Cytokine 120 (2019) 210–219

Contents lists available at ScienceDirect

Cytokine

journal homepage: www.elsevier.com/locate/cytokine

Cytokine expression profiles in cervical mucus from patients with cervical cancer and its precursor lesions

Sayaka Otani[a,1], Takuma Fujii[a,*,1], Iwao Kukimoto[b], Naoki Yamamoto[c], Tetsuya Tsukamoto[d], Ryoko Ichikawa[a], Eiji Nishio[a], Aya Iwata[a]

[a] Department of Obstetrics and Gynecology, Fujita Health University, School of Medicine, 1-98, Dengakugakubo, Kutsukakecho, Toyoake, Aichi 470-1192, Japan
[b] Pathogen Genomics Center, National Institute of Infectious Diseases, 4-7-1, Gakuen, Musashi-murayama, Tokyo 208-0011, Japan
[c] Regenerative Medicine Support Promotion Facility, Center for Research Promotion and Support, Fujita Health University, School of Medicine, 1-98, Dengakugakubo, Kutsukakecho, Toyoake, Aichi 470-1192, Japan
[d] Department of Pathology, Fujita Health University, School of Medicine, 1-98, Dengakugakubo, Kutsukakecho, Toyoake, Aichi 470-1192, Japan

ARTICLE INFO

Keywords:
Chemokine
Immune response
Cervix
Papillomavirus

ABSTRACT

Human papillomavirus (HPV) infection can persist in the cervical epithelium without provoking a strong host immune response, leading to the development of cervical cancer. Cytokines, which mediate innate and adaptive immune activities, are secreted in the cervical mucus; however, there is currently no appropriate method for assessing cytokine levels in mucus specimens. Here, we employed multiplexed bead-based immunoassays to examine cytokine levels in cervical mucus using both weighted-volume and total protein concentration methods to adjust for different specimen volumes in individual patients. Out of 18 cytokines initially examined in the primary cohort patient group (n = 28), 14 were detected in more than 10% of the samples. Of these 14 cytokines, expression levels of interferon (IFN)-γ, granulocyte–macrophage colony-stimulating factor (GM-CSF), RANTES, and eotaxin were significantly increased with the disease severity in the secondary cohort patient group (n = 235). We also examined associations between cytokine levels and clinical parameters, such as cytology and HPV genotype. Of the 14 cytokines, granulocyte colony-stimulating factor (G-CSF) was down-regulated in HPV-positive specimens. Examination of co-expression patterns of cytokines in relation to HPV infection status revealed that several pairs of cytokines were simultaneously upregulated in HPV-positive cases, including INF-γ and interleukin (IL)-17A, GM-CSF and monocyte chemoattractant protein-1 (MCP-1), GM-CSF and RANTES, IL-17A and RANTES, and MCP-1 and eotaxin. Interestingly, upregulation of GM-CSF and RANTES might reflect a shift in immuno-regulatory cytokines in HPV-positive specimens, potentially associated with more severe cervical neoplasia.

1. Introduction

Human papillomavirus (HPV) infection in the cervix causes cervical intraepithelial neoplasia (CIN). Temporal HPV infections cause low-grade squamous intraepithelial lesions/CIN1, whereas persistent HPV infection results in high-grade squamous intraepithelial lesions/CIN2–3, which represent precursor lesions of cervical cancer. Although some viral infections elicit strong host immune responses and are consequently eliminated by the host, HPV effectively evades such immune recognition, thereby allowing the establishment of persistent viral infection [1].

One of the main functions of cervical mucus is to prevent infections from microorganisms. This mucus includes cytokines, which mediate many innate and adaptive immune activities. However, the association between cervical neoplasia and mucosal cytokines is controversial and poorly understood. We therefore aimed to detect and identify cytokines in cervical mucus using multiplexed bead-based immunoassays to identify cytokines, chemokines, and growth factors, based on information from previous investigations [2–6] and the availability of specific antibodies.

However, specimen volumes vary among patients, and the use of different adjustment methods has resulted in inconsistent results among earlier studies [2,3]. We therefore used two different measurement methods to adjust for inter-individual differences: the weighted volume

* Corresponding author at: Department of Obstetrics and Gynecology, Fujita Health University, School of Medicine, 1-98, Dengakugakubo, Kutsukakecho, Toyoake, Aichi 470-1192, Japan.
E-mail address: fujiit44@fujita-hu.ac.jp (T. Fujii).
[1] Equal contributions.

https://doi.org/10.1016/j.cyto.2019.05.011
Received 30 October 2018; Received in revised form 13 May 2019; Accepted 13 May 2019
Available online 20 May 2019

(WV) method and the total protein concentration (TPC) method. We also measured cytokine levels in cell lines to determine if cervical cancer cells alone could produce candidate cytokines.

2. Materials and methods

2.1. Study subjects

The specimens were collected from patients, aged 24–89 years, who attended the outpatient clinic at Fujita Health University Hospital, Aichi prefecture, Japan, for routine gynecological examinations from October 2014 to September 2017. We excluded patients who (a) were younger than 20 years; (b) were pregnant; (c) had undergone previous treatment with chemotherapy, radiation, or surgery for any cancer or CIN; or (d) took medication for sexually transmitted diseases. Details of the pathological results and HPV genotypes are shown in Table S1. The study protocol was approved by the ethics committees of Fujita Health University and the National Institute of Infectious Diseases. Written informed consent was obtained from each patient.

We performed a series of experiments to compare cytokine profiles with clinical parameters, such as cytology and HPV genotype. Cervical mucus specimens for cytokine analysis were collected using Merocel™ cervical sponges (Medtronic Xomed, Inc., Jacksonville, FL, USA) and stored at −80 °C. We did not use any specimens collected during menstruation. Cytological interpretation was classified according to the Bethesda 2001 system. HPV genotype assays were performed by polymerase chain reaction with PGMY primers followed by reverse line blot hybridization [7]. This assay can detect 31 HPV genotypes, including HPV 6, 11, 16, 18, 26, 31, 33, 34, 35, 39, 40, 42, 44, 45, 51, 52, 53, 54, 55, 56, 57, 58, 59, 66, 68, 69, 70, 73, 82, 83, and 84.

2.2. Sample groups and strategy

We initially evaluated 18 cytokines in mucus specimens from 28 patients in the primary cohort. The cytology results were classified as negative for intraepithelial lesion or malignancy (NILM; $n = 10$), low-grade squamous intraepithelial lesion (LSIL; $n = 3$), high-grade squamous intraepithelial lesion (HSIL; $n = 7$), or cancer (invasive squamous cell carcinoma or adenocarcinoma; $n = 8$). We then evaluated the 14 detectable cytokines in specimens from 235 patients (including the initial 28 patients) in the secondary cohort. Their cytology results were classified as NILM ($n = 35$; median age: 37 years), LSIL ($n = 31$; median age: 38 years), HSIL ($n = 113$; median age: 37 years), and cancer ($n = 56$; median age: 56 years). Their HPV status was classified as positive ($n = 192$; median age: 39 years) or negative ($n = 43$; median age: 38 years).

2.3. Protein extraction from cervical sponges

Protein for cytokine analysis was extracted from Merocel™ cervical sponges, using previously described methods [3]. First, the wet weight of each sponge was recorded, and each sponge was then placed in a 2-ml Spin-X centrifuge filter tube (Corning Inc., Corning, NY, USA), and 300 µl of extraction buffer (phosphate-buffered saline, PBS, Sigma-Aldrich, St. Louis, MO, USA), 256 mM NaCl, and 100 µg/ml aprotinin (Wako, Amagasaki, Japan) were slowly added. The sponges were incubated at 4 °C for 2 h and then centrifuged at 14,000 rpm for 15 min at 4 °C, followed by the addition of 30 µl of fetal bovine serum to the 270 µl of extract. The sample was then vortexed briefly, aliquoted, and frozen at −80 °C until further testing. The remaining extracts were stored at −80 °C until the time of total protein measurement.

2.4. Cell cultures

Eleven human cervical cancer cells (C33a, SKG-1, SKG-2, SKG-3a, SKG-3b, SiHa, HeLa, HT-3, ME-180, CaSki, and C-4I) and two breast cancer cell lines (MCF7 and MDA-MB231) were obtained from Keio University, Japan (C33a, SKG-1, SKG-2, SKG-3a, SKG-3b, SiHa and HeLa), or the American Type Culture Collection (HT-3, ME-180, CaSki, C-4I, MCF7, and MDA-MB231). The identities of the analyzed cell lines obtained from Keio University were confirmed by short terminal repeat genotyping, which revealed a correspondence of > 80% of the markers tested. Among the cervical cancer cells, the HPV16 genome was found in SKG-3a, SKG-3b, SiHa, and CaSki cells, the HPV18 genome in SKG-1, SKG-2, HeLa, and C4I cells, and the HPV68 genome in ME-180 cells. HT-3 and C33a cells were HPV-negative. The cell lines were cultured in Ham's F-12 (Wako; C33a, SKG-1, SKG-2, SKG-3a, SKG-3b, SiHa, HeLa), RPMI-1640 (Gibco, Life Technologies, UK; CaSki), D-MEM (Wako; MCF7 and MDA-MB231), McCoy's 5a (Gibco; HT-3 and ME-180), or Waymouth MB 752/1 (Gibco; C4I), supplemented with 10% fetal bovine serum (Gibco), 10 µg/ml penicillin, and 10 µg/ml streptomycin (Wako) and maintained at 37 °C in 5% CO_2. All cell lines were seeded in 6-well plates and allowed to grow for 24 h. After 48 h in serum-free medium conditions, supernatants and cells were collected, resuspended in 300 µl of PBS, and lysed by repeated freeze–thaw cycles. Supernatants and lysates were stored at −80 °C until use.

2.5. Cytokine measurements using cytometric bead array

The following cytokines, chemokines, and growth factors were measured using multiplexed bead-based immunoassays (Cytometric Bead Array; CBA) according to the manufacturer's protocol (BD Biosciences, Franklin Lakes, NJ, USA): interleukin (IL)-1α (Cat# 560153), IL-1β (Cat# 558279), IL-2 (Cat# 558270), IL-4 (Cat# 558272), IL-6 (Cat# 558276), interferon (IFN)-α (Cat #560379), IFN-γ (Cat# 558269), tumor necrosis factor (TNF)-α (Cat# 558273), granulocyte–macrophage colony-stimulating factor (GM-CSF) (Cat# 558335), granulocyte colony-stimulating factor (G-CSF) (Cat# 558326), IL-10 (Cat# 558274), IL-8 (Cat# 558277), IL-17A (Cat# 560383), IL-21 (Cat# 560358), monocyte chemoattractant protein (MCP)-1 (Cat# 558287), macrophage inflammatory protein (MIP)-1α (Cat# 558325), RANTES (Cat# 558324), and eotaxin (Cat# 558329). Cervical extracts were thawed and diluted 1:1 to 1:1000 in extraction buffer depending on the cytokine levels. Briefly, a 10-point standard curve ranging from 0 to 2500 pg/ml for each cytokine was prepared using the cytokine standard provided in each kit. Samples and cytokine standards were incubated in the capture bead mixture for 1 h and phycoerythrin-conjugated antibodies against each cytokine were added to the sample-bead mixture for 2 h of incubation at room temperature. All buffers used were from the CBA human soluble protein master buffer kit (Cat# 558265, BD Biosciences). Beads were washed and analyzed using a BD FACSCalibur flow cytometer (BD Biosciences). Mean fluorescence intensity for each bead cluster was converted into cytokine concentrations based on the 10-point standard curve, using FCAP Array™ software (BD version 3.0.1).

2.6. Adjustment of cytokine expression levels

Cytokine expression levels were adjusted by WV or TPC, according to a previous report [2]. To compare differences in sponge weights after specimen collection, the dilution factor was calculated as $[(x - y) + 300\text{ mg of buffer}]/(x - y)$, where x equals the weight of the sponge after collection and y is the weight of the dry sponge. Each cytokine measured was multiplied by this dilution factor to obtain weight-normalized values. TPC was measured in each extract using a bicinchoninic acid assay according to the manufacturer's protocol (Pierce, Rockford, IL, USA). Specimens were diluted 1:10 and 1:100 and run in duplicate. Total protein concentration was estimated using an 8-point standard curve from 0 to 1000 µg/ml. The ratio of cytokine concentration to TPC was then calculated and expressed as [ng of immune marker]/[mg of total protein].

Table 1
Detectable cytokines in the primary cohort patient group (n = 28).

Cytokines	Number of samples within detectable limits	Rate of specimen within detectable limits (%)	Median cytokine level (pg/ml)
IL-1α	28/28	100%	519.7
IL-1β	28/28	100%	1557.7
IL-2	0/28	0%	0.0
IL-4	0/28	0%	0.0
IL-6	28/28	100%	541.8
IFN-α	2/28	7%	0.0
IFN-γ	5/28	18%	0.0
TNF-α	25/28	89%	34.3
GM-CSF	11/28	39%	0.0
G-CSF	28/28	100%	961.4
IL-10	24/28	86%	8.3
IL-8	28/28	100%	26,596.1
IL-17A	13/28	46%	0.0
IL-21	0/28	0%	0.0
MCP-1	28/28	100%	209.1
MIP-1α	28/28	100%	203.6
RANTES	28/28	100%	39.9
Eotaxin	9/28	32%	0.0

Footnote: 18 cytokines were selected in multiplexed bead-based immunoassays, Cytometric Bead Array (CBA).

2.7. Statistical analysis

All statistical analyses were performed using SPSS for Windows (ver. 22.0.0.0; IBM Corp, Armonk, NY, USA). The Jonckheere–Terpstra trend test was used to identify significant trends among disease-category groups, and the Kruskal–Wallis one-way analysis of variance on ranks was used to compare overall differences among disease-category groups. Data were analyzed by two-tailed Mann–Whitney U tests with Bonferroni correction. Spearman's rank correlation after Sidak's correction for multiple comparisons was estimated for each pair of cytokines to identify patterns of co-expression. We defined $p < 0.05$ as significant.

3. Results

3.1. Determination of relevant candidate cytokines in cervical mucus

In the primary cohort patient group (n = 28), we examined expression levels of 18 candidate cytokines within detectable limits (%). Four cytokines, IL-2, IL-4, IFN-α, and IL-21, were detected in fewer than 10% of the samples (Table 1) and were therefore omitted from analysis in the secondary cohort patient group.

3.2. Associations between cytokine levels and cervical disease severity

We explored the associations between cervical cytology and expression levels of the 14 cytokines in the secondary cohort patients (n = 235). Detailed pathological results and HPV genotypes are shown in Table S1. The detection rates of the cytokines in patient samples ranged from 21% to 100%. Cytokine levels were adjusted by TPC and WV [2,3] to compensate for differences in quantities of cervical mucus among individual patients. The results of these two methods were similar, but not identical. The results adjusted by TPC showed that levels of five cytokines, IL-6, INF-γ, GM-CSF, RANTES, and eotaxin, increased with increasing disease severity, from NILM/LSIL to HSIL and cancer. Interestingly, G-CSF was inversely correlated with disease severity. These were statistically significant by Jonckheere–Terpstra trend tests. Levels of eight cytokines, IL-1α, IL-1β, INF-γ, GM-CSF, G-CSF, IL-8, RANTES, and eotaxin, varied significantly among disease categories by Kruskal–Wallis, Mann–Whitney U test with Bonferroni correction (Table 2, Fig. 1). When the results were adjusted by the WV method, levels of 10 cytokines, INF-γ, TNF-α, GM-CSF, IL-10, IL-8, IL-17A, MCP-1, MIP-1α, RANTES, and eotaxin, increased with increasing disease severity, and G-CSF was inversely correlated as above.

These were statistically significant by Jonckheere–Terpstra trend tests. Integrated analysis by TPC and WV methods revealed that expression levels of IFN-γ, GM-CSF, RANTES, and eotaxin increased significantly and G-CSF decreased significantly with disease severity.

3.3. Association between cytokine levels and HPV infection status

TPC showed downregulation of IL-6, G-CSF and MCP-1, and WV showed downregulation of G-CSF and upregulation of IL-1α and IL-8 in HPV-positive specimens (Table 3, Fig. 2). Notably, G-CSF was shown to be downregulated by both methods.

3.4. Cytokine co-expression patterns in relation to HPV infection status

Cytokine expression levels are mutually regulated through the complex immune network, and we therefore explored their co-expression patterns in relation to HPV infection status. The two adjustment methods initially gave varying results (Fig. 3A, B), and we therefore identified concordance patterns between the two methods (Fig. 3C). Proinflammatory cytokines, including IL-1α, IL-1β, IL-6, and TNF-α, and the inhibitory cytokine, IL-10, were expressed simultaneously in patients with or without HPV infection. However, some combinations of cytokines were associated with HPV infection, including INF-γ + IL-17A, GM-CSF + MCP-1, GM-CSF + RANTES, IL-17A + RANTES, and MCP-1 + eotaxin. In contrast IL-1α + MCP-1, and G-CSF + INF-γ were inversely related to HPV infection. Inconsistently, WV showed the combination of IL-8 + RANTES to be positively correlated with HPV infection, whereas the TPC method showed them to be inversely correlated (Table S2 and Fig. 3C).

3.5. Cytokine expression in cultured cells

Cytokines, chemokines, and growth factors are generated by various blood cells, stromal cells, and epithelial cells including tumor and non-tumor cells. It is therefore difficult to identify which cells in cervical mucus, which presumably contains various candidate cells, are responsible for producing the cytokines. We therefore explored cytokine levels in cell lysates or supernatants from various cultured cell lines.

The expression levels of proinflammatory cytokines including IL-1α, IL1-β, and IL-6 were relatively high in most cervical cells. These cytokines were measurable in the cell supernatants, suggesting that the cells secreted the cytokines into the media. MCP-1 and RANTES were moderately expressed in eight of the 11 cervical cell lines (Fig. 4) and were also secreted into the media. About half of the cervical cells secreted GM-CSF.

Cytokines were measured as described in the Materials and methods section and their levels were adjusted by protein concentration. INF-γ and IL-17A were omitted because they were not measurably expressed. X-axis: cell lines; Number 1: specimen derived from cell lysate; Number 2: specimen derived from cell supernatant. Cell line details are given in the Materials and methods section.

4. Discussion

The absence of viremia and cytolysis associated with cervical HPV infection contributes to the difficulty in defining the immune mechanisms that regulate HPV clearance [1]. Cytokine activation is presumed to occur shortly after establishment of HPV infection, leading to mucosal expression of candidate antiviral (INF-α), type-1 (IFN-γ and IL-12), regulatory (IL-10), and proinflammatory (IL-1α, IL-1β IL-6, IL-8, MIP-1α, and TNF-α) cytokines [6]. However, associations between local cytokine levels and the severity of cervical neoplasia are poorly understood, due to a lack of knowledge about the natural history of the

Table 2
Cytokine profiles for cervical mucus and their associations with cytology in the secondary cohort patient group (n = 235) adjusted by total protein concentration method and by weighted volume method.

Cytokines	No. of samples within detectable limits	Rate of specimen within detactable limits (%)	Median cytokine level (pg/ml)	Adjustment by protein concentration (ng/mg)					
				NILM + LSIL	HSIL	cancer	Kruskal Wallis	Mann-Whitney U	
				(n = 66)	(n = 113)	(n = 56)	p-Value	NILM + LSIL vs HSIL p-Value	NILM + LSIL vs cancer p-Value
IL-1α	235/235	100%	528.8	0.16	0.24	0.12	0.006*	0.276	0.194
IL-1β	235/235	100%	1714.1	0.47	0.87	0.27	0.001*	0.214	0.118
IL-6	235/235	100%	497.9	0.24	0.15	0.14	0.061	0.067	0.239
IFN-γ	50/235	21%	0.0	0	0	0.000046	1.1E-15*	0.216	9.9E-11*
TNF-α	211/235	90%	22.4	0.0050	0.0077	0.0070	0.177	0.185	1.034
GM-CSF	87/235	37%	0.46	0.00012	0.00014	0.00018	0.026*	2.803	0.037*
G-CSF	235/235	100%	483.5	0.37	0.21	0.02	4.8E-20*	7.8E-05*	4.6E-16*
IL-10	184/235	78%	8.2	0.0021	0.0016	0.0024	0.166	1.555	1.246
IL-8	235/235	100%	34,844.0	10.2	13.6	9.3	0.033*	0.928	0.474
IL-17A	88/235	37%	0.0	0	0	0	0.153	0.844	0.142
MCP-1	235/235	100%	289.9	0.143	0.087	0.080	0.118	0.134	1.147
MIP-1α	229/235	97%	133.9	0.041	0.039	0.047	0.694	1.435	2.903
RANTES	233/235	99%	33.1	0.010	0.012	0.093	1.3E-10*	0.708	3.7E-09*
Eotaxin	193/235	82%	3.3	0.0009	0.0009	0.0016	0.001*	1.137	0.063

Cytokines	Adjustment by protein concentration (ng/mg)		Adjustment of weighted volume (ng/mL)							
	Mann-Whitney U	Jonckheere-Terpstra	NILM + LSIL	HSIL	cancer	Kruskal Wallis	Mann-Whitney U			Jonckheere-Terpstra
	HSIL vs cancer						NILM + LSIL vs HSIL	NILM + LSIL vs cancer	HSIL vs cancer	
	p-Value	p-Trend	(n = 66)	(n = 113)	(n = 56)	p-Value	p-Value	p-Value	p-Value	p-Trend
IL-1α	0.008*	0.215	2.8	3.9	3.8	0.091	0.090	0.383	2.976	0.066
IL-1β	0.001*	0.128	7.7	12.6	7.6	0.066	0.069	0.811	0.816	0.242
IL-6	2.657	0.038*	3.8	2.6	5.1	0.004*	0.846	0.092	0.003*	0.060
IFN-γ	8.4E-11*	2.4E-13*	0	0	0.0022	7.6E-16*	0.216	9.1E-11*	5.8E-11*	1.9E-13*
TNF-α	1.500	0.271	0.07	0.14	0.26	0.002*	0.079	0.002*	0.209	4.6E-04*
GM-CSF	0.054	0.028*	0.0019	0.0025	0.0057	6.E-09*	0.890	2.0E-07*	1.2E-07*	4.3E-08*
G-CSF	1.2E-13*	2.4E-21*	4.9	3.2	0.8	2.8E-13*	0.007*	4.0E-11*	2.1E-09*	5.8E-14*
IL-10	0.125	0.323	0.032	0.032	0.087	2.5E-04*	2.410	0.010*	1.2E-04*	0.002*
IL-8	0.025*	0.197	141.9	226.6	287.3	0.003*	0.124	0.003*	0.203	0.001*
IL-17A	0.865	0.056	0	0	0	0.060	0.882	0.057	0.326	0.020*
MCP-1	0.874	0.340	1.4	1.3	2.9	7.0E-05*	1.210	0.005*	4.9E-05*	0.004*
MIP-1α	1.462	0.944	0.7	0.7	1.4	2.0E-04*	2.911	0.003*	2.1E-04*	0.001*
RANTES	9.2E-09*	1.7E-09*	0.1	0.2	4.2	5.1E-14*	0.198	3.0E-12*	6.7E-11*	6.1E-13*
Eotaxin	4.3E-04*	0.020*	0.015	0.016	0.081	4.2E-09*	2.492	1.3E-06*	9.8E-09*	1.4E-06*

Footnote: NILM: negative for intraepithelial lesion or malignancy; LSIL: low-grade squamous intraepithelial lesion; HSIL: high-grade intraepithelial lesion; Cancer: squamous cell carcinoma and adenocarcinoma. Mann–Whitney U: Mann–Whitney U tests with a Bonferroni correction.

* $p < 0.05$ was statistically significant.

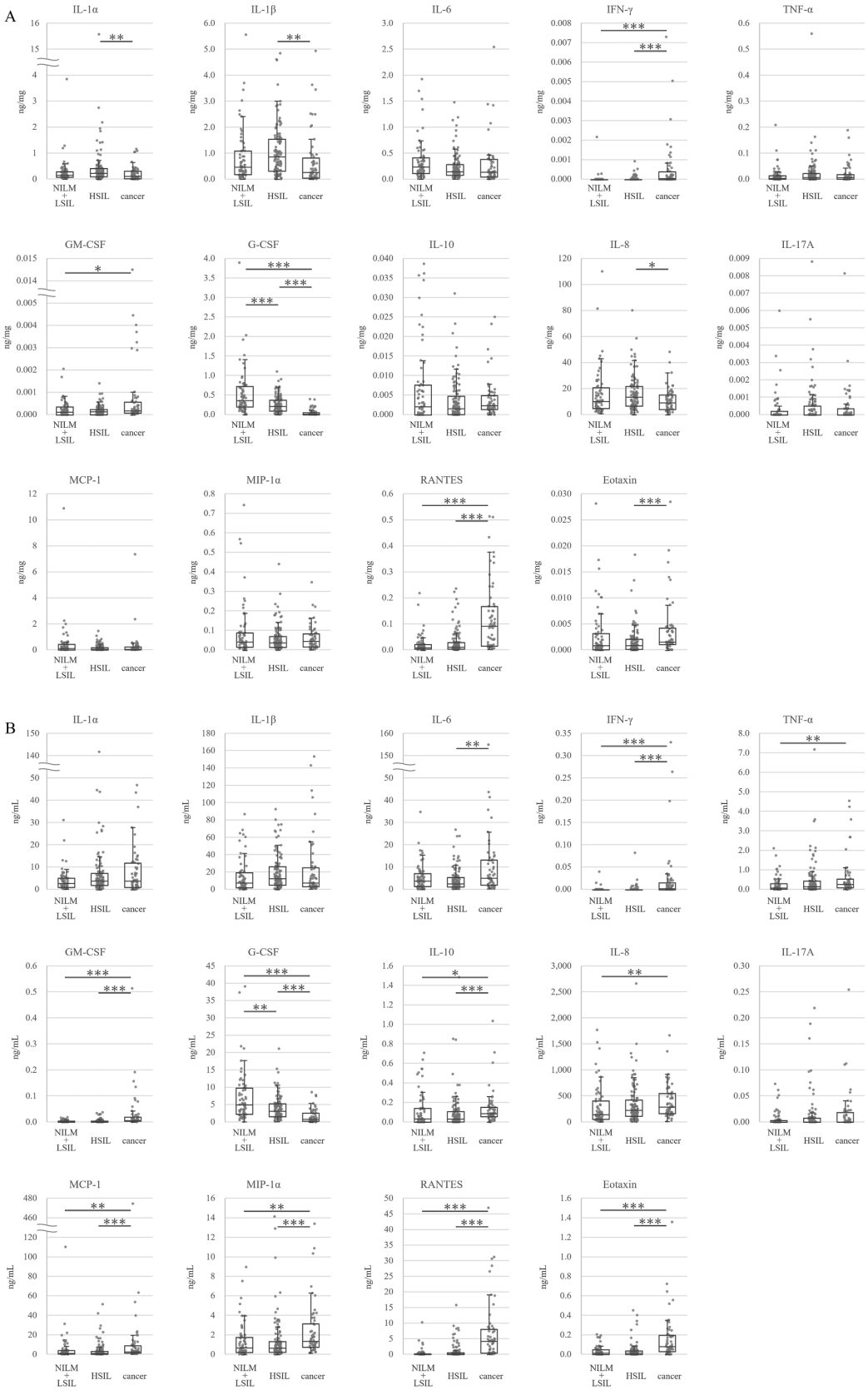

Fig. 1. Associations between cytology results and cytokine levels, adjusted by (A) total protein concentration and (B) weighted volume methods. HSIL: high-grade intraepithelial lesion; LSIL: low-grade squamous intraepithelial lesion; NILM: negative for intraepithelial lesion or malignancy. Statistical analysis by Mann–Whitney U tests with Bonferroni correction: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Table 3
Associations between cytokine profiles in cervical mucus and HPV infection status in the secondary cohort patient group (n = 235) adjusted by total protein concentration and by weighted volume methods.

Cytokines	No. of samples within detectable limits	Rate of specimen within detectable limits (%)	Median cytokine level (pg/ml)	Adjustment by protein concentration (ng/mg)			Adjustment of weighted volume (ng/mL)		
				HPV negative (n = 43)	HPV positive (n = 192)	Mann-Whitney U p-Value	HPV negative (n = 43)	HPV positive (n = 192)	Mann-Whitney U p-Value
IL-1α	235/235	100%	528.8	0.14	0.20	0.183	2.0	3.9	0.004*
IL-1β	235/235	100%	1714.1	0.45	0.59	0.566	6.6	11.5	0.079
IL-6	235/235	100%	497.9	0.25	0.15	0.004*	4.2	3.3	0.147
IFN-γ	50/235	21%	0	0	0	0.278	0	0	0.276
TNF-α	211/235	90%	22.4	0.0050	0.0070	0.436	0.10	0.15	0.178
GM-CSF	87/235	37%	0.46	0.00011	0.00015	0.453	0.0022	0.0030	0.092
G-CSF	235/235	100%	483.5	0.54	0.16	2.1.E-08*	6.7	2.5	3.9.E-06*
IL-10	184/235	78%	8.2	0.0024	0.0019	0.161	0.057	0.054	0.504
IL-8	235/235	100%	34,844.0	9.2	11.5	0.228	146.0	228.4	0.008*
IL-17A	88/235	37%	0	0	0	0.437	0	0	0.361
MCP-1	235/235	100%	289.9	0.218	0.076	0.002*	3.0	1.6	0.121
MIP-1α	229/235	97%	133.9	0.064	0.039	0.283	0.85	0.88	0.951
RANTES	233/235	99%	33.1	0.016	0.015	0.813	0.19	0.25	0.212
Eotaxin	193/235	82%	3.3	0.0012	0.0011	0.973	0.018	0.022	0.391

Footnote: HPV: human papillomavirus. Mann–Whitney U: Mann–Whitney U tests with a Bonferroni correction.
* $p < 0.05$ was statistically significant.

HPV life cycle, the specific host immune response, and other factors.

Although quantifying cytokine levels in cervical mucus specimens is a critical issue, the optimal method for obtaining specimens is unclear. Cervicovaginal lavage is convenient to use but may produce diluted specimens, with consequently undetectable levels of some target cytokines. Indeed, four cytokines (IL-2, IL-4, IFN-α, and IL-21) were excluded from further analysis in the current study because they were undetectable even without dilution. We obtained specimens directly using Merocel ophthalmic sponges, as described previously [2,3,8]. Another problem included the need to adjust the cytokine results to account for different amounts of mucus in samples from different individuals. In this study, we evaluated two adjustment methods, TPC and WV [2,3]. Cytokine levels were adjusted by WV because the volume of cervical mucus varies greatly among patients, depending on factors such as age, estrogen hormone status, and bacterial infection. In contrast, adjustment by TPC was not influenced by such conditions but could be influenced by the presence of non-human proteins or by immune response to foreign proteins, or even by other endogenous cytokines [8]. Interestingly, Koshiol et al. preferred the WV method while Marks et al. used TPC for adjustment [2,3]. We recognised no consensus regarding the best adjustment method, and we therefore analyzed the associations between cytokine levels and disease severity according to cytology using both adjustment methods. Although the results from the two methods were not always consistent, four cytokines, including IFN-γ, GM-CSF, RANTES, and eotaxin, were positively associated with disease severity and G-CSF was inversely correlated according to both methods.

Some studies have reported associations between disease severity and other cytokines [3,4,9–14]. However, information on the associations between expression levels of the four cytokines identified in the current study and disease severity is lacking. Peghini et al. reported that IFN-γ increased with disease severity, but not significantly (P = 0.09), possibly reflecting the small sample size (n = 126) [9]. Levels of IFN-γ were shown by immunohistochemistry to increase from normal tissue to CIN, but then decreased again from CIN to cancer [11]. However, we found no such decrease from CIN to cancer in the current study (data not shown). This apparent discrepancy may be due to differences between the studies in terms of methodology or clinical stage.

Th1 differentiation is driven mainly by IFN-γ and occurs in response to microbes that activate dendritic cells, macrophages, and NK cells [15]. Immune responses against HPV and CIN arise from Th1 differentiation. Topical application of GM-CSF to patients with LSIL induced recruitment of immune cells, such as NK and T cells, which produced INF-γ [16]. The current results indicated that mucus levels of GM-CSF were related to disease severity. Furthermore, RANTES was previously shown to enhance antitumor immunity induced by a GM-CSF-transduced tumor vaccine in a mouse model [17]. Transduction of the *GM-CSF* gene into mouse tumor cells was associated with a seven-fold increase in RANTES [18], and RANTES was also shown to be highly expressed in a cervical cancer specimens [19]. RANTES is associated with chronic inflammation and may directly affect angiogenesis [20], and its expression has been demonstrated in various cancers, including breast cancer, melanoma, and papillary thyroid carcinoma. RANTES has also been associated with viral infections including HPV [21]. Based on these findings, we suggest that IFN-γ, GM-CSF, and RANTES interact in cervical neoplasia. To the best of our knowledge, this study provides the first evidence indicating an inverse correlation between G-CSF expression in cervical mucus and disease severity.

We also examined the associations between cytokine levels and HPV infection status. Although several previous studies have used similar multiplex bead assays [2–4], their results were inconsistent, possibly reflecting variations in experimental conditions, including the method used for adjusting cytokine expression levels, antibodies, and patient factors, such as age, race, smoking status, hormone status, intake of oral contraceptives, and sexual behavior. The number of specimens and clinical conditions, including grading of dysplasia or squamous intraepithelial lesions, would also affect the results of statistical analysis.

The associations between HPV infection status and cytokine expression levels identified in the present study were not conclusive, because the two adjustment methods gave some discrepant results. However, both methods showed that G-CSF was downregulated in HPV-positive specimens. Downregulation of G-CSF abolishes its effects, including stimulating neutrophil development and differentiation. Neutrophils are phagocytic cells that play a critical role in the innate immune response to microbes that cross the epithelial barrier. Our data suggest that HPV can escape the immune response by suppressing G-CSF expression.

Cytokine networks associated with HPV infection are important in the local immune response, and cytokines produced in response to HPV infection may promote or inhibit the production of other cytokines. Furthermore, viral infection may induce chemokines either directly or indirectly. We therefore examined concomitant expression of cytokine levels, using Spearman's rank correlation after Sidak's correction for multiple comparisons. The two adjustment methods gave obviously

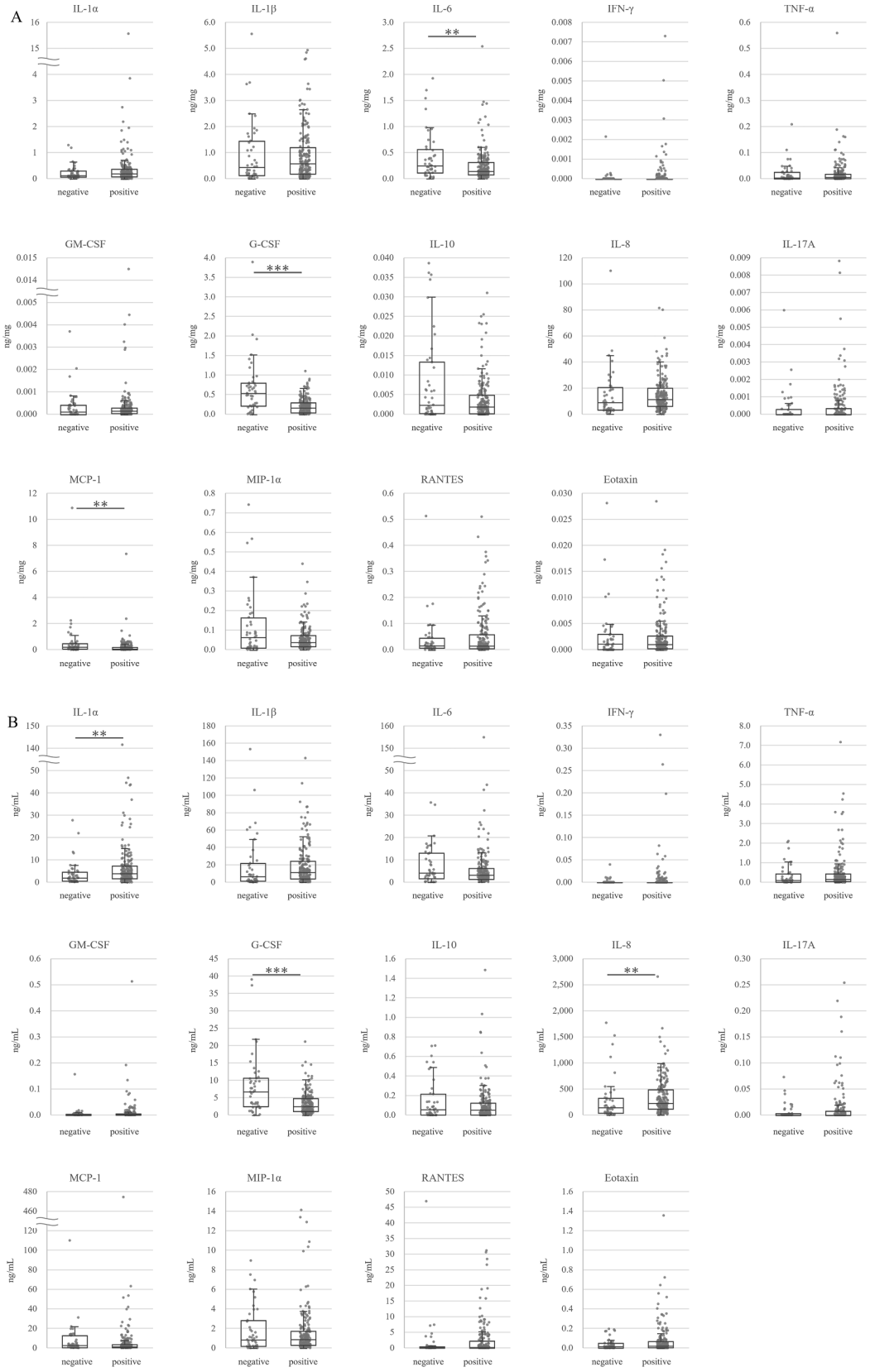

Fig. 2. Expression levels of 14 cytokines and correlations with HPV infection status in the secondary cohort patients, adjusted by (A) total protein concentration and (B) weighted volume methods. Negative: HPV-negative; positive: HPV-positive. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (Mann–Whitney U tests with Bonferroni correction).

different results, and we therefore tried to integrate the results from the two methods. We detected some co-expression patterns regardless of the presence or absence of HPV infection, and our analysis of the correlations between cytokine levels and HPV-positive status significantly identified RANTES, MCP-1, GM-CSF, eotaxin, and IL-17. Marks et al. also observed that GM-CSF and IL-17 were highly expressed in HPV-positive women [8]. Notably, RANTES and GM-CSF expression level were also proportional to disease severity.

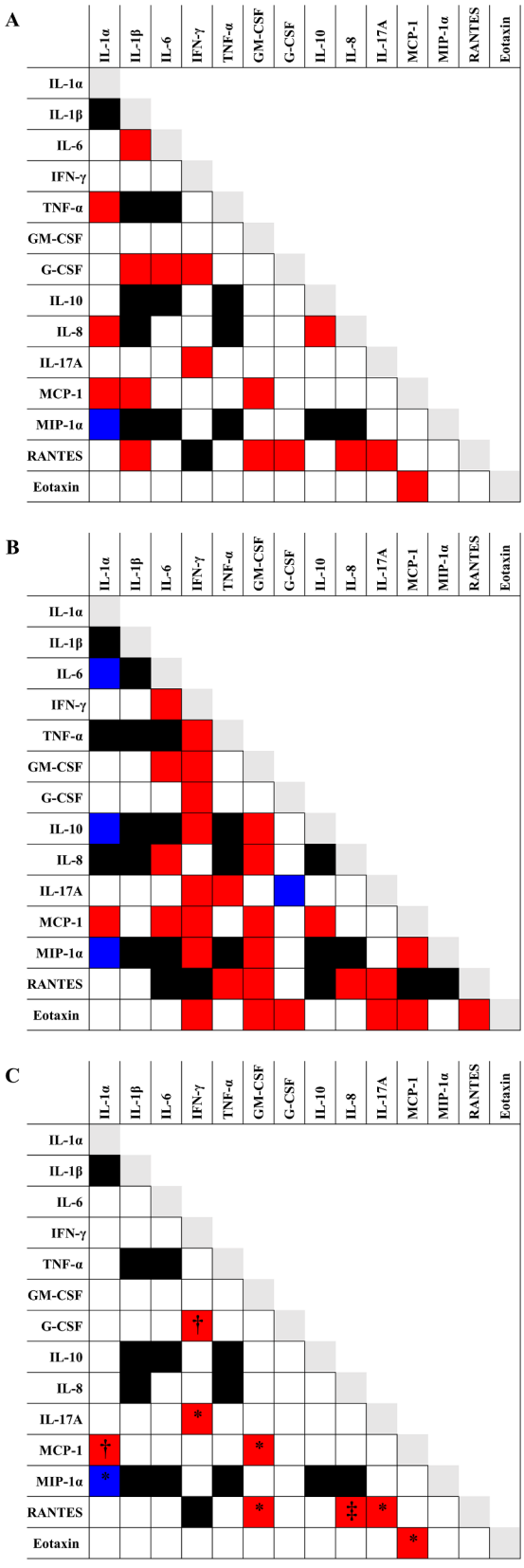

Fig. 3. Multiple comparisons were estimated (Spearman's rank correlation after Sidak's correction) for each pair of cytokines, to identify co-expression patterns adjusted by (A) total protein concentration and (B) weighted volume methods, and (C) their concordance using both adjustments. $p < 0.05$; black: significant in HPV-negative and -positive specimens; white: not significant in either HPV-negative or -positive specimens; red: significant in HPV-positive only; blue: significant in HPV-negative only. *positive correlation, †negative correlation, ‡inconsistent results by adjustment methods. Correlation levels are described in Table S2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Cytokines are secreted by dendritic cells, macrophages, epithelia, and fibroblasts [22], and identifying the components of cervical neoplasia (such as hematopoietic cells, epithelia, or surrounding stromal cells) responsible for secreting specific cytokines is difficult. Proinflammatory or inhibitory cytokines are secreted from cervical neoplastic cells [12,23–26], and chemokines, such as MCP-1 and RANTES, are secreted from monocytes, macrophages, fibroblasts, T cells [22], and cancer cells [10,27,28]. The production of these cytokines by tumors results from complex interactions between tumor and non-tumor cells, with both cell types contributing to cytokine production [27]. In the current study, MCP-1 and RANTES were secreted by most cultured cervical cancer cells that contained high-risk HPVs (HPV16 and 18). Notably, GM-CSF, RANTES, and eotaxin were measurable in cultured cells, while IFN-γ was not. These results suggest that IFN-γ might be produced by hematopoietic or stromal cells.

HPV infection is known to induce the expression of RANTES and MCP-1 [21]. RANTES is expressed in a variety of cancers, such as breast cancer, melanoma, and papillary thyroid carcinoma, and mediates cancer progression by controlling leukocyte recruitment and angiogenesis. IL-1β, TNF-a, RANTES, and MCP-1 are also known to act synergistically to promote breast cancer progression [29]. Therapies that block RANTES and its receptors could thus potentially provide the basis for cancer treatments [20]. However, to the best of our knowledge, there have been no previous reports on the effects of RANTES and MCP-1 on cervical cancer cells, and further studies are warranted.

This study had some limitations. This was a single-institution study and may thus have had some selection bias related to the enrolled population. In particular, no cytology results were obtained from the general population. Further investigations are therefore needed, using larger datasets obtained from population-based screening, including healthy women. In addition, the candidate cytokines examined were limited by the available antibodies. Finally, additional patient information was lacking, including histories of sexually transmitted infections, smoking status, or intake of oral contraceptives.

However, despite their limitations, our study also had some strengths, including the use of a relatively large cohort ($n = 235$), which allowed analysis of the association between disease severity and cytokine expression levels using the two different adjustment methods. Information about cytology, histology, and HPV genotype were also available for the enrolled patients, and cytokine expression levels were examined in both clinical specimens and cell cultures.

5. Conclusion

Expression levels of the cytokines IFN-γ, GM-CSF, RANTES, and eotaxin were significantly elevated with the increasing severity of cervical neoplasia. Cytokine interactions, particularly between GM-CSF and RANTES, might indicate changes in HPV infection associated with increased disease severity. Furthermore, GM-CSF and RANTES are produced by cancer cells.

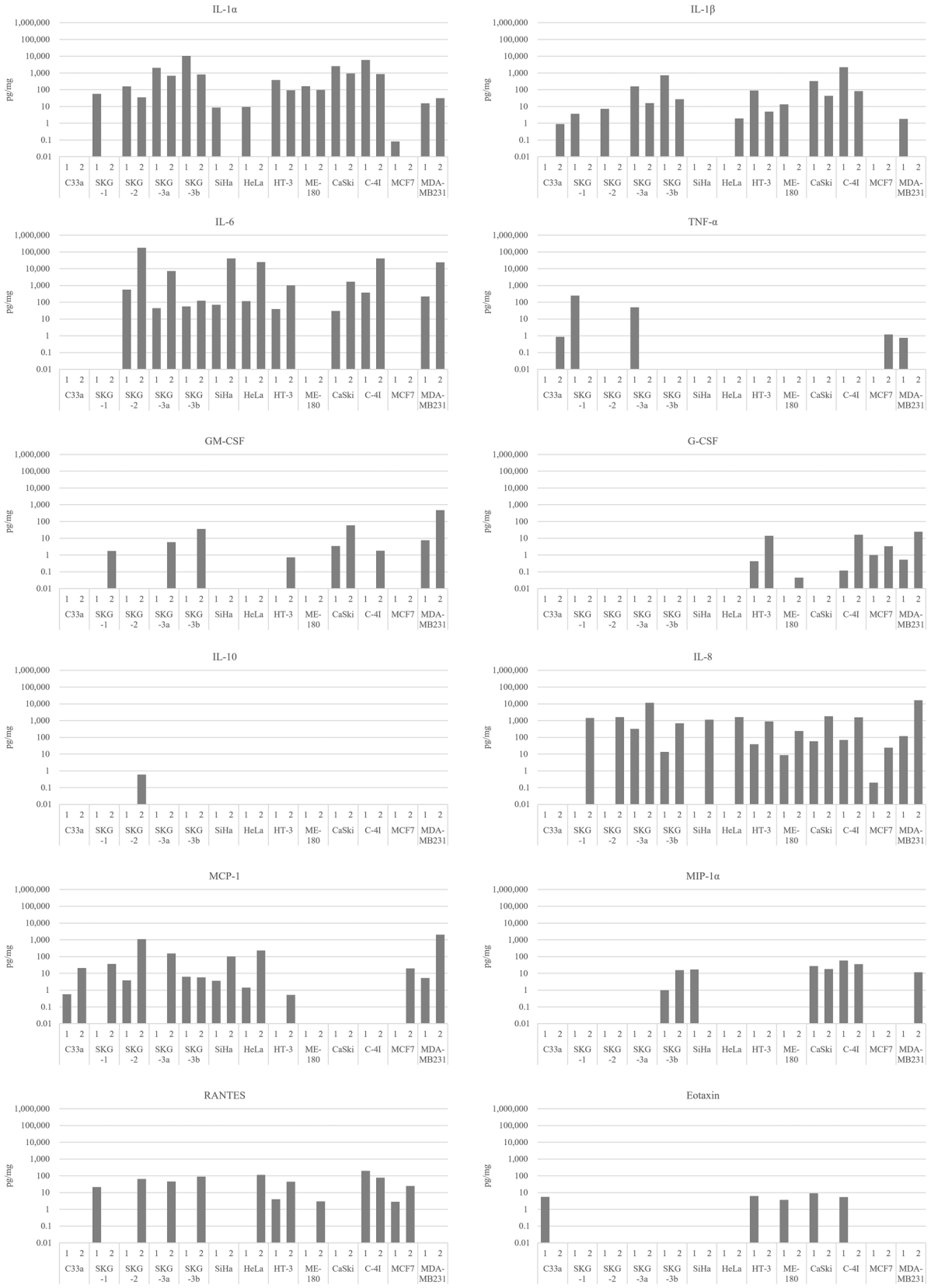

Fig. 4. Expression levels of cytokines in cultured cells.

Authors' contributions

Designed the experiments: TF.
Performed the experiments: AI, NY, IK.
Analyzed the data: TF, SO, AI, IK.
Contributed reagents/materials/analysis tools: SO, IK, NY, TT, RI, EN.
Contributed to the writing of the manuscript: TF, AI, IK, SO.
All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declared that there is no conflict of interest.

Acknowledgements

This work was partly supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan (grant no. 17K11300) awarded to TF. We thank Ms. Nakagawa, Department of Obstetrics and Gynecology, Fujita Health University, for editing the manuscript. We thank Ellen Knapp, PhD, Marla Brunker, and Susan Furness, PhD, from Edanz Group (www.edanzediting.com/ac) for editing a draft of this manuscript. The authors also thank Dr. T. Iwata, of Keio University for providing the cell line.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cyto.2019.05.011.

References

[1] M.A. Stanley, J.C. Sterling, Host responses to infection with human papillomavirus, Curr. Probl. Dermatol. 45 (2014) 58–74.
[2] M.A. Marks, Y. Eby, R. Howard, P.E. Gravitt, Comparison of normalization methods for measuring immune markers in cervical secretion specimens, J. Immunol. Methods 382 (1–2) (2012) 211–215.
[3] J. Koshiol, M. Sklavos, N. Wentzensen, T. Kemp, M. Schiffman, S.T. Dunn, S.S. Wang, J.L. Walker, M. Safaeian, R.E. Zuna, A. Hildesheim, R.M. Pfeiffer, L.A. Pinto, Evaluation of a multiplex panel of immune-related markers in cervical secretions: a methodologic study, Int. J. Cancer 134 (2) (2014) 411–425.
[4] T. Iwata, T. Fujii, K. Morii, M. Saito, J. Sugiyama, H. Nishio, T. Morisada, K. Tanaka, T. Yaguchi, Y. Kawakami, D. Aoki, Cytokine profile in cervical mucosa of Japanese patients with cervical intraepithelial neoplasia, Int. J. Clin. Oncol. 20 (1) (2015) 126–133.
[5] J.A. Lieberman, A.B. Moscicki, J.L. Sumerel, Y. Ma, M.E. Scott, Determination of cytokine protein levels in cervical mucus samples from young women by a multiplex immunoassay method and assessment of correlates, Clin. Vaccine Immunol. 15 (1) (2008) 49–54.
[6] M.E. Scott, Y.B. Shvetsov, P.J. Thompson, B.Y. Hernandez, X. Zhu, L.R. Wilkens, J. Killeen, D.D. Vo, A.B. Moscicki, M.T. Goodman, Cervical cytokines and clearance of incident human papillomavirus infection: Hawaii HPV cohort study, Int. J. Cancer 133 (5) (2013) 1187–1196.
[7] Y. Azuma, R. Kusumoto-Matsuo, F. Takeuchi, A. Uenoyama, K. Kondo, H. Tsunoda, K. Nagasaka, K. Kawana, T. Morisada, T. Iwata, D. Aoki, I. Kukimoto, Human papillomavirus genotype distribution in cervical intraepithelial neoplasia grade 2/3 and invasive cervical cancer in Japanese women, Jpn. J. Clin. Oncol. 44 (10) (2014) 910–917.
[8] M.A. Marks, R.P. Viscidi, K. Chang, M. Silver, A. Burke, R. Howard, P.E. Gravitt, Differences in the concentration and correlation of cervical immune markers among HPV positive and negative perimenopausal women, Cytokine 56 (3) (2011) 798–803.
[9] B.C. Peghini, D.R. Abdalla, A.C. Barcelos, L. Teodoro, E.F. Murta, M.A. Michelin, Local cytokine profiles of patients with cervical intraepithelial and invasive neoplasia, Hum. Immunol. 73 (9) (2012) 920–926.
[10] S. Hazelbag, G.J. Fleuren, J.J. Baelde, E. Schuuring, G.G. Kenter, A. Gorter, Cytokine profile of cervical cancer cells, Gynecol. Oncol. 83 (2) (2001) 235–243.
[11] A. Kobayashi, V. Weinberg, T. Darragh, K. Smith-McCune, Evolving immunosuppressive microenvironment during human cervical carcinogenesis, Mucosal Immunol. 1 (5) (2008) 412–420.
[12] T.T. Prata, C.M. Bonin, A.M. Ferreira, C.T. Padovani, C.E. Fernandes, A.P. Machado, I.A. Tozetti, Local immunosuppression induced by high viral load of human papillomavirus: characterization of cellular phenotypes producing interleukin-10 in cervical neoplastic lesions, Immunology 146 (1) (2015) 113–121.
[13] N. Jacobs, I. Renard, W. Al-Saleh, P. Hubert, J. Doyen, W. Kedzia, J. Boniver, P. Delvenne, Distinct T cell subsets and cytokine production in cultures derived from transformation zone and squamous intraepithelial lesion biopsies of the uterine cervix, Am. J. Reprod. Immunol. 49 (1) (2003) 6–13.
[14] K. Kleine-Lowinski, R. Gillitzer, R. Kuhne-Heid, F. Rosl, Monocyte-chemo-attractant-protein-1 (MCP-1)-gene expression in cervical intra-epithelial neoplasias and cervical carcinomas, Int. J. Cancer 82 (1) (1999) 6–11.
[15] A.K. Abbas, A.H. Lichtman, S. Pillai, Differentiation and Functions of CD4 + Effector T Cells, in: A.K. Abbas, A.H. Lichtman, S. Pillai (Eds.), Cellular and Molecular Immunology, Elsevier, Philadelphia, PA, USA, 2017, pp. 225–242.
[16] P. Hubert, J. Doyen, X. Capelle, M. Arafa, V. Renoux, B. Bisig, L. Seidel, B. Evrard, L. Bousarghin, C. Gerday, J. Boniver, J.M. Foidart, P. Delvenne, N. Jacobs, Local applications of GM-CSF induce the recruitment of immune cells in cervical low-grade squamous intraepithelial lesions, Am. J. Reprod. Immunol. 64 (2) (2010) 126–136.
[17] H. Inoue, M. Iga, M. Xin, S. Asahi, T. Nakamura, R. Kurita, M. Nakayama, Y. Nakazaki, K. Takayama, Y. Nakanishi, K. Tani, TARC and RANTES enhance antitumor immunity induced by the GM-CSF-transduced tumor vaccine in a mouse tumor model, Cancer Immunol. Immunother. 57 (9) (2008) 1399–1411.
[18] Y. Nakazaki, H. Hase, H. Inoue, Y. Beppu, X.K. Meng, G. Sakaguchi, R. Kurita, S. Asano, Y. Nakamura, K. Tani, Serial analysis of gene expression in progressing and regressing mouse tumors implicates the involvement of RANTES and TARC in antitumor immune responses, Mol. Ther. 14 (4) (2006) 599–606.
[19] Y. Niwa, H. Akamatsu, H. Niwa, H. Sumi, Y. Ozaki, A. Abe, Correlation of tissue and plasma RANTES levels with disease course in patients with breast or cervical cancer, Clin. Cancer Res. 7 (2) (2001) 285–289.
[20] R.E. Marques, R. Guabiraba, R.C. Russo, M.M. Teixeira, Targeting CCL5 in inflammation, Expert Opin. Ther. Targets 17 (12) (2013) 1439–1460.
[21] W.G. Glass, H.F. Rosenberg, P.M. Murphy, Chemokine regulation of inflammation during acute viral infection, Curr. Opin. Allergy Clin. Immunol. 3 (6) (2003) 467–473.
[22] A.K. Abbas, A.H. Lichtman, S. Pillai, Leukocyte Circulation and Migration into Tissues, in: A.K. Abbas, A.H. Lichtman, S. Pillai (Eds.), Cellular and Molecular Immunology, Elsevier, Philadelphia, PA, USA, 2017, pp. 39–56.
[23] S.L. Giannini, W. Al-Saleh, H. Piron, N. Jacobs, J. Doyen, J. Boniver, P. Delvenne, Cytokine expression in squamous intraepithelial lesions of the uterine cervix: implications for the generation of local immunosuppression, Clin. Exp. Immunol. 113 (2) (1998) 183–189.
[24] L.H. Wei, M.L. Kuo, C.A. Chen, W.F. Cheng, S.P. Cheng, F.J. Hsieh, C.Y. Hsieh, Interleukin-6 in cervical cancer: the relationship with vascular endothelial growth factor, Gynecol. Oncol. 82 (1) (2001) 49–56.
[25] R. Srivani, B. Nagarajan, A prognostic insight on in vivo expression of interleukin-6 in uterine cervical cancer, Int. J. Gynecol. Cancer 13 (3) (2003) 331–339.
[26] Z. Song, Y. Lin, X. Ye, C. Feng, Y. Lu, G. Yang, C. Dong, Expression of IL-1alpha and IL-6 is associated with progression and prognosis of human cervical cancer, Med. Sci. Monit. 22 (2016) 4475–4481.
[27] T. Yoshimura, The chemokine MCP-1 (CCL2) in the host interaction with cancer: a foe or ally, Cell. Mol. Immunol. (2018).
[28] M. Buettner, B. Meyer, S. Schreck, G. Niedobitek, Expression of RANTES and MCP-1 in epithelial cells is regulated via LMP1 and CD40, Int. J. Cancer 121 (12) (2007) 2703–2710.
[29] A. Khalid, J. Wolfram, I. Ferrari, C. Mu, J. Mai, Z. Yang, Y. Zhao, M. Ferrari, X. Ma, H. Shen, Recent advances in discovering the role of CCL5 in metastatic breast cancer, Mini Rev. Med. Chem. 15 (13) (2015) 1063–1072.