Expression of integrin and toll-like receptors in cervical cancer (*in vivo* and *ex vivo* study)

By

Jeffrey Patrick Werner

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Lakehead University
Thunder Bay, Ontario, Canada

Supervised by Dr. Marina Ulanova, MD, PhD

Dr. Ingeborg Zehbe, PhD

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Abbreviations

AB/AM	antibiotic/antimycotic
APC	antigen presenting cell
ATCC	American Type Culture Collection
BSA	bovine serum albumin
BV	bacterial vaginosis
CBC	Canadian Broadcasting Corporation
CCS	Canadian Cancer Society
cDNA	complementary DNA
CFU	colony forming units
CWHN	Canadian Women's Health Network
DMEM	Dulbecco's modified eagle medium
dsDNA	double-stranded DNA
dsRNA	double-stranded RNA
ECM	extracellular matrix
EMT	epithelial to mesenchymal transition
FBS	fetal bovine serum
g	gravitational force
h	hours
HPV	human papillomavirus
HSIL	high-grade squamous intraepithelial lesion
LB	Luria-Burtani medium
LBA	Luria-Burtani agar
LPS	lipopolysaccharide
LSIL	low-grade squamous intraeipithelial lesion
MFI	mean fluorescence intensity
MOI	multiplicity of infection
MRS	de Man Rogosa Sharpe medium
NACI	National Advisory Committee on Immunization
OD_{600}	optical density at 600 nm
PAMP	pathogen associated molecular pattern
PBS	phosphate buffered saline, pH 7.4
PCR	polymerase chain reaction
PolyI:C	polyribosinic:polyribocytidylic acid
PRR	pattern recognition receptor
R-PE	R-Phycoerythrin
rpm	revolutions per minute
ssRNA	single-stranded RNA
STI	sexually transmitted infection
TBRHSC	Thunder Bay Regional Health Science Centre
TLR	toll-like receptor

Abstract

Cervical cancer is the second most common cancer in women world-wide. Although persistent human papillomavirus (HPV) infection is considered the most important causative agent of cervical cancer, the mechanisms toward malignant transformation remain unclear. Some evidence suggests that bacterial vaginosis (BV), a shift in normal vaginal flora toward infection of Gram-negative bacteria, may play a role in the development of cervical cancer. As part of the innate immune system, toll-like receptors (TLR) are important for the early detection and clearance of viral and bacterial infections, and are poised to play an important role in the development of cervical cancer. Integrins are trans-membrane receptors which signal through various pathways to regulate cellular functions such as migration, apoptosis, proliferation, and mitosis, and as such, are currently considered therapeutic targets for some cancers. In order to elucidate the roles integrins and TLRs may play in cervical cancer, we examined expression of these receptors in HeLa, CaSki, SiHa, ME180, and C-33A cervical cancer derived cell lines at protein and genetic levels. Then we examined the surface expression of integrins and TLRs in HeLa cells following stimulation for 4 h with two Gram-negative bacteria, *Escherichia coli* and *Pseudomonas aeruginosa*, and one commensal bacteria associated with normal vaginal flora, *Lactobacillus reuteri*. Frozen cervical tissue biopsies of varying stages of dysplasia were also examined for integrin and TLR expression. Our data reveal there are differences in integrin and TLR expression between the five cell lines. Although we did not detect changes in TLR expression in the biopsied tissue, changes in integrin expression between normal and carcinoma samples were noted. Data from our *ex vivo* infection model show that Gram-negative bacterial affect integrin and TLR expression in HeLa cells, while there is no change in expression with commensal bacteria infection. These changes in integrin and TLR expression associated with

Gram-negative infection may facilitate tumor growth and progression, suggesting that BV may play a role in the development of cervical cancer.

Introduction

The importance of cervical cancer as an issue for women's health cannot be understated, cervical cancer is the second most common cancer in women world-wide (Parkin, Bray, Ferlay, Pisani, 2005). Incidence and prevalence of cervical cancer varies throughout the world and is typically highest in developing countries, and lower in developed countries where cervical screening programs are in place (Govan, Rybicki, Williamson, 2008; Parkin, 2005). Although Canada currently does not have a nationally organized cervical cancer screening program, incidences and mortalities in Canada are lower compared to those in developing countries due to available screening programs (BC Cancer Agency, 2008; Canadian Cancer Society's [CCS] Steering Committee, 2009). Nevertheless, it is estimated that 1400 women per year are diagnosed with cervical cancer, and 400 will succumb to the disease in Canada per year. Within the province of Ontario, approximately 10 women are diagnosed and 3 die from cervical cancer per week (CCS, 2006; Kaplan-Myrth, Dollin 2007). Cervical cancer is the second most common epithelial cancer in women between the ages of 15 to 29 in Canada, and is more common than breast cancer for this age group (CCS, 2009). In addition to the human cost, the financial burden to Canada's health care system may also be significant. In British Columbia alone, the cost to health care for cervical cancer in 2005 was estimated at $36 million (Krueger, 2006).

Human papillomavirus infection and cervical cancer

It has been established that persistent infection of human papillomavirus (HPV) is the main causative agent for cervical cancer (Stanley, Pett, Coleman, 2007; Yee, Krishnan-Hewlett, Baker, Schlegel, Howley, 1985; zur Hausen, 2002). HPV infection is spread by skin-to-skin contact and is the most common sexually transmitted infection (STI) in North America (Dell, Chen, Ahmad, Stwart, 2000). Prevalence of HPV infection within the general population has been shown to vary between 11% and 60% depending on the type of study and sampling techniques, and it is estimated that approximately 80% to 85% of all women will have had an HPV infection in their lifetime (Jenkins, Sherlaw-Johnson, Gallivan, 1996; Kahn, J., Lan, Kahn, R., 2007; Kaplan-Myrth, 2007; National Advisory Committee on Immunization [NACI], 2007).

Although HPV infection is common, only few women infected with HPV will go on to develop cervical cancer, the reasons for which are unclear. Though the specific mechanisms by which HPV infection develops to cervical cancer remain elusive, research has revealed some important aspects of the relationship between HPV infection and cervical cancer (Massimi, Pim, Banks, 1997; Stanley et al, 2007). Primarily, not all HPVs found in the genital tract are considered to cause cervical cancer. HPVs are considered high-risk or low-risk depending on their oncogenic potential and association with cervical cancer (Muñoz et al., 2003; Stanley et al, 2007). Approximately 40 different HPV genotypes are associated with the genital tract, of these, it is estimated that as many as 19 may be considered high-risk HPV (Jensen, Lehman, Antoni, Pereira, 2007; Muñoz, 2003). The progression from HPV infection to cervical cancer may take up to a decade, and in most cases, pre-cancerous lesions are cleared (Canadian Women's Health Network [CWHN], 2007; Castle et al., 2001; Nobbenhuis et al, 2001). It is thought that the

initiation of HPV infection occurs when the virus infects stem cells in the basement membrane via micro-tears in the epithelium (reviewed by Doorbar, 2005; Muñoz, Castellsagué, de Gonzalez, Gissmann, 2006; Stanley et al, 2007). Initially, the copy number of the virus is amplified to approximately 50 to 100 copies per cell in infected proliferating basal cells (reviewed by Stanley et al, 2007). Infected cells then migrate to the suprabasal layer, where typically an uninfected cell would undergo terminal differentiation (Doorbar, 2005). In the suprabasal layer, infected cells do not initiate terminal differentiation, and may proliferate (Doorbar, 2005; Muñoz et al., 2006). It is at this stage that the virus replicates at high rates, and it is suggested that at this stage it is possible for malignant transformation to be initiated by the viral E6 and E7 proteins (Doorbar, 2005; Stanley, et al., 2007). Evidence of this can be found as the E6 and E7 genes from HPV-16 have been used to establish immortalized epithelial cell lines (Ficherova, Rheinwald, Anderson, 1997). The virus is then released as the infected cell continues to migrate upward through the epithelial layers and disintegrates (Muñoz et al, 2006).

The ability of the virus to avoid detection by the immune system is therefore key for initial infection as well as survival. It has been suggested that antigen-presenting cells (APC) present in the epithelial layers may not respond to the presence of the virus, allowing the virus to evade the immune system (Fausch, Da Silva, Rudolf, 2002). It is also thought that viral replication in cells destined for apoptosis may be another mechanism by which the virus avoids clearance (Stanley et al, 2007). Furthermore, it has been suggested that interferon synthesis and signalling, which triggers the adaptive immune system, may be inhibited by HPV in infected cells (Stanley et al, 2007). There is also evidence that the manipulation of toll-like receptors (TLR), part of the innate immune system, is another mechanism used by the virus to evade detection by the immune system (Hasan et al., 2007; Stanley et al, 2007). As cell migration and evasion of the

immune system play key roles in the development of persistent HPV infection, it follows that integrins, molecules associated with cell migration (Breuss, et al.,1995), as well as components of the immune system in epithelial tissues, including TLRs, play important roles in the development of cervical cancer.

Integrins

Integrins are trans-membrane heterodimeric receptors consisting of a specific combination of one alpha and one beta chain. Their ligands are extra cellular matrix (ECM) proteins including collagens, laminins, and fibronectins, and some cell surface adhesion molecules (Stupack & Cheresh, 2002). In addition to regulating cell-to-cell or cell-to-ECM adhesion, integrins signal through various pathways to regulate cellular functions such as migration, apoptosis, proliferation, and mitosis (Breuss, et al., 1995; Stupack & Cheresh, 2002). Integrins are therefore crucial for cell survival and function. During cancer development and progression, integrins have been shown to play important roles in morphogenesis, tumor growth, and angiogenesis (Davis & Senger, 2005; Stupack & Cheresh, 2002). It has also been suggested that some integrins are responsible for allowing cells to metastasize and proliferate without attachment to the ECM (Breuss, et al, 1995; Janes & Watt, 2004). Furthermore, changes in integrin expression have been observed during various stages of cervical cancer progression, and integrins have been shown to facilitate HPV infection in epithelial cell lines (Carico, French, Bucci, Falcioni, Vecchione, Mariani-Costantini, 1993; Fothergill & McMillan, 2006; Hazelbag et al., 2007; Hughes, Rebello, al-Nafussi, 1994; Yoon, Kim, Park, Cheong; 2001). The

relationship between integrin expression and cancer development is so strong that currently, integrins are considered as therapeutic targets in certain cancers (Tucker, 2006).

Toll-like receptors

Toll-like receptors are a group of pattern-recognition receptors (PRR) that are important front-line responders in the innate immune system (reviewed by Takeda & Akira, 2004). TLRs are found in various cell types including cervical epithelial cells, and are activated by pathogen-associated molecular patterns (PAMP) including lipopolysaccharide (LPS), a cell wall component of Gram-negative bacteria, double-stranded RNA (dsRNA) from viruses, and the CpG motif found in bacterial and viral DNA (Herbst-Kralovetz, et al., 2008; reviewed in Takeda & Akira, 2004). Some TLRs such as TLR2 are cell surface receptors, others such as TLR9 are located intracellularly, while it has also been suggested that others such as TLR3 and TLR4 may be expressed either intracellularly or at the cell's surface (Hemmi, et al., 2000; Hornef, Fisan, Vandewalle, Normark, Richter-Dahlfors, 2002; Jiang, Wei, Tian, 2008; reviewed in Takeda & Akira, 2004). Stimulation of TLRs from pathogenic material can result in the delay or eradication of infection, and can trigger the immune system. Therefore, TLRs are poised to play an important role in HPV infection, a pre-requisite for cervical cancer development.

Bacterial vaginosis

Bacterial vaginosis (BV) is characterized by a shift from vaginal flora consisting mainly of commensal bacteria to pathogenic bacteria, including Gram-negative bacteria, accompanied by a pH greater than 4.5 (Cherpes, Marrazzo, Cosentino, Meyn, 2008; Devillard, Burton, Reid, 2005; Donders,et al., 2002; Hillier, Krohn, Rabe, Klebanoff, Eschenbach, 1993; Nugent, Krohn, Hillier, 1991). Prevalence of BV is estimated as 30% within the general population, as high as 50% in some sub-populations, and may occur without overt symptoms (Koumans, et al., 2007). As previously mentioned, the mechanisms for the development of cervical cancer are not clear, and some have suggested that there is an association between cervical cancer development and BV (Castle, et al., 2001; Discacciati, et al., 2006; Kharsany, Hoosen, Moodley, Bagaratee, Gouws, 1993; Mikamo, Sato, Hayasaki, Kawazoe, 1999; Platz-Christensen, Sundström, Larsson, 1994). Yet other investigators were unable to find such connections (Boyle, et al., 2003, Peters, Van Leeuwen, Pieters, Hollema, Quint, Burger, 1995). Given the inconsistencies within the literature regarding the association between BV and cervical cancer, and that the mechanisms for the progression from persistent HPV infection to cervical cancer are not yet elucidated, further investigation into the relationship between BV and the development of cervical cancer may yield important findings.

Objectives

As previously mentioned, integrins and TLRs have the potential to play important roles in the development of cervical cancer. However, gaps in the literature regarding expression patterns of integrins and TLRs in cervical epithelial cells exist. **The first objective of our study was to characterize mRNA and surface protein expression of these molecules in cervical epithelial cells**. We chose 5 established cervical caner-derived cell lines, ME180, CaSki, HeLa, SiHa, and C-33A, to examine integrin and TLR expression. Four of the 5 cell lines, ME180, CaSki, HeLa,, and SiHa contain different HPV genetic material, the C-33A cell line is HPV absent. The ME180 cell line contains genomic material from HPV-68 (Longuet, Beaudenon, Orth, 1996; Reuter, et al., 1998). CaSki cells contain 60 to 600 genome copies of HPV-16 and some HPV18 (American Type Culture Collection [ATCC], Product information sheet for ATCC® CRL-1550; Lin, et al., 2007; Yee, et al., 1985). HeLa cells contain 10 to 50 copies of HPV-18 genome equivalent (ATCC, Product information sheet for ATCC® CCL-2; Lin, et al., 2007), and SiHa cells contain 1 or 2 copies of HPV-16 per cell (ATCC, Product information sheet for ATCC® HTB-35; Mincheva, Gissmann, zur Hausen, 1987). All of the aforementioned HPV genetic material found in these cell lines originated from HPVs considered to be high risk types (Muñoz, et. al., 2003).

Bacterial vaginosis is associated with a shift in flora dominated by commensal bacteria to pathogenic bacteria including Gram-negative strains (Cherpes, et al., 2008; Devillard, Burton, Reid, 2005; Donders, et al., 2002; Nugent, Krohn, Hillier, 1991; Reid, et al., 2003). **The second objective of our study was to investigate whether infection with Gram-negative pathogenic bacteria and commensal bacteria affects integrin and TLR expression in cervical epithelial**

cells. For these experiments, we infected HeLa cells with *Escherichia coli*, *Pseudomonas aeruginosa*, and *Lactobacillus reuteri*. We chose these three bacteria as all have been found in the lower female genital tract, and have been shown to interact with HeLa cells (*L. reuteri* is formerly known as *L. fermentum* [Reid & Hammond, 2005]) (Hillier, Krohn, Rabe, Klebanoff, Eschenbach, 1993; Ma, Forsythe, Bienenstock, 2004; Reid, G., Beuerman, Heinemann, Bruce, 2001; Watt, Goldacre, Loudon, Annat, Harris, Vessey, 1981; Wiedmaier, et al., 2008).

Although expression for some integrins has been previously examined in biopsied cervical tissues (Hazelbag et al., 2007; Hughes et al, 1994), to the best of our knowledge, TLRs have not been examined in this manner. As tissue biopsies contain epithelial cells in addition to immune system derived cells, **our third objective was to examine TLR and integrin expression in normal, low-grade squamous intraepithelial lesion (LSIL), high-grade squamous intraepithelial lesion (HSIL), and carcinoma cervical biopsies**.

Materials and Methods

Cell lines

The cervical carcinoma cell lines HeLa (catalogue #CCL-2™), CaSki (catalogue #CLR-1550), SiHa (catalogue #HTB-35), C-33A (catalogue #HTB-31™), and ME180 (catalogue #HTB-33™) were purchased from the American Type Culture Collection (ATCC, Rockville, MD). Frozen stocks of cells were stored in liquid nitrogen until thawed for culturing. All cells were maintained at 37°C with 5% CO_2 in 75 cm^2 flasks containing Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich, Oakville, ON, Canada) supplemented with 10% (v/v) heat-inactivated Fetal Bovine Serum (FBS, HyClone, Logan, Utah, USA) and 100 U of penicillin, 100 µg of streptomycin, 0.25 µg amphotericin B per ml ([1 X AB/AM], catalogue #15240-062, Gibco, Grand Island, NY, USA). Cell lines were passaged every 2 days to sustain a 60% to 80% confluent monolayer. The cells used in experiments were passaged from 3 to a maximum of 7 times from originally purchased frozen stocks.

Bacterial strains

Escherichia coli 25922 (ATCC catalogue #25922; Vermeulen, Keeler, Nandakumar, Kanavillil, Leung, 2008), *Pseudomonas aeruginosa* PAK (Pasloske, Finlay, Paanchych 1985), and *Lactobacillius reuteri 23272* (ATCC catalogue #23272; Forsythe, Inman, Bienenstock, 2007) were kindly provided by Dr. Kam Leung, Lakehead University, Thunder Bay, ON, Dr. R. J.

Irvin, University of Alberta, Edmonton, AB, and Dr. P. Forsythe, McMaster University, Hamilton, ON, respectively.

Single colonies of *E. coli* and *P. aeruginosa* were transferred under sterile conditions into 20 ml of Luria-Burtani (LB) medium (catalogue #L7275, Sigma-Aldrich), and were grown in a shaking incubator at 37°C at 150 rpm. After 16 h, 1 ml of each bacterial culture was transferred to 20 ml of fresh LB medium, and allowed to grow for 1 h. Aliquots of 1 ml freezer stocks of *E. coli* and *P. aeruginosa* for long-term storage were made by adding an equal volume of sterile glycerol to the suspended bacteria and freezing at -80°C. Prior to experiments, frozen *E. coli* and *P. aeruginosa* cultures were thawed at room temperature, then grown in LB medium as previously described. A sterile loop was dipped into the culture and streaked onto a plate containing Luria- Burtani agar (catalogue #L7025LBA, Sigma-Aldrich), and grown for 16 to 20 h at 37°C until single colonies appeared.

A single colony of *L. reuteri* was transferred under sterile conditions to a 15 ml conical tube containing 15 ml of MRS medium (catalogue #69966, Sigma-Aldrich, Steinheim, Switzerland). The bacteria were grown in a GasPak100™ system (catalogue #271040, Becton, Dickinson and Company, Sparks, Maryland, USA) at 37°C in anaerobic conditions for 16 h. Indicator strips within the sealed anaerobic jar confirmed that the oxygen was displaced. *L. reuteri* were then gently resuspended in MRS broth, and aliquots were kept at -20°C as freezer stocks.

Preparation of bacteria for experiments

E. coli and *P. aeruginosa* cultures were grown at 37°C for 16 h in a shaking incubator at 150 rpm. Then, 1 ml of each culture was transferred to 20 ml of fresh LB medium, and allowed to grow for an additional 1 h. Frozen *L. reuteri* stock was thawed at room temperature, and under sterile conditions, 50 µl of *L. reuteri* culture was gently mixed in 15 ml of MRS medium into a 15 ml conical tube. The tube containing the bacteria was transferred to a GasPak100™ system and grown at 37°C in anaerobic conditions for 16 h.

Each bacterial culture was centrifuged at 3500 x g for 20 min at 4°C, and pellets were washed 3 times with 10 ml of cold, sterile phosphate buffered saline, pH 7.4 (PBS). For each bacteria, the final pellet was re-suspended in PBS to achieve optical density at 600 nm (OD_{600}) of 0.39-0.44. This corresponds to 2 X 10^8 CFU/mL for *E. coli*, 5 X 10^7 CFU/mL for *L. reuteri*, and 4 X 10^8 CFU/ml for *P. aeruginosa* as determined through multiple drop plating assays.

Bacterial infection of HeLa cells

HeLa cells were trypsinized for a maximum of 5 min at 37°C [0.05% trypsin-EDTA (Invitrogen, Grand Island, NY)], washed with sterile PBS and counted using Vi-CELL™ XR viability analyzer (Beckman Coulter, Mississauga, ON), resuspended in culture medium and seeded at 2 x 10^6 cells per 75 cm^2 flask 24 h prior to each experiment.

Medium from the flasks was removed, and the cells were rinsed 2 times with 5 ml of sterile PBS. The appropriate volume of bacterial suspension calculated for the desired multiplicity of

infection (MOI) was added to the treatment flask. A volume of PBS equal to the volume of suspended bacteria in the treated flask was added to the control flask. Serum and antibiotic/anitmycotic free medium was added to each flask to achieve a final volume of 15 ml. Both flasks were then incubated at 37°C, 5% CO_2 incubator for 4 h. Although numbers of colony forming units (CFU) corresponding to specific OD_{600} were determined during optimization of the assay through drop plating on LBA for *E.coli* and *P. aeruginosa*, and on MRS agar for *L. reuteri*, the MOI was confirmed in all the experiments with counting HeLa cells in control flasks and serial dilutions and drop plating of each bacteria.

LPS stimulation of ME180 cells

In some experiments, HeLa and ME180 cells were stimulated for 24 h with lipopolysaccharide from the *E. coli* 0111:B4 strain at concentrations of 10 μg/ml and 100 μg/ml (catalogue #tlrl-pelps, InvivoGen, San Diego, CA). Cells seeded 24 h prior to treatment were washed 2 times with 2 ml of PBS. During the treatment period, medium was changed from DMEM supplemented with 10% FBS and 1 x AB/AM to DMEM supplemented with 5% FBS and 1 x AB/AM. Following stimulation, cells were prepared for flow cytometry.

Preparation of cells for flow cytometry analysis

Following 4 h of bacterial infection or LPS stimulation, the medium from each flask was transferred to a 15 ml conical tube. The flasks were washed 2 times with 2 ml of PBS, with each wash retained in appropriately labeled 15 ml conical tubes. The remaining adherent cells in the flasks were then trypsinized, after which an equal amount of DMEM medium containing 10% FBS was added to neutralize the trypsin. The remaining cells from the flasks were transferred to

tubes containing their respective PBS washes. From this point on, all samples and buffers were kept on ice. Cells were centrifuged at 500 x g for 6 min and washed two times with PBS at 4°C.

For analysis of unstimulated cells, all 5 cell lines were seeded 24 h prior to each experiment. Medium was removed from each flask, and the cells were washed 2 times with 5 ml of PBS. Cells were trypsinized and washed as described above. A sample from each cell line was used for counting with the Vi-CELL™ XR.

For immunostaining, the cell pellets were re-suspended in 1.5 ml of PBS containing 0.1% bovine serum albumin (BSA, Sigma catalogue #A1470-100G), and 100 µl aliquots containing 2 x 10^5 cells were transferred to flow cytometry tubes. Appropriate antibodies (table 1) were added to each tube at a dilution factor of 1:50, the tubes were gently vortex mixed, covered, and incubated in the dark at 4°C for 1.5 h. Following incubation, 1 ml of PBS was added to each test tube. The samples were then centrifuged at 500 x g for 6 min at 4°C, the supernatant decanted and 1 ml of fresh PBS was added to each tube. Washes were repeated two more times. Cells immunostained with antibodies conjugated to R-Phycoerythrin (R-PE) were re-suspended in 700 µl of PBS and analyzed by flow cytometry. Cells immunostained with unconjugated primary antibody were re-suspended in 100 µl of PBS and secondary antibody conjugated to R-PE was added at a dilution ratio of 1:50. These tubes were then covered and incubated in the dark at 4°C for 1.5 h. Following incubation, cells were washed as previously described, then re-suspended in 700 µl of PBS, and analyzed by flow cytometry.

Permeabilization for intracellular immunostaining of HeLa cells.

Medium was removed from the flask, the cells were washed twice with 2 ml of PBS. Cells were then trypsinized as described above, after which an equal amount of DMEM medium containing 10% FBS was added to neutralize the trypsin. Cells were counted and split into two 15 ml conical tubes, then centrifuged at 500 x g for 6 min at 4°C and washed 3 times with PBS. Cells were re-suspended in 1 ml of 1 x permeabilization buffer solution (catalogue #347692, BD Biosciences, San Jose, CA) or in 1 ml of PBS for 10 min at room temperature, after which 5 ml of cold PBS was added to each tube. From this point on, all buffers and samples were kept on ice. The cells in both tubes were then centrifuged at 500 x g for 6 min, supernatant was decanted, and cells were re-suspended in 1 ml of PBS containing 0.1% BSA. An appropriate volume containing 2.5 x 10^5 cells was transfered to a flow cytometry tube. The volume was then brought up to 200 µl with PBS containing 0.1% BSA. Antibodies were added to each tube at a dilution factor of 1:50, the tubes were gently vortex mixed, covered, and incubated in the dark for 1.5 h. Following incubation, 1 ml of PBS was added to each test tube. The samples were then centrifuged at 500 x g for 6 min, the supernatant decanted and 1 ml of fresh PBS was added to each tube. Washes were repeated two more times. Cells immunostained with TLR3 and TLR9 antibody were re-suspended in 100 µl of PBS and secondary antibody conjugated to R-PE was added at a dilution ratio of 1:50. These tubes were then covered and incubated in the dark at 4°C for 1.5 h. Following incubation, cells were washed as previously described, then re-suspended in 700 µl of PBS, and analyzed by flow cytometry.

Table 1. Antibodies used for immunostaining

Antibodies specific to TLR4 (eBiosciences, San Diego, CA) and β_1, β_3, β_4, α_v, α_3, α_5, and α_6 integrins are directly conjugated with R-PE. Antibodies specific to TLR3, TLR9, and β_6 integrin required incubation with a secondary antibody conjugated with R-PE. Unconjugated antibody to TLR4 (Santa Cruz Biotechnology, Santa Cruz, CA) was used for immunostaining of LPS stimulated HeLa and ME180 cells, and required incubation with a secondary antibody conjugated with FITC.

Specificity	Clone	Isotype	Format	Source	Catalogue number
β_1	MAR4	Mouse IgG1, κ	PE conjugated	BD Pharmingen	555443
β_3	VI-PL2	Mouse IgG1, κ	PE conjugated	BD Pharmingen	555754
β_4	439-9B	Rat IgG2b, κ	PE conjugated	BD Pharmingen	555720
β_6	442.5C4	Mouse IgG	unconjugated	Calbiochem	407317
α_v	13C2	Mouse IgG1	PE conjugated	Chemicon	CBL490P
α_3	C3 II.1	Mouse IgG1, κ	PE conjugated	BD Pharmingen	556025
α_5	IIA1	Mouse IgG1, κ	PE conjugated	BD Pharmingen	555617
α_6	GoH3	Rat IgG2a, κ	PE conjugated	BD Pharmingen	555736
TLR3	TLR3.7	Mouse IgG1, κ	unconjugated	Santa Cruz	sc-32232
TLR4	HTA125	Mouse IgG2a, κ	PE conjugated	eBiosciences	12-9917-73
TLR9	5G5	Mouse IgG2a	unconjugated	Santa Cruz	sc-47723
TLR4	HTA125	Mouse IgG2a	unconjugated	Santa Cruz	sc-13593
isotype control	DD13	Rat IgG2a	PE conjugated	Chemicon	CBL605P
isotype control	MOPC-31C	Mouse IgG1, κ	unconjugated	BD Pharmingen	550878
isotype control	polyclonal	Mouse IgG(H+L)	PE conjugated	eBiosciences	12-4012-87

Acquisition and analysis

Flow cytometry analysis was performed using FACSCalibur instrument (BD Biosciences, San Jose, CA). For each test, 10,000 gated events have been analyzed. Instrument settings were retained throughout all experiments. Geometric mean fluorescence intensity was measured and normalized according to the corresponding isotype control.

The following calculations were used:

1) Fluorescence Intensity = ln(Antibody geo mean) - ln(Isotype control geo mean).

As variability increased with higher mean fluorescence intensity, data were analyzed using a natural log transformation (Howell, 2007, p. 318).

Statistical significance for flow cytometry results for bacterial infection was tested using 2-tailed Student's t-test with SPSS 16.0 for Mac software. Statistical significance comparing 5 different cell lines was tested with ANOVA followed by Tukey's test with Prism Graph software.

Isolation of RNA and real-time PCR analysis

Isolation of RNA and real-time PCR was conducted as previously described (DeCarlo et al, 2008). In brief, all cells were trypsinized as described above, centrifuged at 150 x g for 5 min at 4°C, and washed 2 times with PBS. Cell pellets were then frozen and stored at -80°C until the RNA extraction process, a maximum of 48 h. RNA extraction was done using the RNAqueous®-4PCR kit (catalogue # AM1914, Ambion Inc., Austin, TX), following the recommended protocol

for mammalian cells. RNA quantity and integrity was determined by loading RNA into standard-sensitivity chips for the Bio-Rad Experion Automated Electrophoresis System (Bio-Rad, Hercules, CA) (Fig.1). In addition to calculated 28s/18s ratio, electrophoregram and digital gel images were considered to assess RNA integrity. RNA isolated from samples was reverse transcribed in 18 µl volumes to complementary DNA (cDNA) using the High Capacity cDNA Archive Kit (catalogue #4322171, Applied Biosystems, Foster City, CA) according to manufacturer's directions, with random hexamer primers. RNA from cell lines required cDNA amplification and was reverse transcribed at 4 ng/µl. Fifty nanograms of cDNA was amplified using the TaqMan® PreAmp Master Mix Kit (catalogue #4384267Applied Biosystems, Foster City, CA). Gene amplification was analyzed for uniformity within each sample by comparing unamplified versus amplified gene expression between two genes using the $\Delta\Delta C_T$ method as suggested by the manufacturer (Applied Biosystems TaqMan® PreAmp Master Mix Kit Protocol). In brief, ΔC_T values (average C_T uniformity gene values subtracted from C_T target gene values) for cDNA and preamplfied cDNA were calculated. The $\Delta C_{T(cDNA)}$ values were subtracted from the $\Delta C_{T(Preamplified)}$ values. Values close to zero plus or minus 1.5 indicated preamplification uniformity (Applied Biosystems TaqMan® PreAmp Master Mix Kit Protocol).

CT values designate the number of amplification cycles required for detection of the fluorescent signal above threshold level, and is inversely related to the amount of starting template (Invitrogen, 2008, p.9). Therefore, higher CT values indicate lower amounts of starting template (cDNA) (Fig. 3, 4, and 5).

Immunohistochemical analysis of cervical tissue biopsies

Frozen cervical tissues were obtained with written consent from the Thunder Bay Regional Health Science Centre (TBRHSC) between 2006 and 2007. A portion of each sample was removed, examined, and graded by a pathologist, Dr. Nicholas Escott as normal, low-grade squamous intraepithelial lesion (LSIL), high-grade squamous intraepithelial lesion (HSIL), or carcinoma. Six of the samples were considered normal, five were LSIL, 3 were HSIL, and 4 samples were carcinomas. Tissues destined for immunostaining were immediately snap frozen in liquid nitrogen and embedded in Tissue Tek™ embedding medium (O.C.T. Compound, Sakura Finetek, Torrance, California, USA). Tissues were kept for long term storage at -80°C. Serial sections (7 μm thick) were cut in a Leica CM1850 cryostat and mounted onto Fisherbrand Colorfrost®/Plus precleaned microsocope slides (catalogue #12-550-20, Fisher Scientific, Pittsburgh, PA). Slides with cut sections were stored at -20°C prior to staining.

Tissues were fixed immediately before staining by immersing the sections in 100% methanol at -20°C for 10 minutes. Immunostaining was performed according to DakoCytomation EnVision+ Dual Link System-HRP (DAB+) (catalogue # K4065, DakoCytomation, Carpinteria, CA). In brief, tissue sections were incubated in Dual Endogenous Enzyme Block for 10 minutes, followed by 3 washes for 5 min each in PBS. Concentrations for primary antibodies were optimized and diluted in Dako ChemMate antibody diluent (catalogue #S2022, Dako, Denmark) at 1:50 for all TLRs, 1:100 for α_v, β_3, and β_4, and 1:1000 for β_6. Sections were incubated with primary antibodies in the dark at 4°C in a humidity chamber for 16 hours, then washed with PBS as previously mentioned. One to two drops of Labeled Polymer-HRP was added to each section and incubated at room temperature for 30 min, followed by 3 PBS washes. Sections were then

covered with 50 µl of DAB+ Substrate-Chromagen mixture and incubated for 10 minutes at room temperature. DAB+ Substrate-Chromagen mixture was removed, and sections were rinsed for 15 minutes in gently running reverse osmosis water. The slides were immediately transferred to the pathology lab at the TBRSC for hematoxylin staining. Sections were automatically mounted with an acetate slip for protection and preservation. Sections designated as negative controls were treated in parallel with the other sections, without the addition of primary antibodies.

Tissue sections were examined using a Nikon DXM light microscope with a Nikon Pan fluor 10 X objective. Classification of staining was conducted independently by myself and Dr. Ingeborg Zehbe and ranged from weak (+), to moderate (++), to strong (+++), to very strong (++++) when compared to negative control sections.

Results

Expression of toll-like receptor and integrin mRNA in unstimulated cervical cancer cell lines.

Human Papillomavirus (HPV) is considered a causative agent in the development of cervical cancer, yet the mechanism for malignant transformation is not clear (Ahn, et al., 2004; al-Saleh, et al., 1998). Both TLRs and integrins have been shown to play a role in the development and progression of squamous cell carcinomas (Koopman Van Aarsen, et al., 2008; Kurokawa, et al., 2008; Lee, et al., 2007). To investigate this relationship, we first analyzed mRNA from 5 cervical cancer cell lines using real-time PCR. Four of the cell lines reportedly contain genomic material from high-risk HPVs in differing quantities and of different genotypes, while the fifth cell line contains no HPV genomic material. The SiHa cell line contains 1 to 2 genome copies of HPV-16 per cell, CaSki cell line contains 60 to 600 genome copies of HPV-16 and some HPV-18 (ATCC, Product information sheet for HTB-35; ATCC, Product information sheet for CRL-1550). ME180 cell line contains HPV-68 DNA (Longuet, et al., 1996; Reuter, et al., 1998), the HeLa cell line contains 10 to 50 copies of HPV-18 genome equivalent (Lin, et al, 2007). The C-33A cell line is negative for HPV genetic material (ATCC, Product information sheet for HTB-31).

Analysis of mRNA expression using real time PCR revealed that TLR -1, -2, -5, -6, -7, -9 were detected in the cell lines studied (Fig. 2). However, TLR3 was not detected in C-33A cells, the cell line without HPV genetic material. TLR8 was not detected in any of the studied cell lines, except C-33A (low level). TLR4 was expressed at high levels in HeLa and SiHa cells, was

detected at low levels in C-33A cells, and was not detected in ME180 and CaSki cell lines. TLR2 appeared to be expressed higher in CaSki compared to other cell lines (Fig. 2 and 3).

In all cell lines, mRNA for all integrins examined was detected (Fig. 4). No difference in integrin expression between cell lines was observed for β_1 and α_v. The most notable differences in integrin expression between cell lines were for β_6. CaSki cells had the highest amounts of integrin β_6, while C-33A cells expressed the lowest amount of this integrin. There were slight variations in expression between the cell lines for the remaining integrins (Fig. 4). Our data show that gene expression of TLRs and integrins differs among cervical cancer-derrived cells.

Effect of LPS stimulation on integrin expression in ME180 cells

Bacterial vaginosis caused by Gram-negative bacteria such as *E. coli* can promote cervical cancer development (Kharsany, et al., 1993; Platz-Christensen, et al., 1994; Reid, et al., 2003). Lipopolysaccharide (LPS), is the major virulence factor of Gram-negative bacteria. It was shown that LPS can up-regulate integrin receptor expression, and it has been suggested that cancer development can be associated with the effect of LPS on integrin expression (Andrews, Wang, Winter, Laug, Redmond, 2001; Wang, et al., 2003). We therefore asked the question of whether LPS, the major virulence factor and cell-wall component of Gram-negative bacteria, affects integrin expression in cervical cancer cells. To answer this question, we stimulated the cervical cancer-derrived ME180 cells with either 10 μM or 100 μM LPS for 24 h. We found that surface expression of integrins α_3, α_5, α_6, α_v, β_1, β_3, and β_4 on ME180 cells was not affected by LPS stimulation. It was also noted that integrin β_3, which has been suggested to be associated with cervical cancer progression, was practically undetectable on the surface of ME180 cells

(Fig. 5) (Gruber, et al., 2005). This experiment was also conducted on HeLa cells. As with ME180 cells, no discernible differences in integrin expression were observed between treated and untreated HeLa cells (n=1, data not shown). However, differences in the profiles of surface integrin expression, specifically with integrins α_5 and β_3, were observed between untreated ME180 and HeLa cells.

The surface protein expression of integrins and toll-like receptors in unstimulated cervical cancer cell lines

Next, we investigated the surface protein expression for integrins, TLR3 and TLR4 of unstimulated cervical cancer-derived cell lines using flow cytometry. Because our mRNA data showed the greatest variability for integrin β_6 among the studied cell lines, and some literature also suggests that β_6 is associated with cervical cancer development (Hazelbag, et al., 2007 Koopman Van Aarsen, et al., 2008), we included the examination of this subunit for subsequent experiments. Likewise, as TLR4 and TLR3 mRNA was undetected in some cell lines and expressed in others, these two TLRs were also included in further experiments.

Corresponding with real-time PCR findings, flow cytometry data revealed that the surface expression of β_1 integrin was highest compared to other subunits in each cell line (Fig. 6, 7, and 8). Integrin β_3 was expressed at the lowest level compared to other subunits in C-33A, SiHa, and ME180 cell lines ($p < 0.05$), and was not detected in CaSki cells.

In the HeLa cell line, integrin β_6 was expressed the lowest ($p < 0.05$). The C-33A cells expressed the lowest levels of α_3, α_6, and β_1 integrins among the studied cell lines ($p < 0.05$).

The CaSki cell line expressed the lowest amount of α_v and the highest amounts of β_6 integrin ($p < 0.05$), corresponding to mRNA data. HeLa cells expressed higher levels of integrins β_1, β_3, α_v, and α_5 compared to the other cell lines ($p < 0.05$), and generally have high integrin expression levels. ME180 cells expressed the lowest levels of α_5 and the highest level of α_6, β_4 and TLR4 ($p < 0.05$). Although expression for TLR3 was also highest in ME180 cells, surface expression for both TLRs examined was extremely low in all cell lines. SiHa cells exhibit all integrins examined at low to moderate levels in comparison to the other cell lines (Fig. 6, 7, and 8).

Expression of TLR -3 -4 and -9 in permeabilized HeLa cells

It has been shown that the expression of TLR-3 and -9 in cervical epithelial cells is almost exclusively intracellular (Andersen, Al-Khairy, Ingalls, 2006). In addition, some evidence suggests that TLR4 may be expressed intracellularly in intestinal epithelial cells (Hornef, et al., 2002). Therefore, to detect TLR-3, -4, and -9 intracellularly, we examined the protein expression for these molecules in permeabilized HeLa cells. Results confirm that HeLa cells exhibit TLR-3 and -9 intracellularly and that TLR4 expression is absent from HeLa cells (Fig. 9).

Effect of bacterial infection on the expression of integrins and toll-like receptors

Because many bacteria use adhesion molecules including integrins to adhere to and invade epithelial cells, we hypothesized that the expression of integrins can be altered as a result of bacterial infection (Breuss,et al., 1995; Schmid, et al., 2004; Tsuda, et al., 2008). To test this

hypothesis, we stimulated HeLa cells with either Gram-negative pathogens *P. aeruginosa* or *E. coli*, or the non-pathogenic Gram-positive bacterium *L. reuteri*. Expression of α_3, α_5, α_6, α_v, β_1, β_3, and β_4 integrin subunits on HeLa cells significantly decreased after 4 h infection with *P. aeruginosa* and *E. coli* ($p < 0.05$) (Fig. 10-13). In contrast, expression of β_6 integrin and TLR4 increased after infection with both pathogenic bacteria ($p < 0.05$). In addition, *E. coli* infection induced expression of TLR3 in HeLa cells ($p < 0.05$), although TLR3 was barely detectable prior to the infection (Fig. 10-13). However, no changes in integrin, TLR3 or TLR4 expression were detected after 4 h infection with *L. reuteri* (Fig. 12 and 13). Examination under a microscope revealed physical changes to the cells infected with *P. aeruginosa* and *E. coli* following 4 h infection. These included cell detachment and a more spherical appearance. There were no obvious differences in the appearance of cells following 4 h infection with *L. reuteri* (Fig. 14).

Integrin and TLR expression in cervical biopsies

We then hypothesized that changes in integrin and TLR expression may occur in cervical tissues *in vivo* during various stages of dysplasia. Frozen cervical biopsies were sectioned and immunostained for integrins β_4, β_6, α_v, β_3, and TLR -3 and -4.

There were no obvious changes in staining patterns for the expression for TLR -3, -4, and -9 between normal, LSIL, HSIL, or carcinoma tissues (Fig. 15). The strength of staining was compared to negative control samples. Weak staining was observed in both epithelium and stroma for TLR3, TLR4 was absent from all samples, and moderate staining was observed throughout all tissues in all samples for TLR9.

Weak to moderate positive staining for integrin β_6 was observed in the stroma for all specimens (Fig. 16). Two of the normal biopsies showed weak staining for β_6, two had moderate staining, while one showed strong staining in the epithelia (one normal sample was damaged during processing). All LSIL and HSIL samples demonstrated moderate to strong staining for β_6 in the epithelial tissues (not shown), while 3 out of the 4 carcinomas displayed strong to very strong staining in epithelia (Fig. 16).

Integrin β_4 was found along the basement membrane of the epithelium in all samples including carcinomas where the basement membrane was visible, and was found focally in patches in 50% of the carcinomas (Fig. 17).

Weak to moderate staining for integrins α_v and β_3 was found almost solely in the epithelium for carcinoma samples, and in the stroma for all other samples (Fig. 17). These results indicate during the development of cervical cancer, noticeable changes occur in integrin, but not TLR expression.

Relative amount of integrin $\alpha_6\beta_4$ in all five cell lines

Invasion as a result of disruption of the basement membrane is important for the development of cervical cancer (Hughes, et al., 1994). It has been demonstrated that integrin $\alpha_6\beta_4$ may play an important role in the invasion of cervical cancer cells (Shen, et al., 2003). For our cell lines, surface expression for both the α_6 and β_4 subunits varied, and although the integrin subunit α_6 may bind to β_1, it has been reported to preferentially bind to the β_4 subunit, forming $\alpha_6\beta_4$ (Hemler, Crouse, Sonnenberg, 1989). Therefore, we wanted to examine integrin $\alpha_6\beta_4$ expression in the five unstimulated cell lines. The relative amount of $\alpha_6\beta_4$ was calculated by dividing the

MFI for β_4 by the MFI for α_6. ANOVA analysis followed by Tukey's post-hoc test for significance revealed that the ratio of β_4 per α_6 was significantly higher in the CaSki, ME180, and HeLa cell lines compared to the SiHa and C-33A cell lines. There were also significant differences between SiHa, HeLa, and CaSki cell lines (Fig. 18).

Discussion

The mechanisms for the development of cervical cancer are not clear, and it has been suggested that bacterial vaginosis (BV), characterized by the dominance of Gram-negative bacteria in the vaginal flora, may be associated with the acquisition of cervical cancer (Ahn, et al., 2004; Nugent, et al., 1991). Nevertheless, due to the oncongenic potential of high-risk human papillomavirus (HPV), and given that HPV is found in almost all cervical cancers, persistent HPV infection is identified as the main causative agent for cervical cancer (Castle, et al., 2001; Gillison & Lowy, 2004; Revaz, et al., 2007; Walboomers, et al., 1999). In order for HPV infection to persist, the virus must avoid detection by the immune system. Toll-like receptors (TLR) are important front-line responders in epithelial tissues to pathogenic microorganisms such as viruses and Gram-negative bacteria, as they initiate innate immune responses, and trigger the adaptive immune system (Akira, Takeda, Kaisho, 2001; Murphy, et al., 2008, Ch2). TLRs are therefore poised to play an important role in the development of cervical cancer.

It has been shown that changes in integrin expression are associated with the development of cervical cancer (Gruber, et al, 2005; Hazelbag, et al., 2007; Hughes, et al., 1994; Janes & Watt, 2006; Valea, Haskill, Moore, Fowler, 1995; Vazques-Ortiz, et al., 2005). This is not entirely unexpected, as integrins play key roles in apoptosis, proliferation, migration, and angiogenesis (Bentz & Yurochko; 2008; Unfried, Sydlik, Bierhals, Weissenberg, Abel, 2007; Yoon, et al., 2001), and changes in integrin expression are linked with tumor growth (Tucker, 2006; Zeng, et al., 2009). Pathogenic microorganisms such as Gram-negative bacteria are known to interact with integrins on epithelial cells (Gu, Wnag, Guo, Zen, 2008; Roger, et al., 1999), and some studies indicate that α_6 may act as a receptor for HPV (Culp, Budgeon, Marinkovich, Meneguzzi,

Christensen, 2006; Fothergill, et al., 2006; Yoon, et al., 2001). Therefore, integrins have the potential to play a role in the development of cervical cancer. Our objective was to characterize integrin and TLR expression in cervical cancer-derived cell lines as well as clinical biopsy material and examine the relationship between integrin and TLR expression for one of these cell lines in a bacterial infection model.

Integrin expression and passage number

Preliminary results from experiments examining integrin expression in ME180 and HeLa cells indicated that passage number affected integrin expression. Data revealed that integrin expression increased between passage 4 and 15 for ME180 cells (n=1, data not shown). Increases were also seen for some integrins in HeLa cells between passages 7 and 11 and 11 and 19, although overall expression for integrins examined in HeLa cells decreased from passage 7 to passage 28 (n=1, data not shown). As a result, all experiments for all cell lines were conducted between 3 and 7 passages from initially purchased stocks.

Integrin expression in 5 cervical cancer-derived cell lines

Our data show that cervical cancer-derived epithelial cells have different integrin expression profiles. The ME180 cell line containing HPV-68 genetic material (Longuet, et al., 1996; Reuter, et al, 1998) displays the lowest amount of the integrin subunit α_5. In contrast, HeLa cells reportedly containing HPV-18 DNA (Lin, et al., 2007) exhibit the highest surface expression of

subunits α_5, α_v, β_1, and β_3. The α_5 subunit only binds to the β_1 subunit, while the β_3 subunit may bind to the α_v subunit. Integrins $\alpha_5\beta_1$ and $\alpha_v\beta_3$ may facilitate the infection of epithelial cells by pathogenic microorganisms including strains of *E. coli* (Frankel, et al., 1996; Gu, et al., 2008; Klotz, Pendrak, Hein, 2001), indicating that these integrin-expressing cells may be more susceptible to bacterial infection. These integrins are also associated with angiogenesis, which is paramount for tumor growth and development, and as such are currently considered as therapeutic targets for some cancers (Bhaskar, et al, 2008; reviewed by Jin & Varner, 2004; reviewed by Tucker, 2006). Indeed, in analyzed biopsy samples, high expression of both α_v and β_3 in epithelial cells was detected in carcinomas. The CaSki cell line containing genome copies of HPV-16 and some HPV-18 exhibited the lowest amount of the a_v subunit and highest amount of subunit β_6. Integrin $\alpha_v\beta_6$ is associated with wound healing, fetal development, and metastatic potential in ovarian and other cancers, and will be discussed in depth further (Breuss, et al., 1995; Janes & Watt, 2004; Janes & Watt, 2006; Koopman Van Aarsen, et al., 2008). For our biopsy samples, we also noted that a_v integrin was up-regulated in carcinomas compared to normal samples. Hence, we have shown that integrin expression varies between these 5 cervical cancer-derived epithelial cell lines.

Our data also show that cell lines containing HPV genetic material typically have higher surface expression of integrins than the HPV-negative C-33A cell line. HeLa and CaSki cell lines which contain high amounts of HPV genetic material display higher surface expression for integrins than that of SiHa cells, which contain low amounts of HPV DNA. For example, the C-33A cell line exhibits the lowest surface expression for 6 of the 8 integrins examined, and the SiHa cell line exhibits the lowest expression for 3 out of 8, while the CaSki and HeLa cell lines only exibit 1, and 2 out of the 8 integrins examined at the lowest levels, respectively. In addition, the CaSki

and HeLa cell lines exhibit the highest surface expression for 4 out of 8 and 6 out of 8 integrins, respectively (fig. 8). Although the shortcomings of our experimental design cannot allow us to conclude that amount of HPV genetic material found in these cell lines influences integrin expression, our results indicate that there may be an intriguing positive relationship between the amount of HPV genetic material and integrin expression. There are indications in the literature that this relationship may exist as well. HPV viral load has been shown to increase as cervical cancer develops (Dalstein, et al., 2003; Wu, et al., 2006), while expression of many integrins is also increased in cervical cancer compared to normal tissue (Breuss, et al., 1995; Chattopadhyay & Chatterjee 2001; Hughes, et al., 1994). Experiments examining integrin expression in cultured cervical epithelial cells infected with HPV at different MOI and without HPV infection may further elucidate this relationship.

Specifially, for one important integrin associated with tumor invasion, $\alpha_6\beta_4$, further investigation into this relationship may be warranted. The invasion of tumorogenic cells from the epithelial layer through the basement membrane is key for the progression of cervical cancer, and is a hallmark for invasive cervical cancer (Hughes, et al., 1994). Integrin $\alpha_6\beta_4$ is found in relation to hemidesmosomes in epithelial basal cells and it regulates adhesion to laminin 5, a basement membrane protein (Mainiero, et al., 1997; reviewed by Nievers, Schaapveld, Sonnenberg, 1999). In addition to cell adhesion, this integrin regulates apoptosis, migration, cell proliferation and differentiation (Jones, Kurpakus, Cooper, Quaranta, 1991; Nievers, et al., 1999). Furthermore, the production of matrix metallaprotease (MMP) -9, which degrades basement membrane proteins allowing tumor cells to penetrate the membrane, can also be affected by the activation of $\alpha_6\beta_4$ (Davis & Senger, 2005; Shen, et al., 2003). As indicated in the literature and also apparent from our analysis of biopsies, β_4 is up-regulated in squamous carcinomas, and changes its

expression from basal cells in pre-cancerous lesions to focal points along the tumor-stroma interface in cervical carcinomas (Hughes, et al., 1994; Nievers, et al., 1999). Although the α_6 subunit may bind to β_1, it preferentially binds to the β_4 subunit, and β_4 only binds to the α_6 subunit (Beer & Schwaiger, 2008; Hemler, et al., 1989). Given this, our data reveal that the ratio of β_4 per α_6 is significantly increased in the CaSki, ME180, and HeLa cell lines. As stated previously, these three cell lines contain higher amounts of HPV DNA than the SiHa cell line. Furthermore, primitive basal epithelial cells bound to the basement membrane are targeted by HPV, which has been shown to use laminin 5 and $\alpha_6\beta_4$ to gain access into the cell (Culp, et al., 2006; Fothergill, et al., 2006; Stanley, et al., 2007). Further exploration as to whether HPV has developed a mechanism to exploit integrin $\alpha_6\beta_4$ expression allowing infection to persist, and whether HPV infection may influence tumor invasion through the basement membrane may provide critical clues as to how cervical cancer develops.

The relationship between integrin expression and HPV infection in cervical tissues has not yet been elucidated. Due to the limitations of this study we cannot directly link integrin expression to HPV genotype or viral load. However, other studies have demonstrated that viral infections affected integrin expression in endothelial and epithelial tissues, and different serotypes of viruses had differential effects on integrin expression (Gavrilovskaya, Peresleni, Geimonen, Mackow, 2002; Halasz, Holloway, Turner, Coulson, 2008). As genetic material from different HPV genotypes in differing amounts are present in each of the aforementioned cell lines, further investigation is needed to elucidate whether HPV genotype and viral load influence integrin regulation in a specific manner.

Toll-like receptor expression in unstimulated cells

TLRs are a part of the innate immune system and are important in early detection of microbial pathogens. The data obtained from the examination of C-33A, HeLa, CaSki, ME180, and SiHa cells indicate that HPV infection affects TLR expression at the mRNA level. Our data show that TLR expression was not homogeneous throughout the cell lines, with the largest discrepancies in TLR -3, -4, and -8 between the C-33A cells and the other cell lines containing HPV genetic material. Others have examined TLR expression in cervical tissues, and although expression in these cell lines has not previously been investigated in this manner, existing literature supports our findings (Andersen, et al. 2006; Hasan, 2007; Herbst-Kralovetz, et al., 2008; Jiang, et al., 2008).

TLR -3, -8, and -9 expression in unstimulated cell lines

TLRs -3, -8, and -9 are part of a sub group of the TLR family, which also includes TLR7, specializing in recognition of viral particles. This group of TLRs is typically found intracellularly, although TLR3 has been found on the surface of some cells (Jiang, et al., 2008; Matsumoto, Kikkawa, Kohase, Miyake, Seya, 2002; Matsumoto, et al., 2003). Polyribosinic:polyribocytidylic acid (PolyI:C), a synthetic form of double stranded RNA (dsRNA), is a ligand for TLR3, while TLR8 recognizes single-stranded RNA (ssRNA) (reviewed by Takeda, 2004). In addition to recognizing the CpG motif in double-stranded DNA (dsDNA) in viruses, TLR9 can also recognize dsDNA in bacteria (reviewed in Barton, 2007; Matsumoto, et al., 2002).

Hasan et al. (2007) report up-regulation of TLR3 mRNA in HPV infected cells (Hasan, et al., 2007). In our study, TLR3 mRNA was not detected in the HPV negative C-33A cell line, but was found in all other cell lines suggesting that the presence of HPV genetic material may cause up-regulation of TLR3. Surface expression of TLR3 was detected at very low levels in all cell lines. Nevertheless, we have detected TLR3 in permeablilized unstimulated HeLa cells, demonstrating that TLR3 is typically expressed intracelluluarly for these cell lines. Our findings are consistent with another study examining TLR3 expression in HeLa cells (Jiang, et al., 2008).

TLR8 mRNA was undetected in the cell lines containing HPV genomic material, yet found in very low concentrations in C-33A cells. Andersen et al. (2006) stated that mRNA for TLR8 was absent in *in vitro* epithelial tissues of the lower female reproductive tract. However, the cells used by Andersen et al. (2006) were immortalized with a retroviral vector containing genes from HPV-16 (Andersen, et al., 2006; Ficherova, et al., 1997). Consistent with our findings, Herbst-Karlovetz et al. (2008) detected TLR8 in primary cervical epithelial tissues grown *in vitro* at very low amounts (Herbst-Karlovetz, et al., 2008). Taken together, this implies that the presence of HPV DNA in cervical epithelial cells may affect TLR8 expression.

HPV is a dsDNA virus that contains a CpG motif, the ligand for TLR9 (reviewed by Barton, 2007). Upon examination of HPV infected cell lines infected with different HPV genotypes, Hasan et al. (2007) demonstrated that HPV -16 and -18 down-regulated TLR9 in human primary keratinocytes, and concluded that HPV-16 had a greater effect on TLR9 expression than HPV-18 (Hasan, et al., 2007). They further went on to examine TLR9 expression in SiHa, CaSki, and HeLa cells (Hasan, et al., 2007). Although our results were not identical regarding the relative magnitude of down-regulation of TLR9 in SiHa (HPV-16) and CaSki (HPV-16) cells with respect to HeLa (HPV-18) cells, we show that mRNA expression of TLR9 appears to be lower in

cells containing HPV genetic material compared to the C-33A cell line. Furthermore, CaSki cells, reportedly containing more HPV genetic material than SiHa cells, exhibit lower TLR9 expression than SiHa cells. It has been hypothesized that down-regulation of TLR9 in cervical epithelial cells is a mechanism by which HPV may evade the immune system, allowing infection to persist, which is a prerequisite for the development of cervical cancer (Castle, et al., 2001; Hasan, et al., 2007; Sun, et al., 1997).

TLR4 expression in unstimulated and LPS stimulated cells

The most striking differences in our data for TLR expression were observed with TLR4, whose ligand is lipopolysaccharide (LPS), a cell wall component of Gram-negative bacteria. Three of the five cell lines HeLa, SiHa, and C-33A, displayed mRNA for TLR4. TLR4 was expressed highest in HeLa cells, followed by SiHa, then C-33A cell line. Although TLR4 was detected in the C-33A cell line, it was expressed at the lowest level of all TLRs examined for all cell lines, and was detected in only one out of two C-33A samples. There are inconsistencies in the literature regarding the presence of TLR4 in epithelial tissues (Ficherova, Cronin, Lien, Anderson, Ingalls, 2002; Herbst-Kralovetz, et al., 2008). Supporting our results, Nishimura & Naito (2005) examined mRNA expression for TLRs 1 through 10 in different cell types including HeLa cells, and found TLR4 to be the highest expressed TLR in HeLa cells (Nishimura & Naito, 2005). Herbst-Kralovets et al. (2008) also detected TLR4 in HeLa S3 cells (a clonal derivative of our HeLa cells) at low amounts, but suggested that the (pooled) TLR4 expression in primary cervical cultures belonged only to a subset of samples (Herbst-Kralovetz, et al., 2008). We have examined surface expression for TLR4 in our cell lines, and TLR4 was

barely detected in all samples. However, we were unable to detect mRNA for TLR4 in ME180 cells, yet this cell line exhibited the highest surface expression of TLR4. In support of this finding, Herbst-Kralovetz et al. (2008) did detect mRNA for TLR4 in very low amounts in this cell line. Furhermore, in parallel with our examination of LPS stimulated ME180 and HeLa cells, Herbst-Kralovetz et al. (2008) also state that ME180 and HeLa cells stimulated with agonists for TLR4 (LPS) showed minimal response toward cytokine elaboration (Herbst-Kralovetz, et al., 2008). In our study, no discernible differences in integrin expression were observed in ME180 and HeLa cells following stimulation with LPS. There may be several possibilities for lack of response in these cells to LPS stimulation in our studies. The mere presence of TLR4 is not sufficient for recognition of LPS, and a combination of the molecules CD14, MD-2 and TLR4 are required for responses to LPS (Da Silva Coria, Soldau, Christen, Tobias, Ulevitch, 2001). CD14 and MD-2 were not examined here, and inconsistencies in the literature as to the presence of mRNA for CD14 in cervical epithelial cells coupled with the uncertainty of the availability of MD-2, demonstrate that further study into the presence of these proteins and their activity potential is required (Ficherova, 2002; Herbst-Kralovetz, 2008). Some studies have also demonstrated that LPS from different sources may affect cells in different ways, and the possibility exists that the choice of LPS source may have certain effect (Birkholz, Knipp, Nietzki, Adamek, Opferkuch, 1993; Nakamura, et al., 2008).

Viruses are known to affect adaptive and immune responses. For example, a mechanism by which a retrovirus is able to subvert the immune system and persist has been demonstrated to occur through TLR4 (Jude, et al., 2003). Because TLR4 mRNA was found in high amounts in some of our cell lines and absent from others, the possibility exists that HPV infection may affect TLR4 expression in a specific manner at the translational level. As mentioned previously, there

is strong evidence to suggest that HPV may affect TLR9 expression. To the best of our knowledge, the relationship between TLR4 expression and HPV status in cervical tissues has not been examined, and this may also explain inconsistencies in the literature regarding the presence of TLR4 in these tissues (Ficherova, et al., 2002; Herbst-Kralovetz, et al., 2008; Pioli, et al., 2004). However, given the limitations of our model, further research is necessary to elucidate the relationship between TLR4 expression and HPV infection.

For our biopsy samples, no changes in TLR expression were noted between samples. TLR3 was detected at the lowest level, TLR9 was detected at moderate levels, and TLR4 was not detected in any samples. It has been reported that TLR molecules are typically found in very low amounts in cells, and the possibility exists that our staining method may not be sensitive enough to differentiate between small changes in TLR expression in biopsy tissues (Akira, et al., 2001).

Bacterial infection model with HeLa cells

Bacterial vaginosis is described as an imbalance in the normal vaginal flora and is characterized as a shift from commensal bacteria to pathogenic bacteria, including Gram-negative bacteria, accompanied by a change in pH (Cherpes, et al., 2008; Devillard, et al., 2005; Donders, et al., 2002; Nugent, et al., 1991). For our experiments, we used *E. coli* and *P. aeruginosa*, two pathogenic Gram-negative bacteria, and *L. rueteri*, commensal bacteria, to infect HeLa cells. These three bacteria are found in vaginal flora and may interact with epithelial cells (Antonio, Hawes, Hillier, 1999; Pabich, et al., 2003; Vielfort, Sjölinder, Roos, Jonsson, Aro, 2008; Watt, et al., 1981; Xicohtencatl-Cortes, et al, 2007; Zhang, et al., 2005). Our data reveal that significant

changes occur in integrin and TLR expression in HeLa cells following 4 h infection with Gram-negative, but not commensal bacteria.

Integrin β_6 expression increases in Gram-negative bacteria-infected HeLa cells

It has been demonstrated that Gram-negative bacteria including *E. coli* and *P. aeruginosa* are able to exploit β_1 integrins to adhere to and internalize into epithelial cells (Frankel, et al., 1996; Gu, et al., 2008; Klotz, et al., 2001; Roger, et al., 1999), therefore down-regulation of the surface expression of some integrins due to internalization of the bacteria is not unexpected. Indeed, our data show that surface expression of integrins in HeLa cells is affected by infection caused by *E. coli* and *P. aeruginosa*, two Gram-negative bacteria. In contrast, there was no change in integrin expression with infection of *L. reuteri*, commensal, Gram-positive bacteria.

Following Gram-negative bacterial infection, significant down-regulation of all of the integrin subunits examined was observed, except for the β_6 subunit, for which expression significantly increased. Integrin $\alpha_v\beta_6$ is associated with migration, development and morphogenic events, and is typically absent from normal fully differentiated epithelia, unless associated with wound repair and inflammation (Breuss, et al., 1995). Furthermore, it has also been suggested that this integrin makes an attractive prognostic marker and therapeutic target for squamous cell carcinomas such as oral and colon carcinomas as it is typically not found in normal epithelia, over-expressed in malignant tissues, and associated with poor prognosis (Bates, 2005a, 2005b; Ramos, Dang, Sadler, 2009).

Observed changes for β_6 expression in our biopsy material toward very strong expression in carcinoma samples corroborate our bacterial infection model. Most importantly, however, recent studies examining $\alpha_v\beta_6$ in colon and oral carcinomas concluded that this integrin is associated with epithelial to mesenchymal transition (EMT), and may create an environment conducive to tumor progression (Bates, 2005a; Ramos, et al., 2009). Upregulation of β_6 integrin has also been shown to allow epithelial cells to survive unattached from extracellular matrix, it has been found associated with squamous cell carcinomas including cervical cancer, and has been linked to malignant transformation in intestinal carcinomas (Bates, 2005a; Breuss, et al, 1995; Janes, & Watt, 2006; Koopman Van Aarsen, et al., 2008). The ability of cells to survive unattached to the extracellular matrix is a hallmark of metastatic tumors, and the up-regulation of this integrin (while all other examined integrins were down-regulated), suggests that Gram-negative bacteria associated with BV may provide a mechanism for metastasis of epithelial tumor cells.

TLR4 expression increases in Gram-negative bacteria-infected HeLa cells

A significant increase in TLR4 was observed as a result of Gram-negative bacteria infection with both *P. aeruginosa* and *E. coli*, but not of stimulation with commensal Gram-positive bacteria. TLR4 was found at extremely low levels on the surface of HeLa cells in our experiments, and was not detected in permeabilized cells suggesting that up-regulation of TLR4 surface expression following Gram-negative bacterial infection is due to new protein production. Interestingly, LPS stimulation did not cause any changes in integrin expression in HeLa cells in our study. In another study, LPS stimulation of HeLa cells produced only minimal cytokine responses (Herbst-Kralovetz, et al., 2008). It is for these reasons we suggest that expression of TLR4 in

this cell line as a result of pathogenic bacterial infection occurs through a different mechanism than the binding of LPS from Gram-negative bacteria to TLR4. The increased expression of TLR4 post infection with Gram-negative bacteria may also be of importance to the development of cervical cancer as up-regulation of TLR4 has been shown to assist tumors with evasion of the adaptive immune system (Huang, et al., 2005; Huang, Zhao, Unkeless, Feng, Xiong, 2008; Qian, et al., 2008).

Expression of TLR3 in Gram-negative bacteria-infected HeLa cells

TLR3 is has been cited as being typically expressed intracellularly in epithelial cells, although its surface expression has also been previously detected in HeLa cells and fibroblasts (Andersen, et al., 2006; Jiang, et al., 2008; Matsumoto, et al., 2002). In our studies, we detected TLR3 at extremely low levels on the surface of untreated cells, yet TLR3 was detected in permeabilized HeLa cells, confirming that in these cells, TLR3 is expressed intracellularly. Interestingly, infection of HeLa cells with *E. coli* significantly up-regulated surface expression of TLR3. This indicates that surface expression of TLR3 may be induced by at least one pathogenic microbe associated with BV. The expression pattern of TLR3 mRNA in the five cell lines examined here also implies that for cervical cancer cells, HPV may play a distinct role in the upregulation of TLR3 in cervical tissues. Ligands for TLR3 are currently used as adjuvants in cancer therapies, but have also been shown to directly induce apoptosis in human breast adenocarcinoma cells (Laplanche, et al., 2000; Salaun, Coste, Rissoan, Lebecque, Renno, 2006). Although due to the shortcomings of our model we cannot definitively elucidate the relationship between HPV infection and TLR3 expression, if TLR3 expression is specific to HPV infection, and is up-

regulated on the surface of epithelial cells in the presence of microbes associated with BV, TLR3 may be an attractive and specific therapeutic target for cervical cancer.

Conclusions

This study was intended to elucidate the relationship between integrin and TLR expression with regard to HPV, bacterial infection, and cervical cancer. Studies within the last two decades have repeatedly shown that persistent HPV infection is paramount in the development of cervical cancer (Stanley, et al, 2007; Yee, et al., 1985; zur Hausen, 2002). To underscore the importance of this relationship, recently, a vaccine for the two most common strains of HPV associated with cervical cancer has been developed and touted as a "cancer vaccine for women" (Canadian Broadcasting Corporation [CBC], 2008). Regardless, not all women infected with high-risk HPV go on to develop cervical cancer, and some persistent infections may resolve on their own, indicating that HPV infection alone is not sufficient for the development of cervical cancer (Ahn, et al, 2004; Mitchell, et al. 1996). In addition, the mechanisms for the development of cervical cancer are not yet clear. Some have suggested that bacterial vaginosis may play a role in the development of cervical cancer, while others refute these claims (Boyle, et al., 2003; Castle, et al., 2001; Discacciati, et al., 2006; Kharsany, et al., 1993; Platz-Christensen, et al., 1994). Our data suggest that Gram-negative bacteria associated with BV may simultaneously produce changes in integrin and TLR expression in cervical-derived cancer cells containing HPV genetic material, conducive to malignant transformation and the facilitation of evasion from the immune system. Although short-comings of our study include that this is a cell culture model based on established cell lines derived from carcinomas, and the cells do not contain live HPV, our research calls for closer investigation into the relationship between infections caused by HPV and BV, and integrin and TLR expression.

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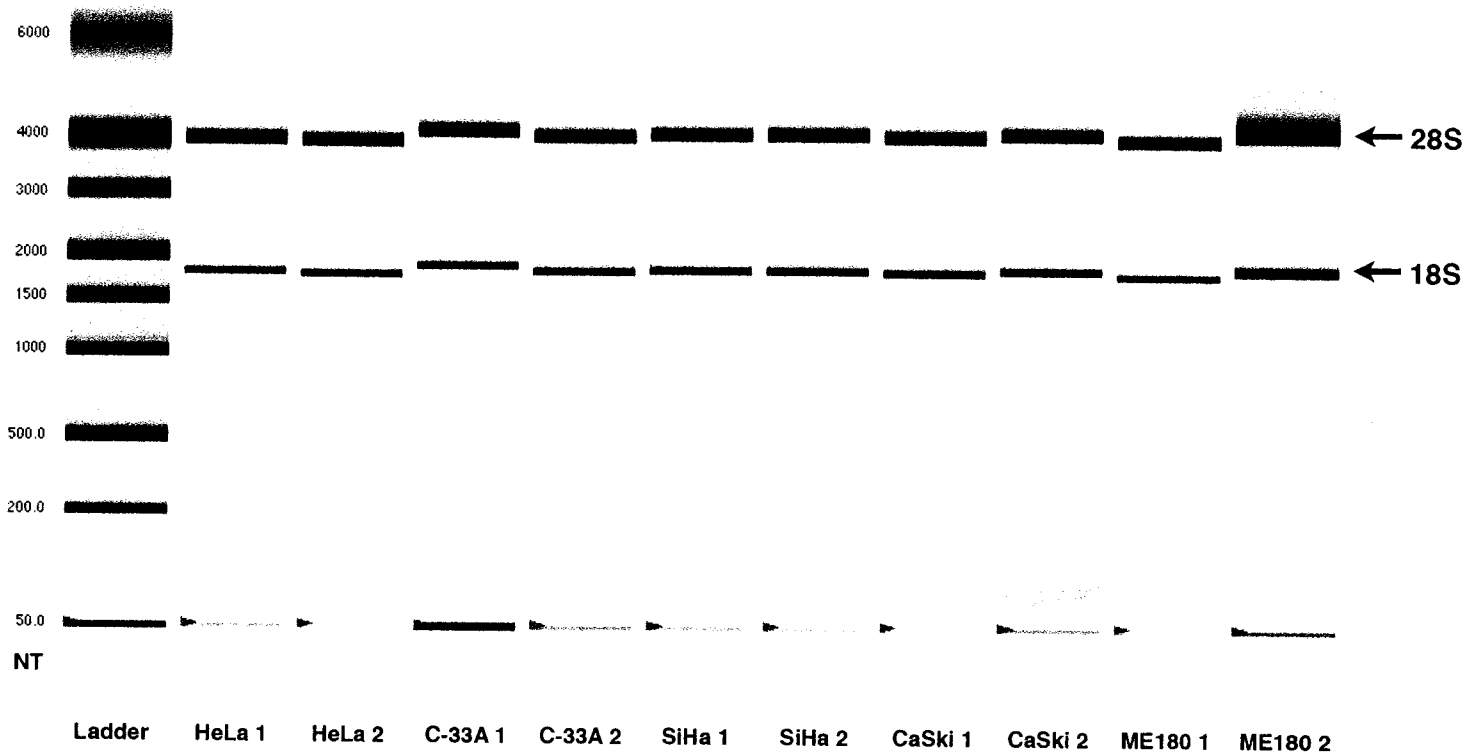


Figure 1: Digital gel image representing RNA integrity for RNA isolated from 5 different cervical cancer-derived cell lines as measured by Bio-Rad Experion Automated Electrophoresis System. Samples for each cell line were loaded into a standard sensitivity chip (n=2). NT: number of nucleotides

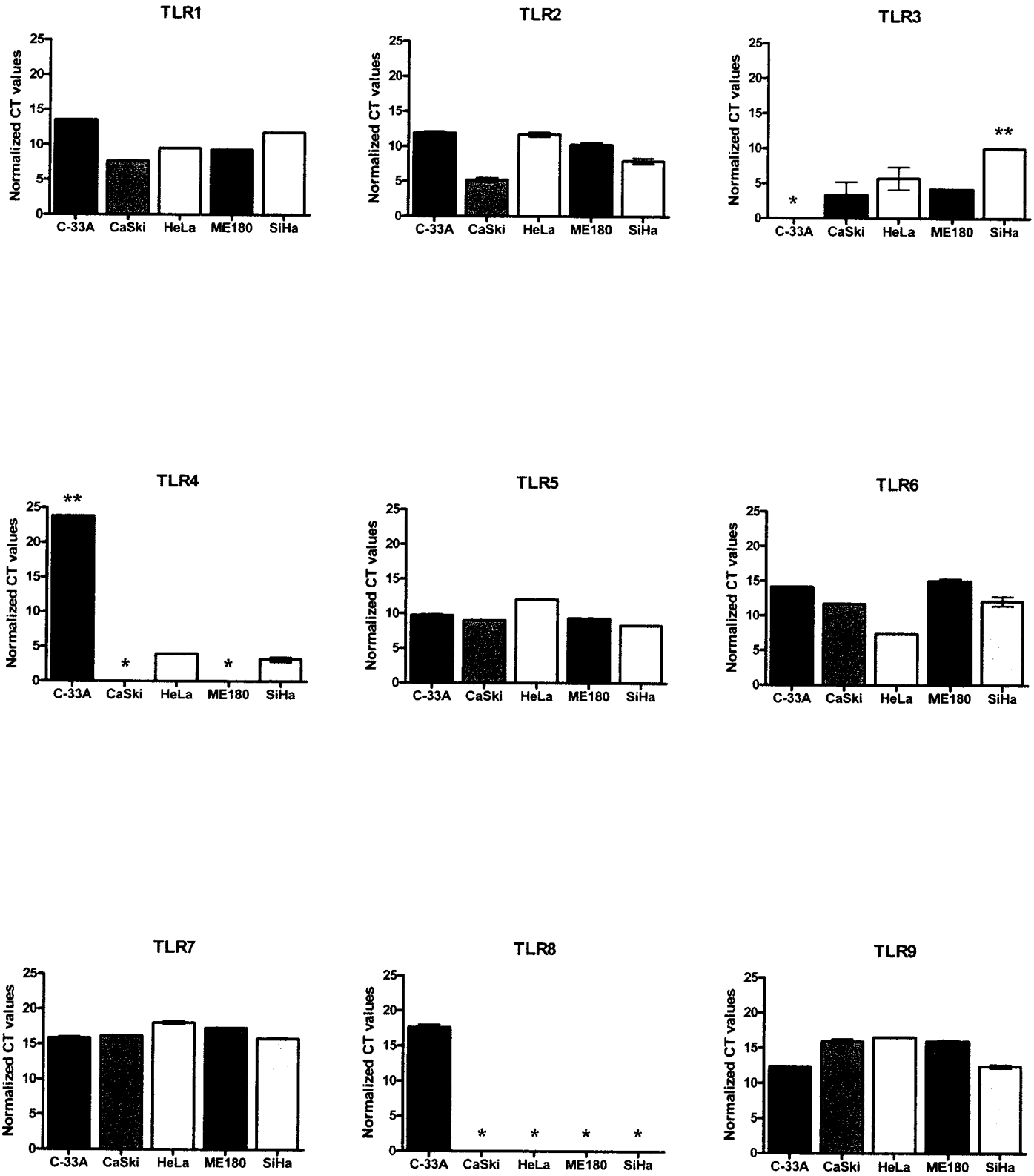


Figure 2: Gene expression of TLRs in cervical cancer cell lines, real-time PCR analysis. * Not detected, ** not detected in one out of 2 samples. Bars represent an average ± range of n=2 experiments

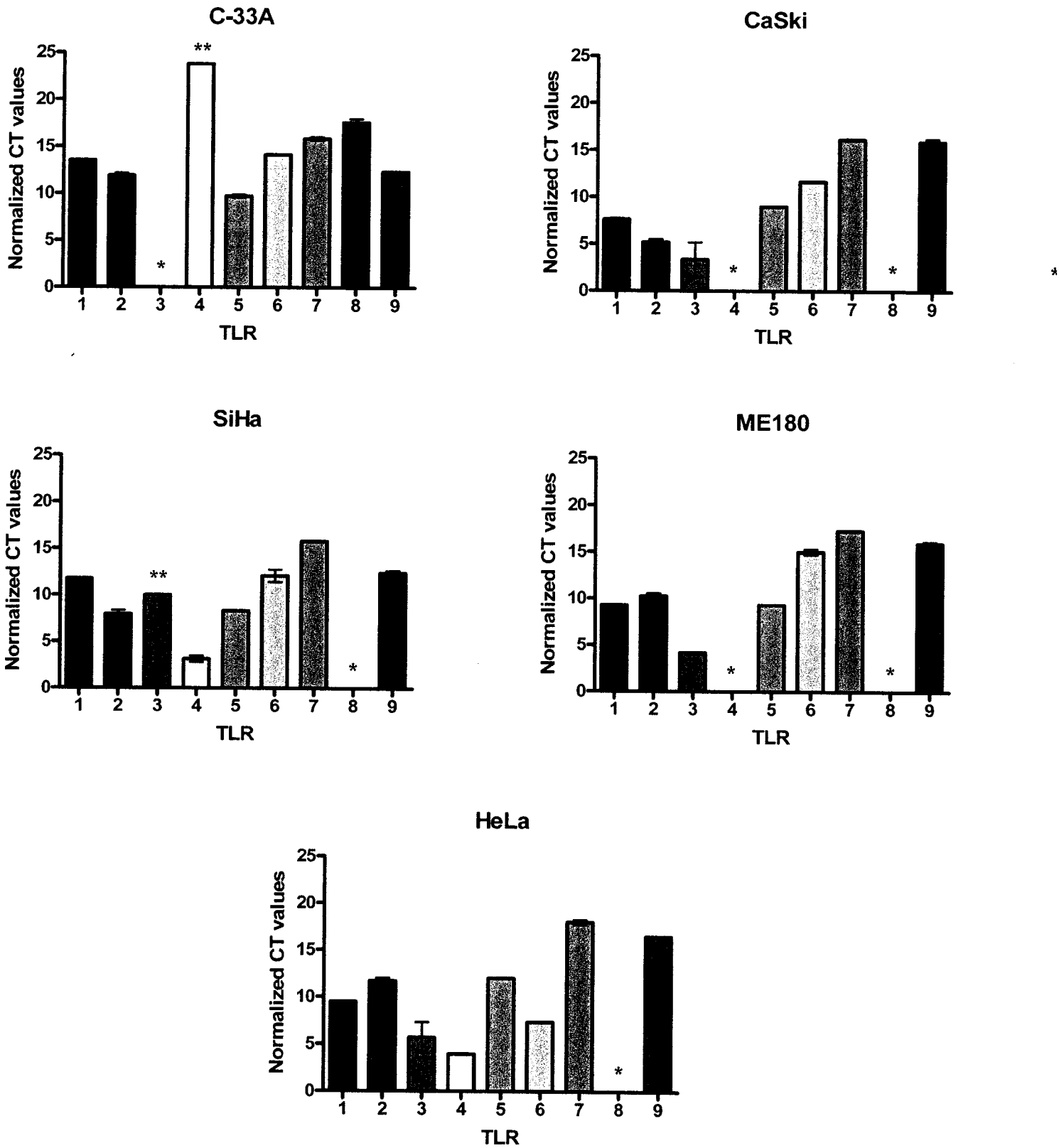


Figure 3: Gene expression of TLRs in cervical cancer cell lines, real-time PCR analysis. * Not detected, ** not detected in one out of 2 samples. Bars represent an average ± range of n=2 experiments

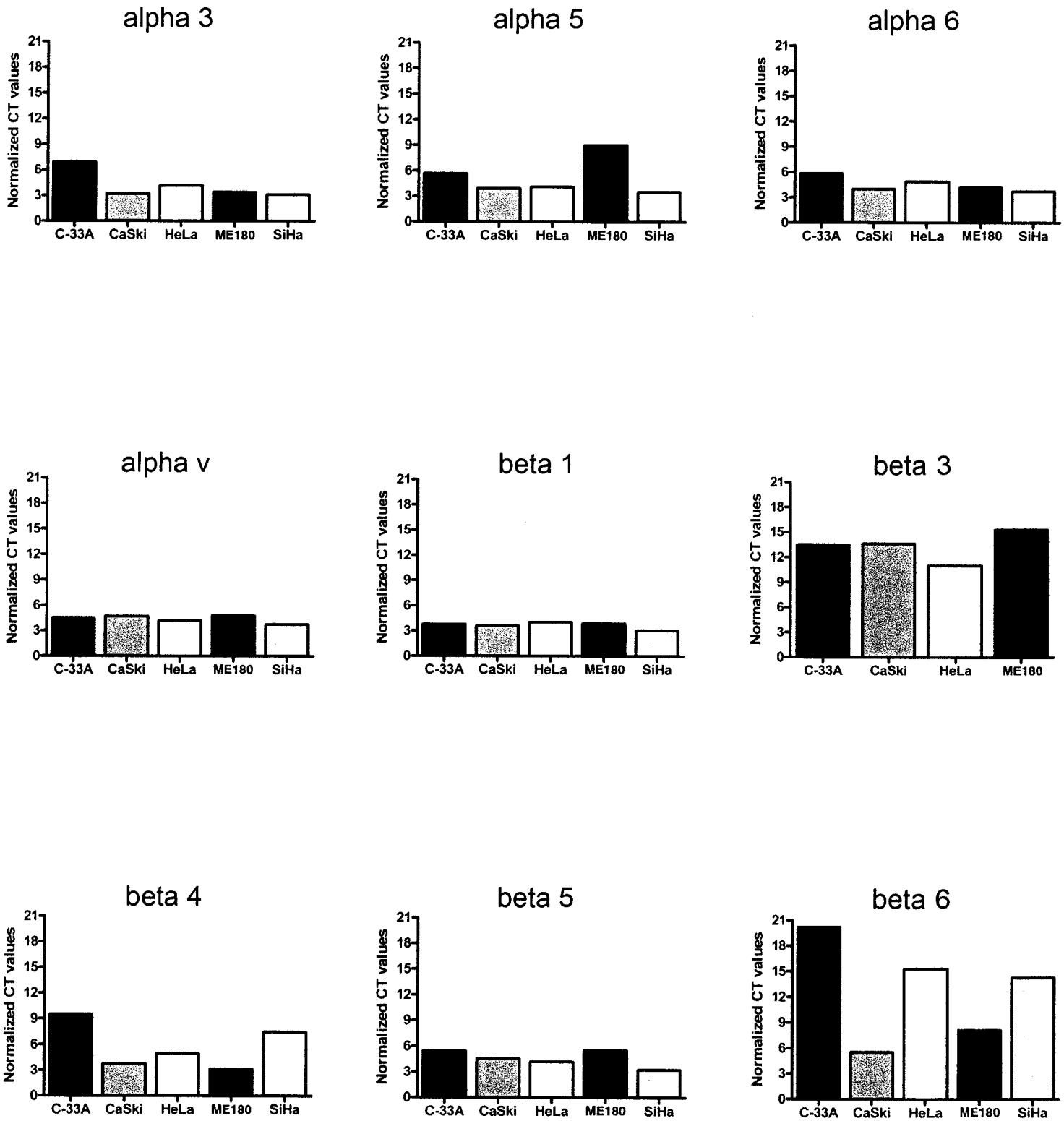


Figure 4: Gene expression of integrin subunits in cervical cancer cell lines, real-time PCR analysis (one experiment).

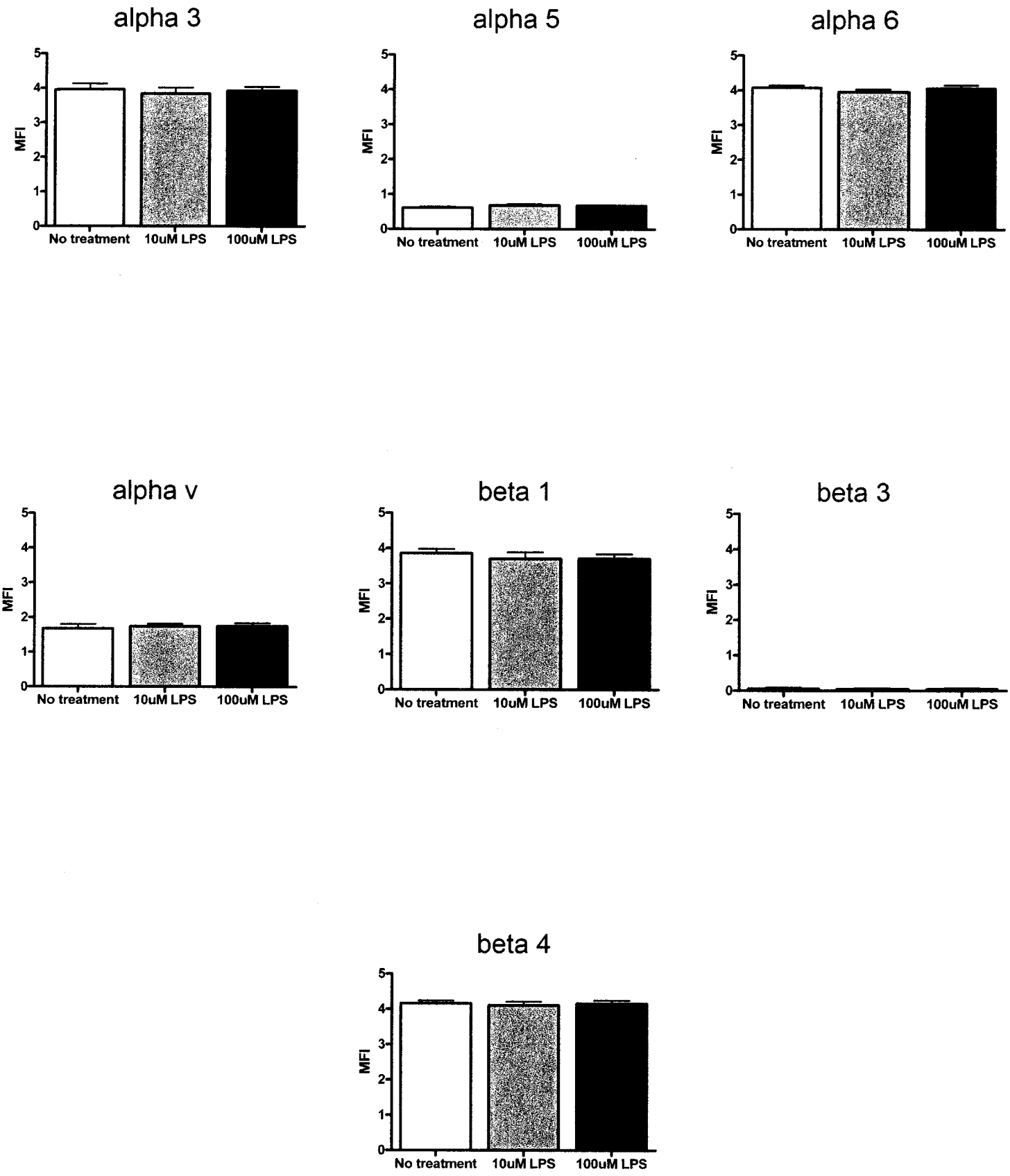


Figure 5: Integrin expression in ME180 cell line stimulated with 10 μM or 100 μM LPS (n=3 experiments). Mean fluorescence intensity is represented as MFI ± SEM

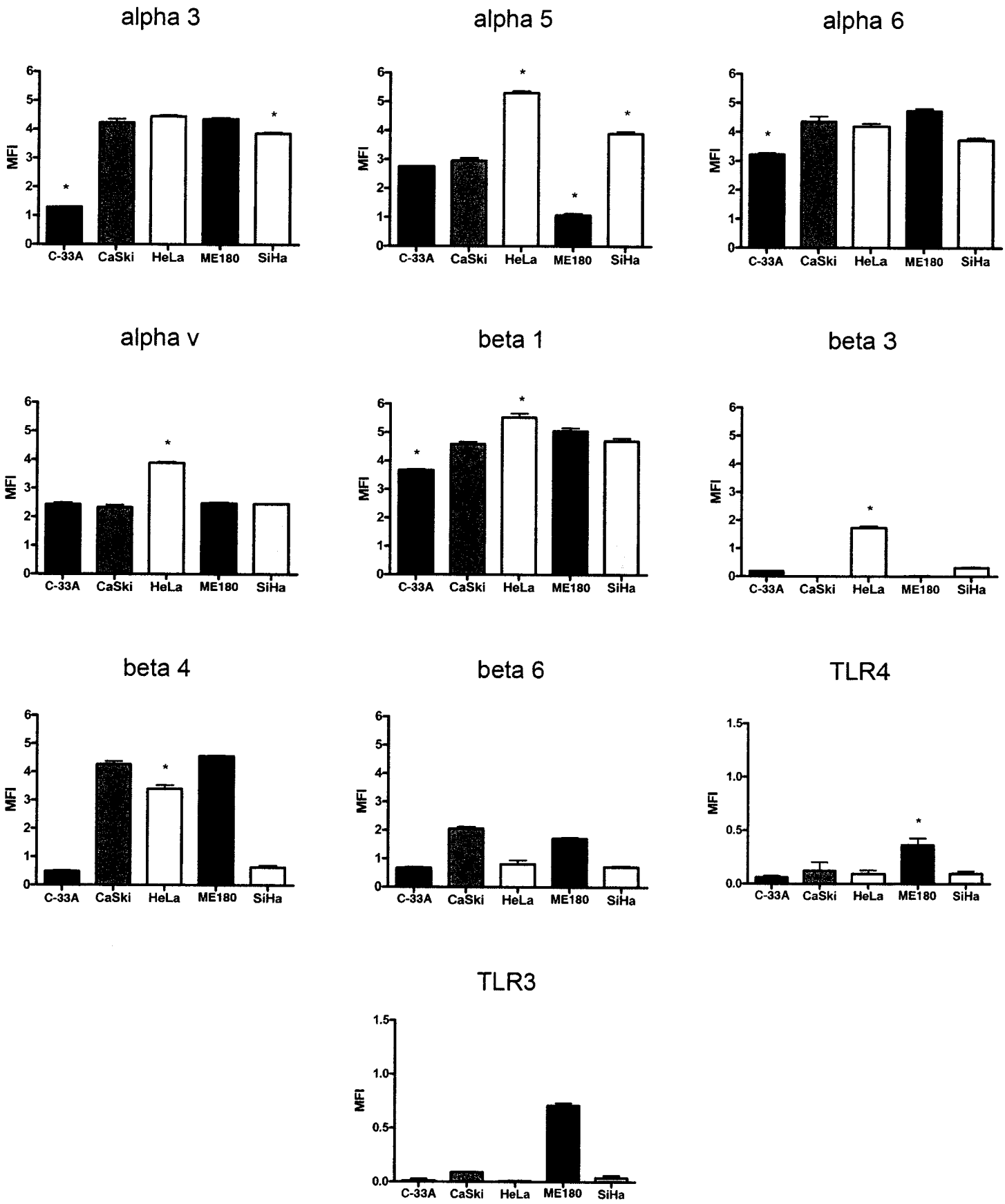


Figure 6: Surface expression of integrin subunits, TLR -3 and -4 in unstimulated cervical cancer cell lines. * Significant differences with respect to all other cell lines ($p < 0.05$, n=3 independent experiments). Mean fluorescence intensity is represented as MFI ± SEM.

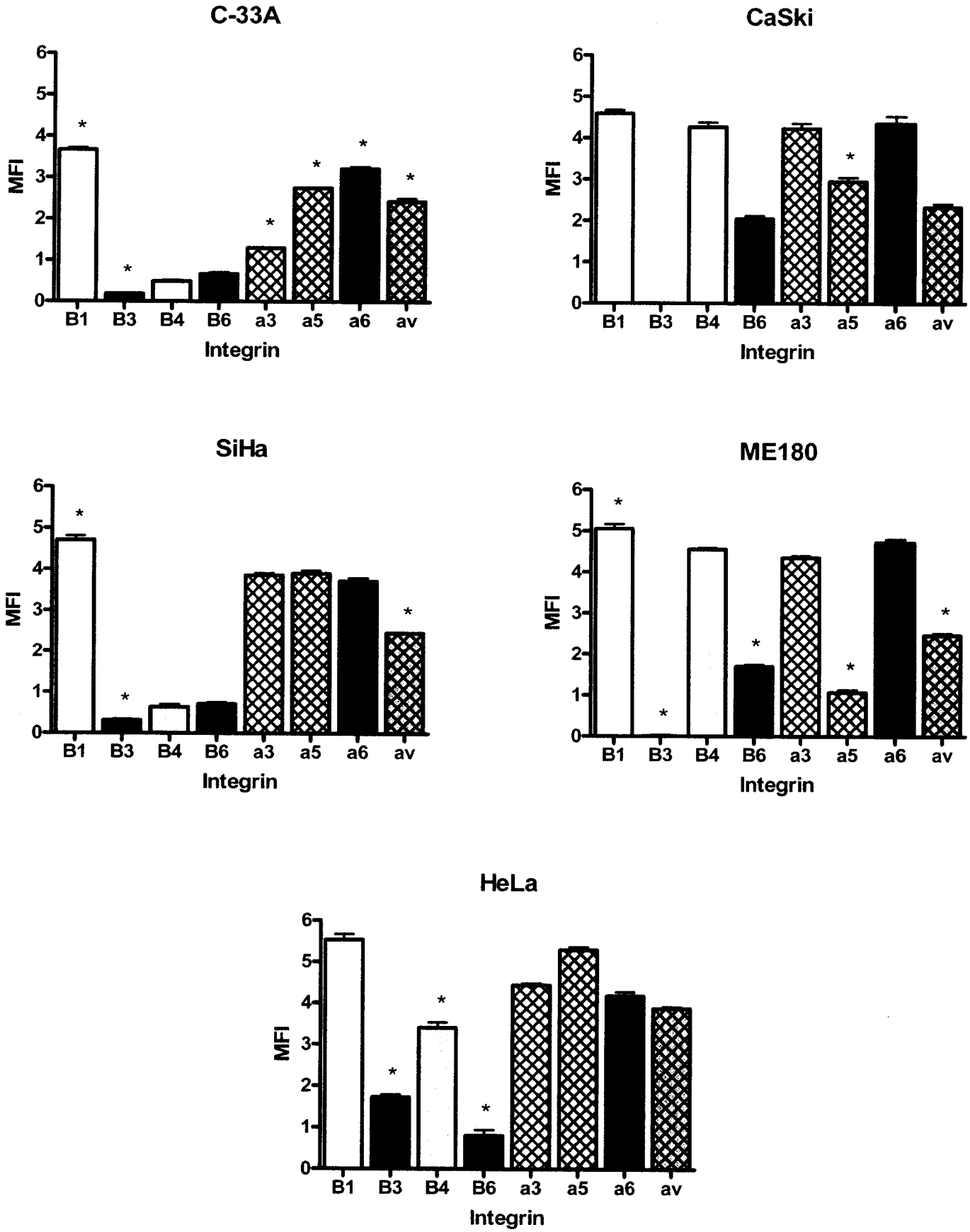
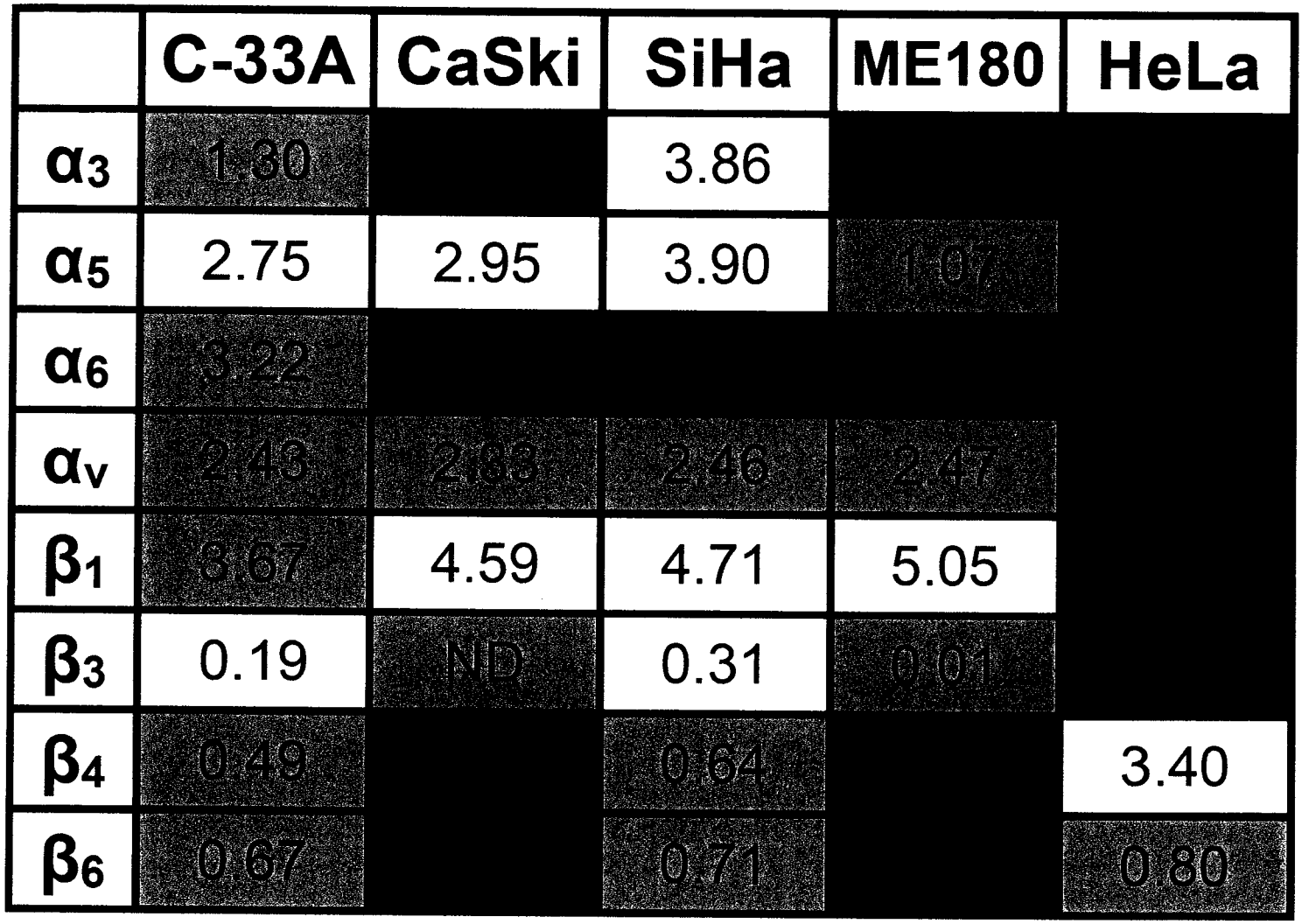


Figure 7: Surface expression of integrin subunits in unstimulated cervical cancer cell lines. * Significant differences with respect to all other integrins ($p < 0.05$, n=3 independent experiments). Mean fluorescence intensity is represented as MFI ± SEM.



	C-33A	CaSki	SiHa	ME180	HeLa
α_3	1.30		3.86		
α_5	2.75	2.95	3.90	1.07	
α_6	3.22				
α_v	2.43	2.03	2.46	2.47	
β_1	3.67	4.59	4.71	5.05	
β_3	0.19	ND	0.31	0.01	
β_4	0.49		0.64		3.40
β_6	0.67		0.71		0.80

Figure 8: Flow cytometry data of integrin expression for 5 cell lines. Values are expressed as transformed mean fluorescence intensity (MFI), with isotype control subtraction. Coloured cells represent groups with significantly lowest (green) expression and significantly highest (purple) expression, $p < 0.05$ (Tukeys multiple comparison test). ND: not detected. n=3.

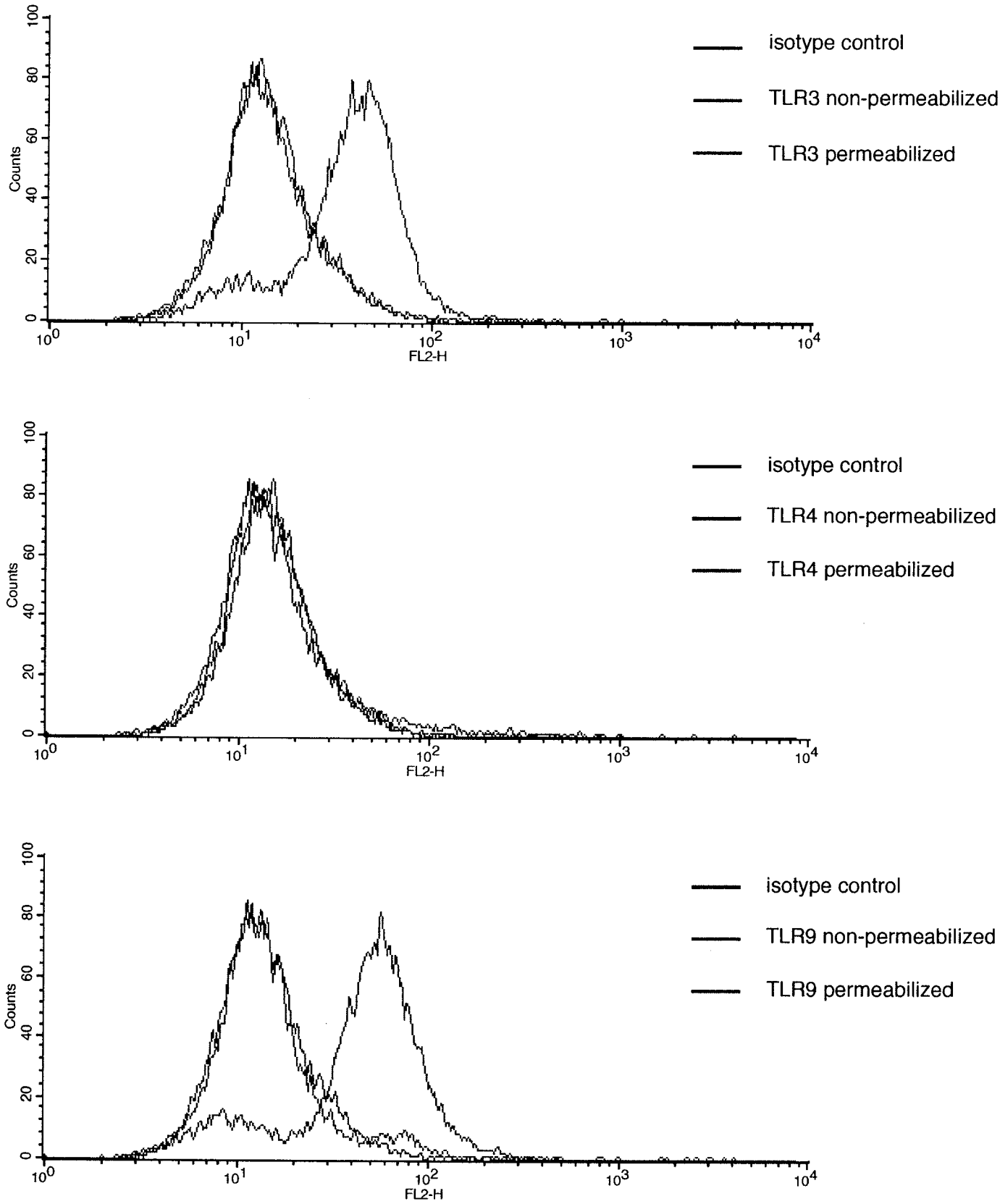


Figure 9: Flow cytometry analysis of TLR -3, -4, and -9 in permeabilized and non-permeabilized HeLa cells. (n=1).

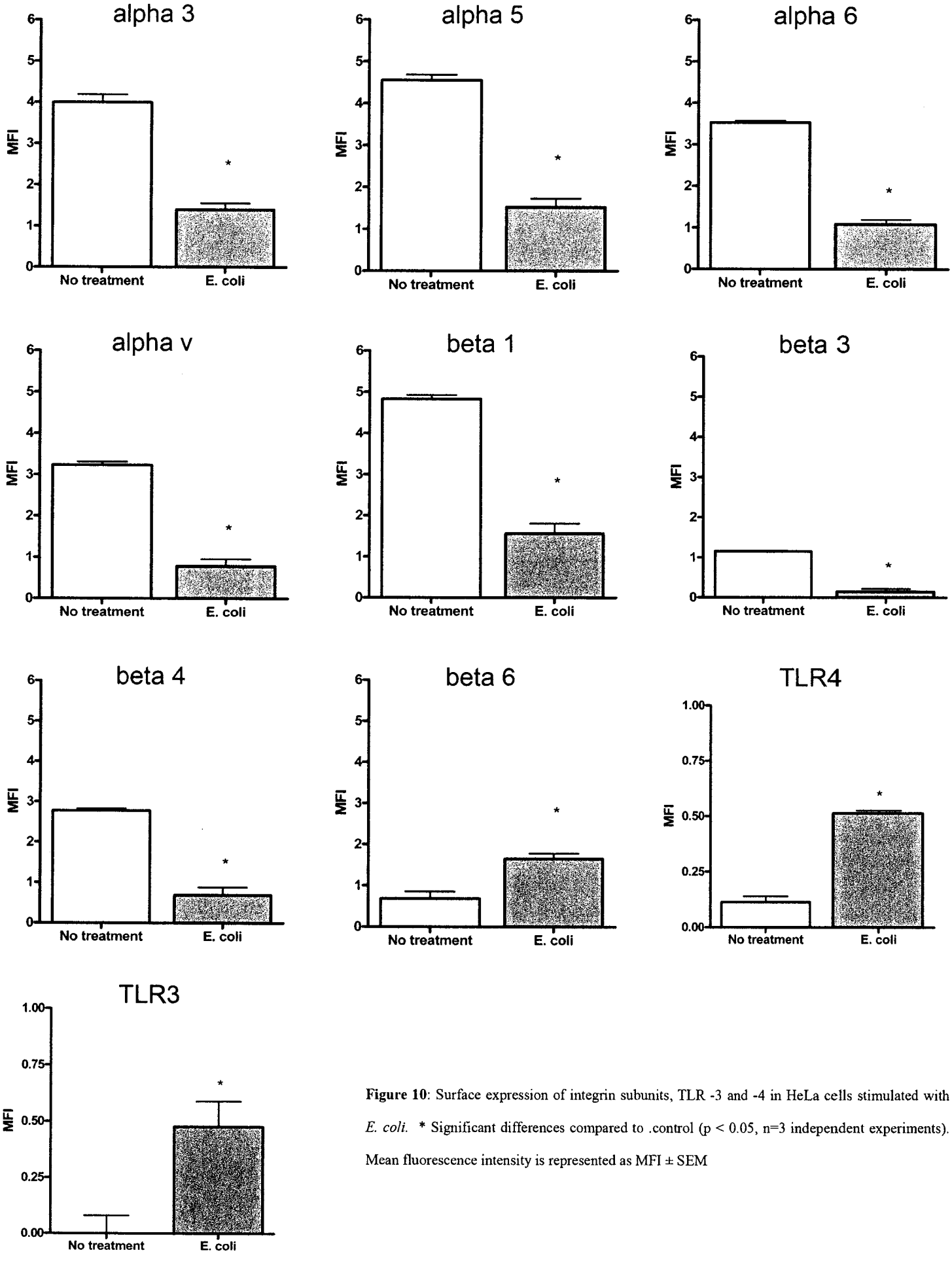


Figure 10: Surface expression of integrin subunits, TLR -3 and -4 in HeLa cells stimulated with *E. coli.* * Significant differences compared to .control ($p < 0.05$, n=3 independent experiments). Mean fluorescence intensity is represented as MFI ± SEM

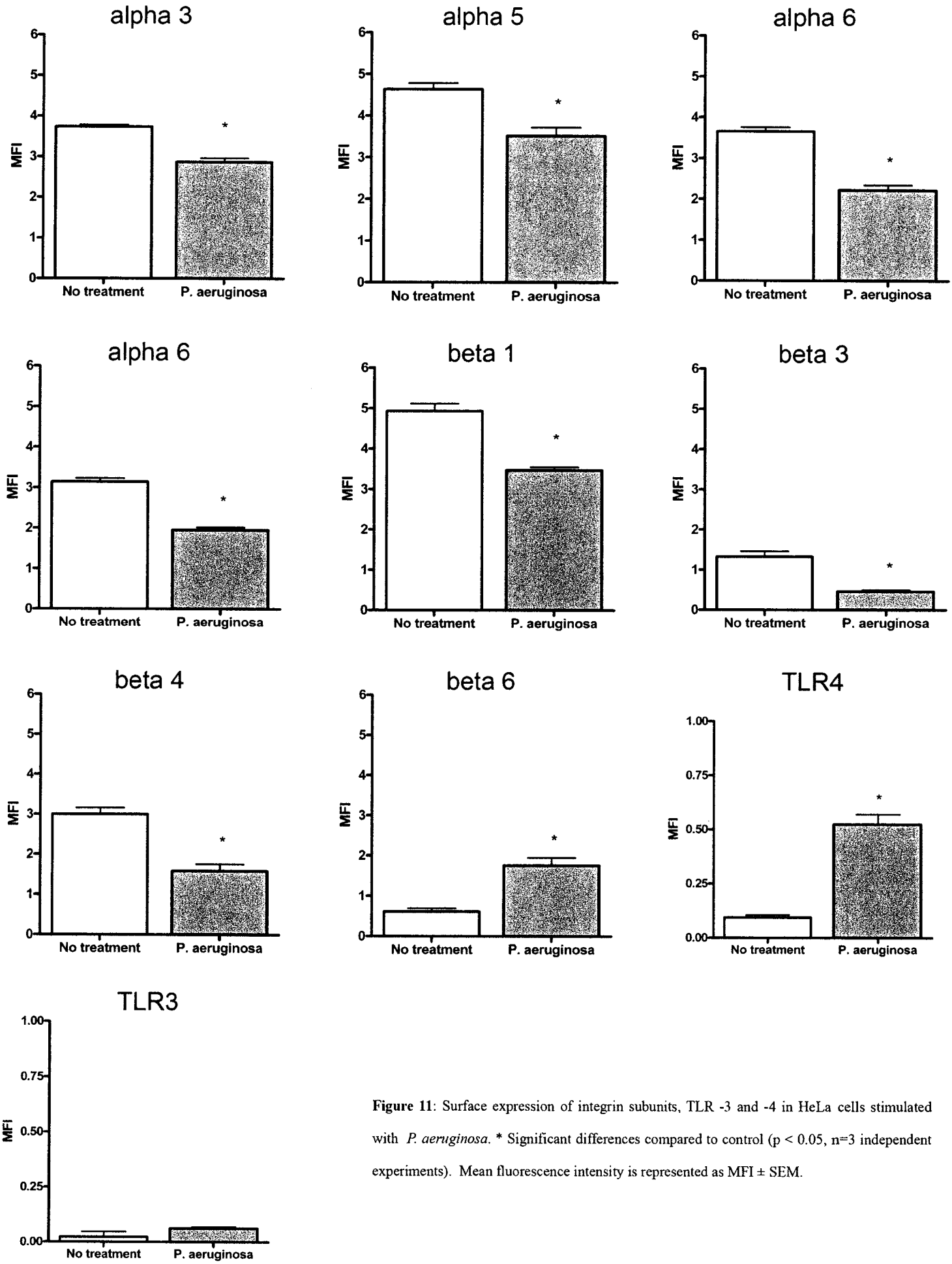


Figure 11: Surface expression of integrin subunits, TLR -3 and -4 in HeLa cells stimulated with *P. aeruginosa*. * Significant differences compared to control ($p < 0.05$, n=3 independent experiments). Mean fluorescence intensity is represented as MFI ± SEM.

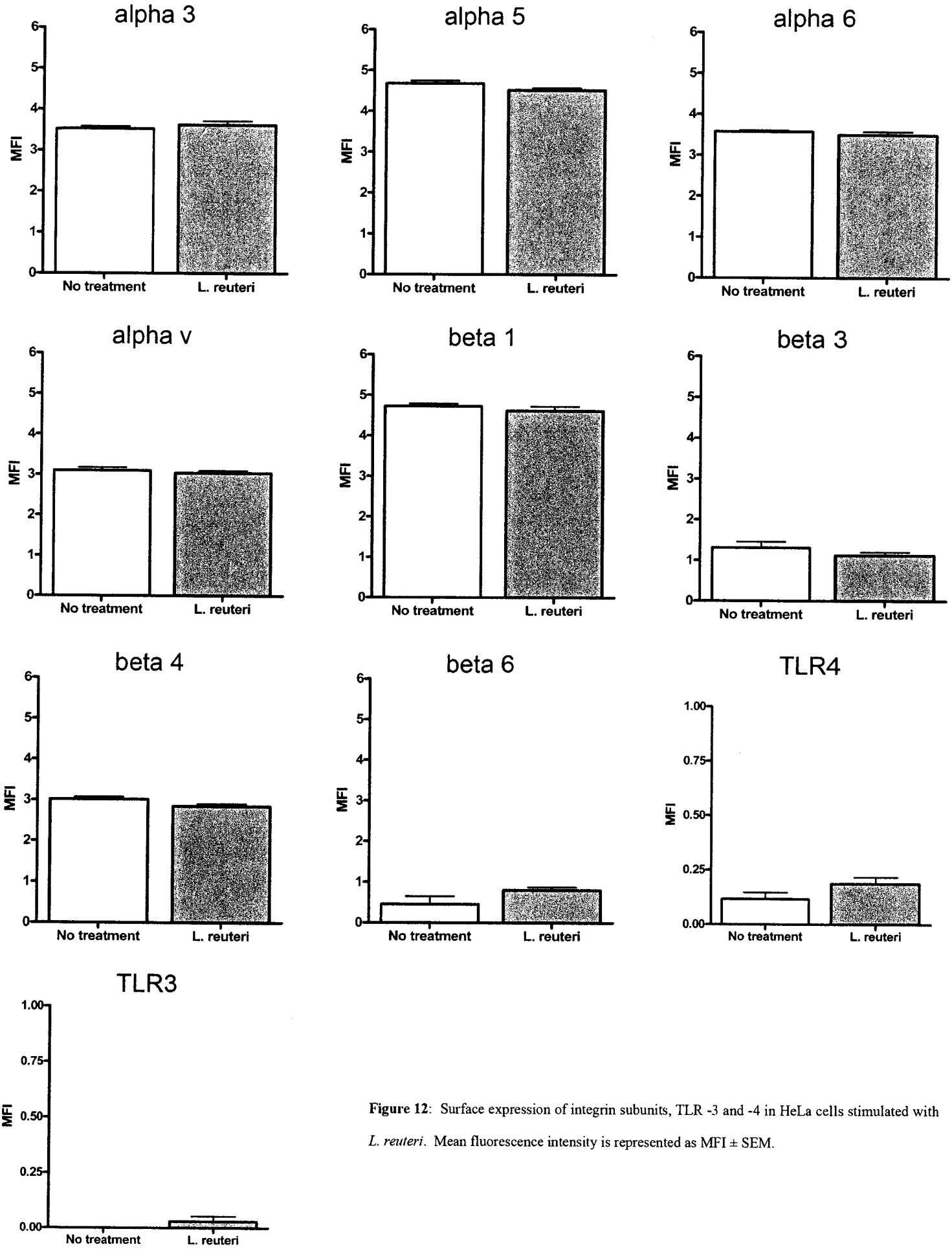


Figure 12: Surface expression of integrin subunits, TLR -3 and -4 in HeLa cells stimulated with *L. reuteri*. Mean fluorescence intensity is represented as MFI ± SEM.

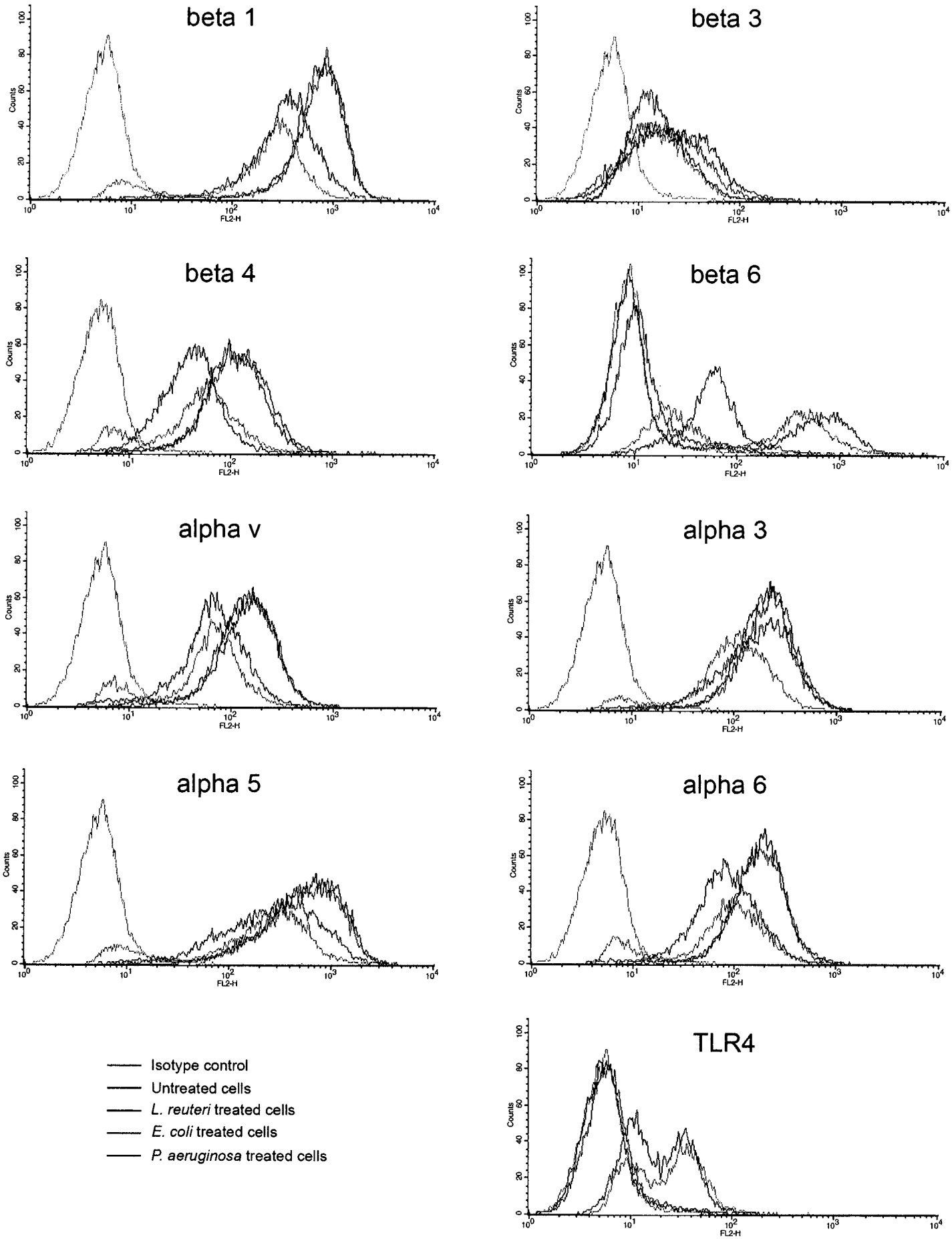


Figure 13: Flow cytometry analysis of integrin and TLR4 expression in HeLa cells, either infected with *E. coli*, *P. aeruginosa*, *L. reuteri*, or untreated. Results of one representative experiment are shown.

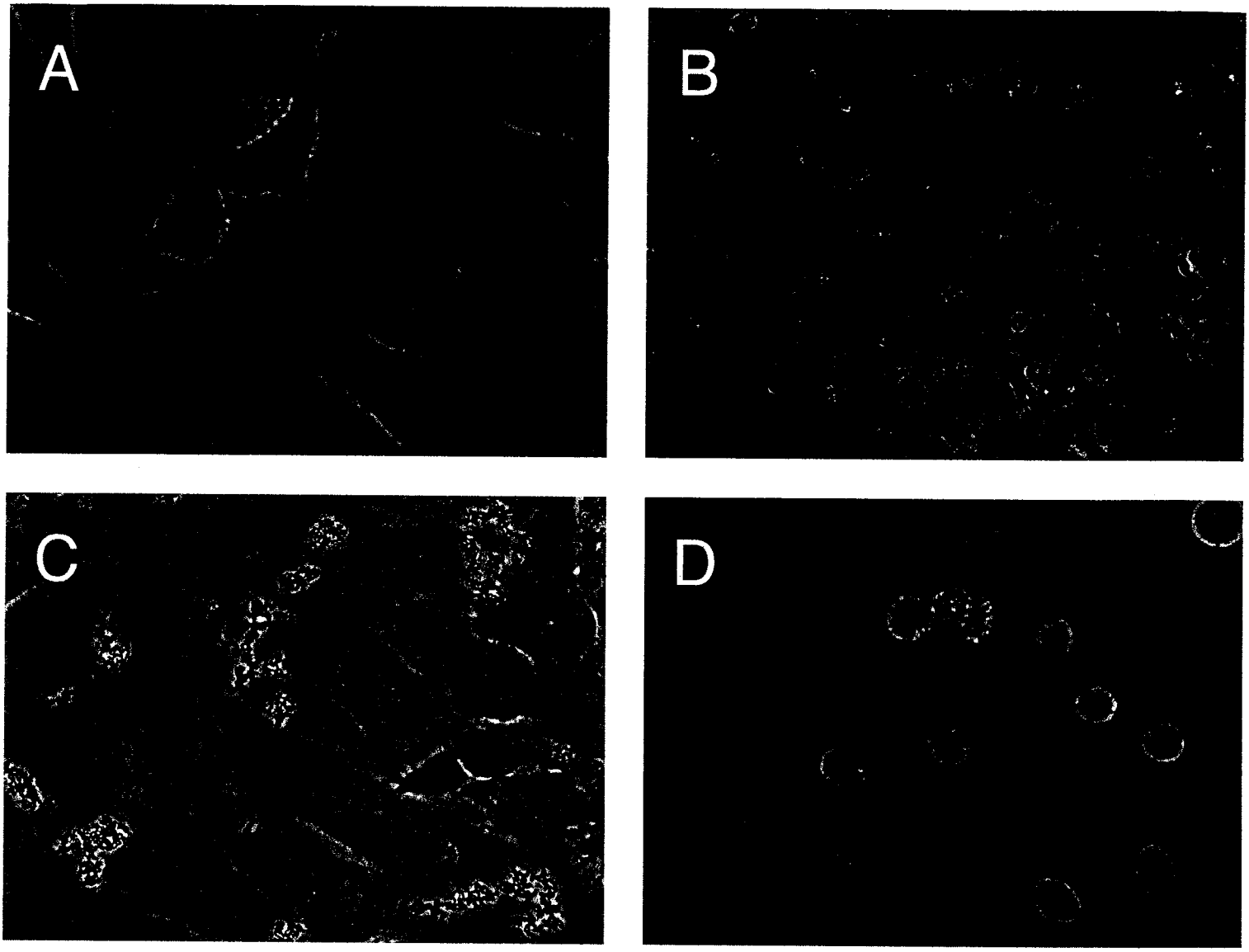


Figure 14. HeLa cells cultured with bacteria at MOI of 50 bacteria per cell. 40x magnification. *E. coli* at 0 h (A), four hour infection with *E. coli* (B), *L. reuteri* (C), and *P. aeruginosa* (D).

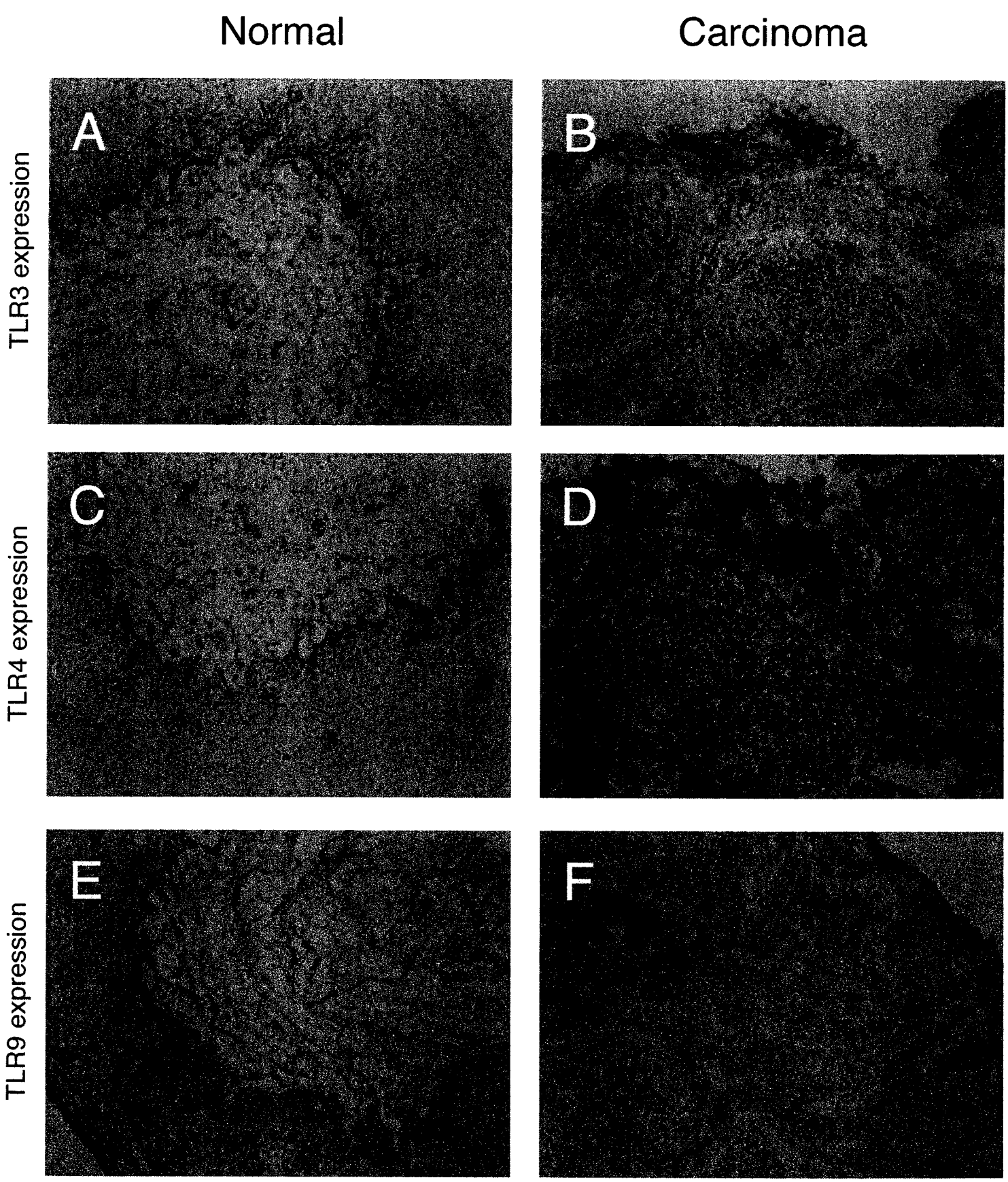


Figure 15. Representative cervical tissue biopsies from normal (left column) and carcinoma (right column) samples stained for TLR3 (A, B) TLR4 (C, D) and TLR9 (E, F). 10x magnification.

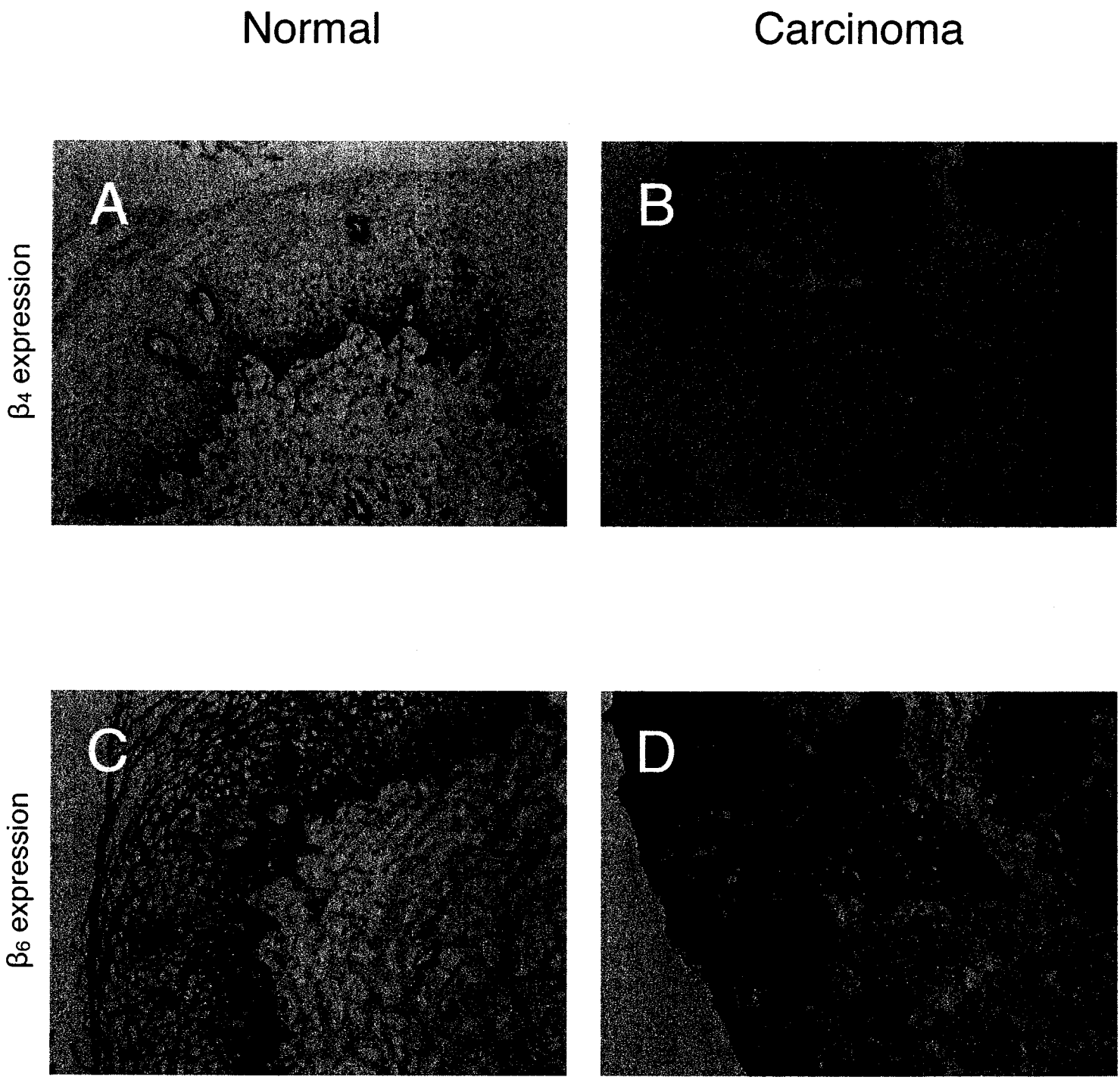


Figure 16. Representative cervical tissue biopsies from normal (left column) and carcinoma (right column) samples stained for integrin β_4 (A, B) and β_6 (C, D). 10x magnification.

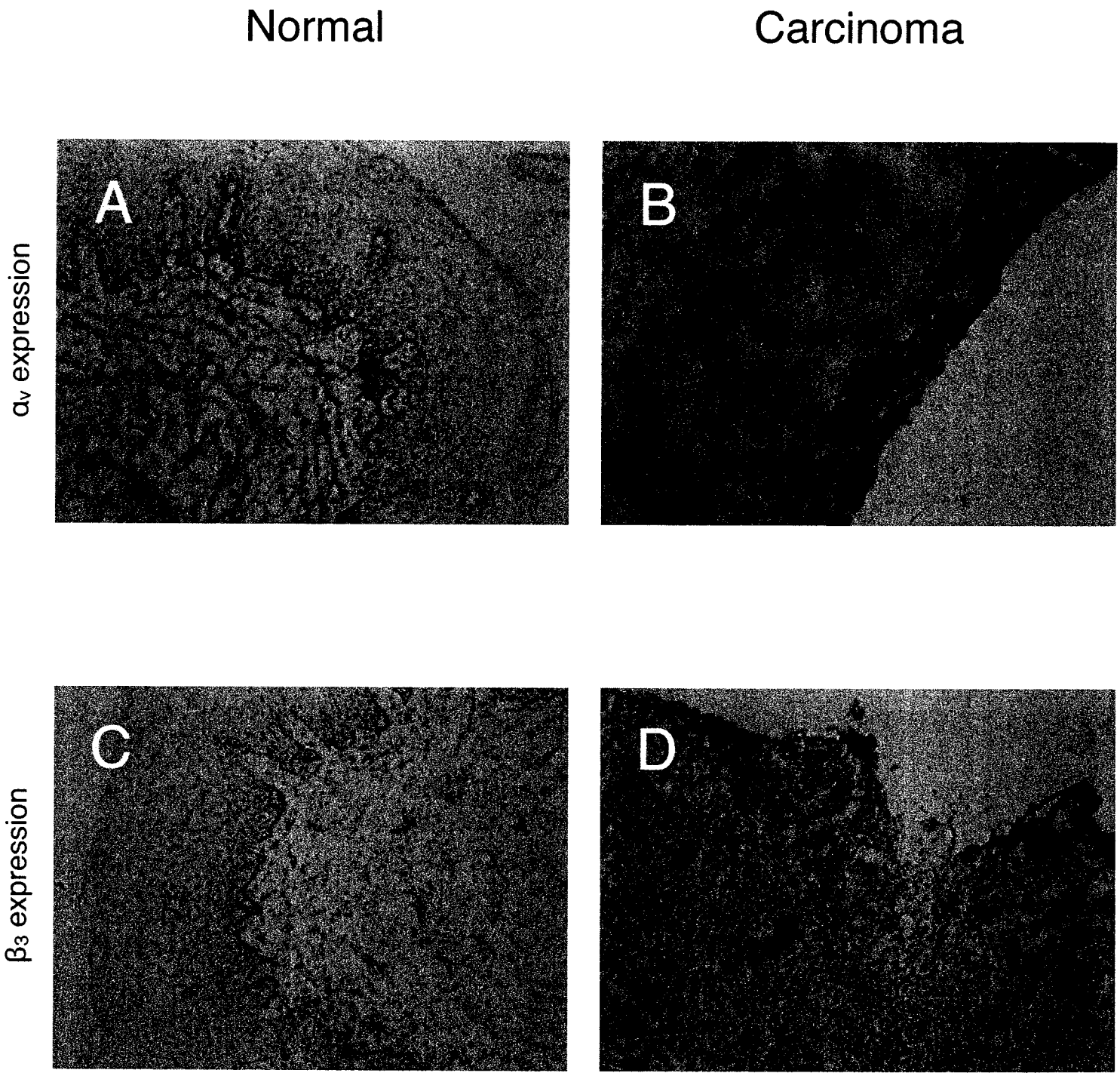


Figure 17. Representative cervical tissue biopsies from normal (left column) and carcinoma (right column) samples stained for integrin α_v (A, B) and β_3 (C, D). 10x magnification.

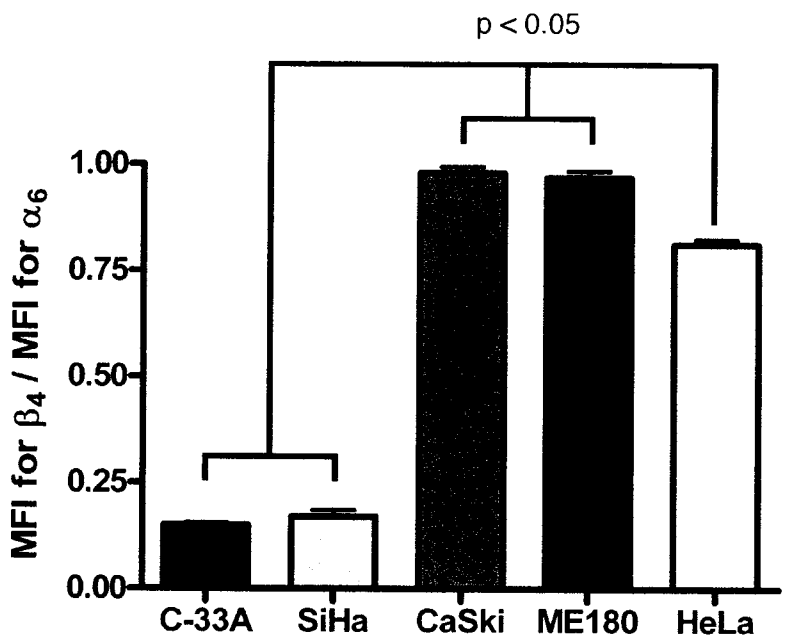


Figure 18. Relative amount of β_4 per α_6 (n=3). Indicated groups are significantly different ($p < 0.05$).