 days before study with progestin, a treatment that produces a diestrous-like state, in which the epithelium is mucified columnar and the lumen contains mucus and desquamated cells (9). Consistent with previous studies (32, 37), our results showed that neutrophils distributed in the lumen and mucosal tissue of the vagina from progestin-treated healthy mice, but macrophages were rarely found. After surfactant treatment, neutrophils did not change significantly, but the percentage of macrophages was raised significantly. It is reported that every one of the three selectins is involved in neutrophil recruitment to skin and mucosal membranes, but P- and E-selectins are especially important. P- and L-selectins play key roles in monocyte infiltration, but the role of E-selectin is less significant (24, 34, 44). Therefore, it is reasonable that there should be a moderate level of E-selectin following influx of a naturally occurring population of neutrophils in healthy mice. The percentage of neutrophils in cervicovaginal inflammation did not change significantly; accordingly, E-selectin had no significant change. However, P- and L-selectins rose significantly, followed by a large influx of macrophages, which was evidenced by P- and L-selectin association with the increased percentage of macrophages. Macrophages are important HIV-1 cell targets *in vivo*; therefore, the large influx of macrophages resulting from microbicide-induced mucosal inflammation would increase the chance of HIV-1 infection. This phenomenon is worth further study.

As shown in Fig. 5, the increased levels of L-selectin and P-selectin in mouse CVL fluid were associated with the presence of an increased number of leukocytes and dilated blood vessels in tissue sections. Migrating leukocytes become the source of soluble L-selectin released into vaginal secretions. Besides activated endothelium and platelets, infiltrating macrophages may be another source of P-selectin because the molecule can also be expressed on macrophages (26, 38). To shed some light on the origin of soluble

isotype control and N9 treatment for one representative experiment to show how macrophage percent was determined; (right) data are represented as mean ± SE value of three replicate tests of one experiment. (C) Soluble factors MCP-1, IL-6, L-selectin, P-selectin, and E-selectin. Increases in L-selectin and P-selectin were detected in CVL fluid from mice treated with all detergents tested; increases in IL-6 and MCP-1 were detected only after treatment with N9 or BZK. Data are represented as mean ± SD for individual mice of one experiment. (D) Pearson's correlation analysis of cumulative (total) mouse vaginal irritation scores and MCP-1, IL-6, L-selectin, P-selectin, and E-selectin for 30 animals. Dashed lines represent the 95% confidence intervals. The correlation coefficients (r) and P values are also shown. (B and C) P values are for detergent group versus PBS control: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

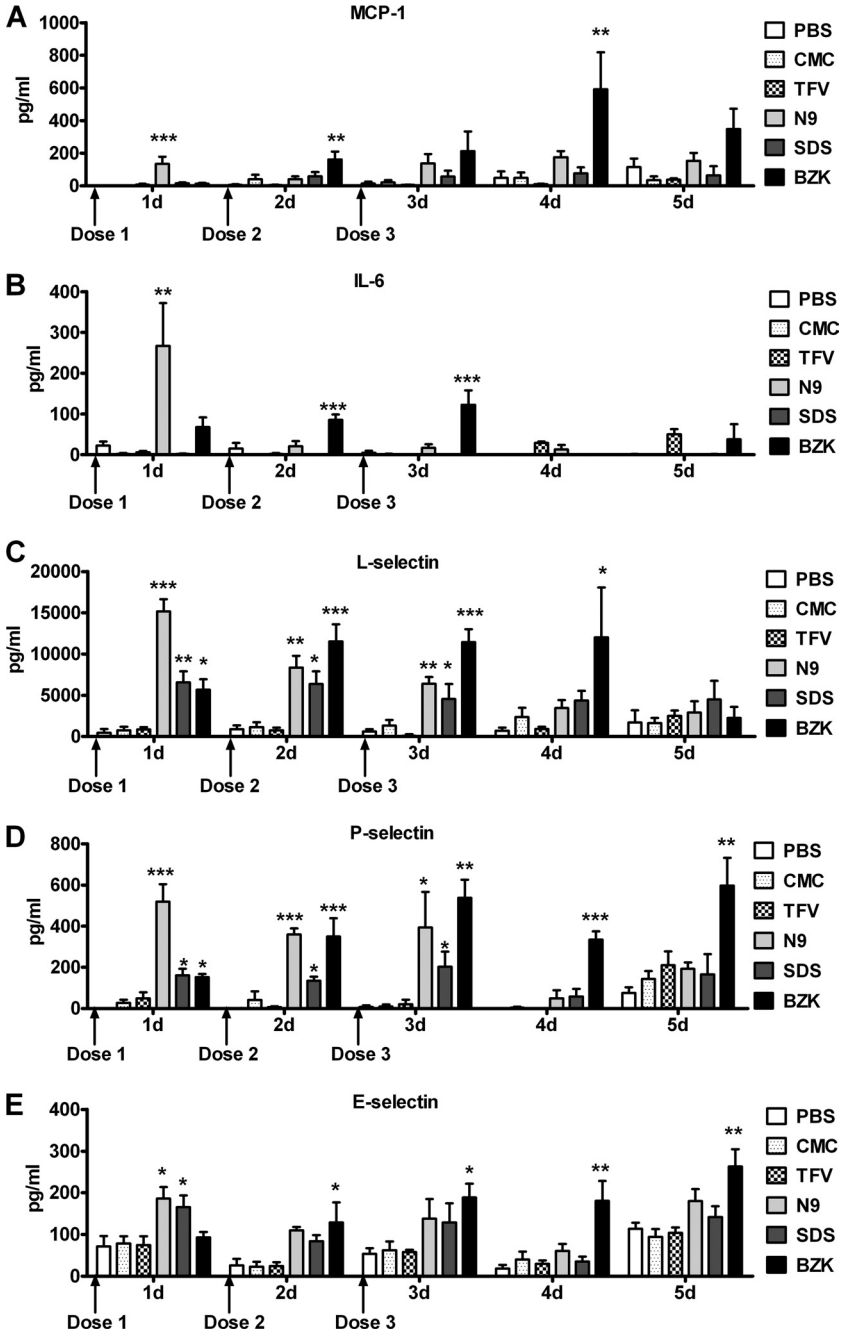

FIG 6 Increase in soluble selectin, MCP-1, and IL-6 levels by consecutive doses of N9, SDS, and BZK. Mice were treated intravaginally with three consecutive doses of 40 mg/ml CMC, 10 mg/ml TFV, 40 mg/ml N9, 40 mg/ml SDS, and 20 mg/ml BZK in PBS or PBS alone. CVL fluid was collected at 4 h after each application and 28 and 52 h after the last application. Supernatant was separated from cell debris by centrifugation and was evaluated for detection of soluble factors by ELISA. Each dose administration is marked by a black arrow. (A and B) Responses of proinflammatory chemokine MCP-1 (A) and IL-6 (B) in mouse CVL fluid samples; (C to E) responses of soluble L-selectin (C), P-selectin (D), and E-selectin (E) in mouse CVL fluid samples. Results represent mean ± SD from five mice for one experiment. *P* values are for the proinflammatory compound treatment group versus PBS control at each time point: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

L-selectin and P-selectin, we checked the surface expression of L- and P-selectins by flow cytometric analysis. As shown in Fig. S2 in the supplemental material, there were low but easily detectable levels of P-selectin expressed on vaginal leukocytes and the subpopulations of neutrophils and macrophages, and the expression was downregulated by N9 treatment. However, L-selectin was expressed at a moderate level, and there was no significant change by N9 treatment. These data gave us some clues that leukocytes and the subpopulation could be the origin of soluble L- and P-selectins.

Since the sole role of selectins is mediating leukocyte rolling and since soluble selectins arise from infiltrating leukocytes and

activated endothelium, we reasoned that the significant increase of these molecules in cervicovaginal fluid could be a good additional predictor of cervicovaginal inflammation.

Until now, the rabbit vaginal irritation (RVI) model focused on histopathological findings is the only animal model recommended for use by the U.S. Food and Drug Administration to assess the toxicity of vaginal products. This model was improved by Fichorova and her colleagues thereafter (13, 39). The refined model was expanded to include soluble markers of inflammation and vascular activation. Besides the classical RVI model, the mouse model is also used in preclinical mucosal safety assessment for microbicide candidates. Despite the limitation that mice are not naturally susceptible to HIV, extensive experience with murine models in immunological studies combined with the low cost and the availability of a wide array of reagents suggested the feasibility of a mouse model in the study of the mucosal safety of microbicides. A few studies examined the mucosal inflammatory response to intravaginally administered N9 and SDS in the mouse model and found exfoliation of epithelia, infiltration of neutrophils and macrophages into the genital lumen and tissue, increases in cytokines and chemokines, and activation of the transcription factor NF-κB (7–9, 16, 32). Catalone and colleagues found that the cervix was the site of N9- and C31G-associated damage and inflammation, while the vaginal epithelium was resistant to the damaging effects of these surfactants (7, 8). However, our study found that the cervix (data not shown) and the vaginal epithelium are both sensitive to N9 irritation. In fact, by pretreatment with progestin, except for the orifice of the vagina, which is covered with the stratified squamous epithelium, the other parts are covered with just single-column epithelia. Accordingly, the former is more resistant and the latter is more sensitive to the irritation from N9 and other proinflammatory agents. Our study also agreed with two other previous studies (9, 16). Despite some discrepancy, our study and the studies of Catalone et al. (7, 8) also had some agreements: N9-associated damage peaked at about 2 to 4 h postapplication and was resolved by 24 to 48 h postexposure. The present study and the previous studies all showed that the murine model is useful for assessment of the mucosal safety of microbicide candidates. As progestins have distinct effects on the cervicovaginal epithelial immune function, results obtained in the medroxyprogesterone-treated mouse model have to be interpreted with caution. The progestin treatment may also be a reason for some discrepancies between the mouse model study and other studies in rabbits and primates. Pretreatment with progestin (medroxyprogesterone) markedly eliminates the influence of the reproductive cycle and enhances the sensitivity to damage, but this cannot be practiced in humans. In nonhuman primate (NHP) or clinical trials, we can carefully record the menstrual cycle of NHPs or women and analyze the collected data grouped by menstrual cycle.

Clinical development of candidate microbicides is expensive and time-consuming; consequently, it is critical to ensure that products be safe and economically viable before moving from the preclinical to the clinical phase. Unfortunately, the current preclinical process is imperfect, and efforts are under way to develop new safety biomarkers and efficacy models (10). This study proposes novel soluble noninvasive markers of cervicovaginal inflammation in a murine model by comparison with the previously described biomarkers MCP and IL-6. Though only epithelial damage-inducing proinflammatory compounds, including N9, SDS, and BZK, are used as positive controls in the mouse model and L-selectin and P-selectin may not be suitable markers for other compounds that are toxic through other mechanisms, the results from this study support the use of soluble L-selectin and P-selectin as additional biomarkers of cervicovaginal inflammation in the mouse model, and L-selectin and P-selectin warrant further assessment in humans. Moreover, the levels of soluble selectins in serum tended to be greater in HIV-1-infected patients, and a positive correlation with virus load and AIDS disease progression was evidenced by several previous studies (6, 21, 31). Therefore, further studies should examine their level in cervicovaginal secretions of healthy controls and HIV-1-infected patients to compare the differences and evaluate the association of levels of soluble selectins and HIV-1 infection. Nonetheless, this study has laid the foundation for the use of soluble L-selectin and P-selectin to monitor the mucosal safety of any microbicide targeted for prevention of HIV-1 and other sexually transmitted infections, and additional measurements of these soluble molecules may enhance the efficiency of further efforts to develop successful novel microbicides to counter HIV-1 infection.

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REFERENCES

1. **Abdool Karim Q, et al.** 2010. Effectiveness and safety of tenofovir gel, an antiretroviral microbicide, for the prevention of HIV infection in women. Science **329**:1168–1174.
2. **Alt C, et al.** 2009. Increased CCL2 expression and macrophage/monocyte migration during microbicide-induced vaginal irritation. Curr. HIV Res. **7**:639–649.
3. **Atashili J, Poole C, Ndumbe PM, Adimora AA, Smith JS.** 2008. Bacterial vaginosis and HIV acquisition: a meta-analysis of published studies. AIDS **22**:1493–1501.
4. **Barkhausen T, Krettek C, van Griensven M.** 2005. L-selectin: adhesion, signalling and its importance in pathologic posttraumatic endotoxemia and non-septic inflammation. Exp. Toxicol. Pathol. **57**:39–52.
5. **Barthel SR, Gavino JD, Descheny L, Dimitroff CJ.** 2007. Targeting selectins and selectin ligands in inflammation and cancer. Expert Opin. Ther. Targets **11**:1473–1491.
6. **Calza L, et al.** 2009. Plasma levels of VCAM-1, ICAM-1, E-selectin, and P-selectin in 99 HIV-positive patients versus 51 HIV-negative healthy controls. J. Acquir. Immune Defic. Syndr. **50**:430–432.
7. **Catalone BJ, et al.** 2004. Mouse model of cervicovaginal toxicity and inflammation for preclinical evaluation of topical vaginal microbicides. Antimicrob. Agents Chemother. **48**:1837–1847.
8. **Catalone BJ, et al.** 2005. Comparative safety evaluation of the candidate vaginal microbicide C31G. Antimicrob. Agents Chemother. **49**:1509–1520.
9. **Cone RA, et al.** 2006. Vaginal microbicides: detecting toxicities in vivo that paradoxically increase pathogen transmission. BMC Infect. Dis. **6**:90.
10. **Cummins JE, Jr, Doncel GF.** 2009. Biomarkers of cervicovaginal inflammation for the assessment of microbicide safety. Sex. Transm. Dis. **36**: S84–S91.
11. **Eckstein P, Jackson MC, Millman N, Sobrero AJ.** 1969. Comparison of vaginal tolerance tests of spermicidal preparations in rabbits and monkeys. J. Reprod. Fertil. **20**:85–93.

12. **Fichorova RN.** 2004. Guiding the vaginal microbicide trials with biomarkers of inflammation. J. Acquir. Immune Defic. Syndr **37**(Suppl 3): S184–S193.
13. **Fichorova RN, et al.** 2004. Interleukin (IL)-1, IL-6, and IL-8 predict mucosal toxicity of vaginal microbicidal contraceptives. Biol. Reprod. **71**: 761–769.
14. **Fichorova RN, et al.** 2011. Baseline variation and associations between subject characteristics and five cytokine biomarkers of vaginal safety among healthy non-pregnant women in microbicide trials. Cytokine **55**: 134–140.
15. **Fichorova RN, Tucker LD, Anderson DJ.** 2001. The molecular basis of nonoxynol-9-induced vaginal inflammation and its possible relevance to human immunodeficiency virus type 1 transmission. J. Infect. Dis. **184**: 418–428.
16. **Galen BT, et al.** 2007. A comprehensive murine model to evaluate topical vaginal microbicides: mucosal inflammation and susceptibility to genital herpes as surrogate markers of safety. J. Infect. Dis. **195**:1332–1339.
17. **Goke M, Hoffmann JC, Evers J, Kruger H, Manns MP.** 1997. Elevated serum concentrations of soluble selectin and immunoglobulin type adhesion molecules in patients with inflammatory bowel disease. J. Gastroenterol. **32**:480–486.
18. **Hillier SL, et al.** 2005. In vitro and in vivo: the story of nonoxynol 9. J. Acquir. Immune Defic. Syndr. **39**:1–8.
19. **Jamieson DJ, et al.** 2001. Longitudinal analysis of bacterial vaginosis: findings from the HIV epidemiology research study. Obstet. Gynecol. **98**: 656–663.
20. **Kelly M, Hwang JM, Kubes P.** 2007. Modulating leukocyte recruitment in inflammation. J. Allergy Clin. Immunol. **120**:3–10.
21. **Kourtis AP, et al.** 2000. Correlation of virus load and soluble L-selectin, a marker of immune activation, in pediatric HIV-1 infection. AIDS **14**: 2429–2436.
22. **Laga M, et al.** 1993. Non-ulcerative sexually transmitted diseases as risk factors for HIV-1 transmission in women: results from a cohort study. AIDS **7**:95–102.
23. **Langer HF, Chavakis T.** 2009. Leukocyte-endothelial interactions in inflammation. J. Cell. Mol. Med. **13**:1211–1220.
24. **Ley K.** 2003. The role of selectins in inflammation and disease. Trends Mol. Med. **9**:263–268.
25. **Ley K, Laudanna C, Cybulsky MI, Nourshargh S.** 2007. Getting to the site of inflammation: the leukocyte adhesion cascade updated. Nat. Rev. Immunol. **7**:678–689.
26. **Li G, Sanders JM, Phan ET, Ley K, Sarembock IJ.** 2005. Arterial macrophages and regenerating endothelial cells express P-selectin in atherosclerosis-prone apolipoprotein E-deficient mice. Am. J. Pathol. **167**:1511–1518.
27. **Li LZ, et al.** 2010. Establishing a Th17 based mouse model for preclinical assessment of the toxicity of candidate microbicides. Chin. Med. J. (Engl.). **123**:3381–3388.
28. **Mania-Pramanik J, Kerkar SC, Salvi VS.** 2009. Bacterial vaginosis: a cause of infertility? Int. J. STD AIDS **20**:778–781.
29. **Mauck CK.** 2009. Biomarkers for evaluating vaginal microbicides and contraceptives: discovery and early validation. Sex. Transm. Dis. **36**: S73–S75.
30. **Mauck CK, Baker JM, Barr SP, Johanson WM, Archer DF.** 1997. A phase I comparative study of three contraceptive vaginal films containing nonoxynol-9. Postcoital testing and colposcopy. Contraception **56**: 97–102.
31. **Miller TL, et al.** 2010. Biomarkers of vascular dysfunction in children infected with human immunodeficiency virus-1. J. Acquir. Immune Defic. Syndr. **55**:182–188.
32. **Milligan GN, Dudley KL, Bourne N, Reece A, Stanberry LR.** 2002. Entry of inflammatory cells into the mouse vagina following application of candidate microbicides: comparison of detergent-based and sulfated polymer-based agents. Sex. Transm. Dis. **29**:597–605.
33. **Mohan JS, Lip GY, Wright J, Bareford D, Blann AD.** 2005. Plasma levels of tissue factor and soluble E-selectin in sickle cell disease: relationship to genotype and to inflammation. Blood Coagul. Fibrinolysis **16**:209–214.
34. **Naruse T, et al.** 2002. P-selectin-dependent macrophage migration into the tubulointerstitium in unilateral ureteral obstruction. Kidney Int. **62**: 94–105.
35. **Ramjee G.** 2010. Microbicide research: current and future directions. Curr. Opin. HIV AIDS **5**:316–321.
36. **Ridker PM, Buring JE, Rifai N.** 2001. Soluble P-selectin and the risk of future cardiovascular events. Circulation **103**:491–495.
37. **Sonoda Y, et al.** 1998. Physiologic regulation of postovulatory neutrophil migration into vagina in mice by a C-X-C chemokine(s). J. Immunol. **160**:6159–6165.
38. **Tchernychev B, Furie B, Furie BC.** 2003. Peritoneal macrophages express both P-selectin and PSGL-1. J. Cell Biol. **163**:1145–1155.
39. **Trifonova RT, et al.** 2007. Biomarkers of leukocyte traffic and activation in the vaginal mucosa. Biomarkers **12**:608–622.
40. **Van Damme L, et al.** 2000. Safety of multiple daily applications of COL-1492, a nonoxynol-9 vaginal gel, among female sex workers. COL-1492 Phase II Study Group. AIDS **14**:85–88.
41. **Van Damme L, et al.** 1998. Safety evaluation of nonoxynol-9 gel in women at low risk of HIV infection. AIDS **12**:433–437.
42. **Van Damme L, et al.** 2002. Effectiveness of COL-1492, a nonoxynol-9 vaginal gel, on HIV-1 transmission in female sex workers: a randomised controlled trial. Lancet **360**:971–977.
43. **Yan MX, Mao HT, Liu Q, Wang WQ, Li YQ.** 2006. Elevated levels of serum soluble E-selectin in patients with chronic hepatitis B: correlation with T lymphocyte subsets, NK cells and liver inflammation. Hepatol. Res. **35**:111–117.
44. **Zarbock A, et al.** 2008. PSGL-1 engagement by E-selectin signals through Src kinase Fgr and ITAM adapters DAP12 and FcR gamma to induce slow leukocyte rolling. J. Exp. Med. **205**:2339–2347.
45. **Zegels G, Van Raemdonck GA, Tjalma WA, Van Ostade XW.** 2010. Use of cervicovaginal fluid for the identification of biomarkers for pathologies of the female genital tract. Proteome Sci. **8**:63.