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TNFα and IL1β Stimulate Differential Gene Expression in Endometrial Stromal Cells

Abha J. Chalpe[1], Chad D. Law[1], Jennifer N. Dumdie[1], Keith A. Hansen[2], Kathleen M. Eyster[1,2*]

[1]Division of Basic Biomedical Sciences, Sanford School of Medicine, The University of South Dakota, Vermillion, SD, USA
[2]Department of Obstetrics and Gynecology, Sanford School of Medicine, The University of South Dakota, Sioux Falls, SD, USA
Email: abhachalpe@gmail.com, clawmdit@gmail.com, jdumdie@ucsd.edu, kahansen@usd.edu, *Kathleen.Eyster@usd.edu

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Abstract

The purpose of this study was to test the hypothesis that specific macrophage-secreted cytokines cause gene expression changes in endometrial stromal cells that reproduce the effects of macrophages in the development of endometriosis. Telomerase-immortalized human endometrial stromal cells (T-HESC) were treated with tumor necrosis factor α (TNFα, 5 ng/ml) and interleukin 1β (IL1β, 1 ng/ml). Differential expression of 249 genes was identified by DNA microarray. Ontologies such as peptidases, cell adhesion, cell death/cell cycle, growth factors, cytoskeletal organization, defense/immune system, signal transduction, and transcriptional regulation which are related to the development of endometriosis were represented by these genes. The up-regulation of interleukin 8 (IL8), interleukin 6 (IL6), IL1β and matrix metalloproteinase 3 (*MMP*3) in response to TNFα ± ILIβ in T-HESC cells was confirmed by real time RT-PCR. TNFα ± ILIβ did not affect the migration or invasion of T-HESC cells. This study reinforces our previous investigations on communication between cells of the immune system and endometrial stromal cells and their potential role in the development of endometriosis.

Keywords

Cytokines, Endometriosis, Endometrium, Endometrial Stromal Cells, Microarray, Gene Expression

*Corresponding author.

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1. Introduction

Endometriosis is an inflammatory disease in which endometrial tissue implants and grows outside the uterus [1]. The inflammatory nature of the disease, and the large number of genes related to the immune system/inflammation that are up-regulated in endometriosis [2] have led to the concept that factors from the immune system exacerbate the development of endometriosis instead of destroying ectopic endometrial tissue [3]-[8].

Factors secreted by macrophages and other immune system cells that are implicated in the development of inflammatory diseases such as endometriosis include the cytokines. It has been proposed that cytokines may promote neovascularization and attachment of endometrial cells to the peritoneum in the process of development of endometriosis [1] [4] [7]-[11].

Previous studies from our laboratory have demonstrated that reciprocal communication occurs between macrophages/monocytes and endometrial stromal cells in cell culture [5] [6]. These studies demonstrated that factors secreted by macrophages/monocytes caused differential gene expression in telomerase-immortalized human endometrial stromal cells (T-HESC) and vice-versa. In the current project we tested the hypothesis that specific cytokines and growth factors secreted by macrophages (tumor necrosis factor α (TNFα), interleukin 1β (IL1β), interleukin 6 (IL6), and interleukin 8 (IL8), and the growth factor, transforming growth factor β (TGFβ)) cause gene expression changes in T-HESC cells that reproduce the effects of macrophage conditioned medium. We also tested whether TNFα and IL1β increased the migratory and invasive properties of T-HESC cells.

This study used human telomerase-immortalized endometrial stromal cells [5] [6] [12] as a model of the early stages of endometriosis. Endometrial stromal cells are considered to be a critical cell type in the establishment of endometriosis lesions [13]. Simplifying our model to include only this single cell type allowed us to more clearly analyze the effects of cytokines and their potential role in endometriosis in a carefully controlled environment.

2. Materials and Methods

2.1. Experimental Design

Concentration-response curves were carried out to identify the concentrations of TNFα, IL1β, TGFβ, IL8, and IL6 that achieved the best response from cultured endometrial stromal cells. DNA microarrays were then used to analyze differential gene expression in endometrial stromal cells in response to TNFα ± IL1β. The ability of TNFα ± IL1β to modify migration and invasion of endometrial stromal cells was also assessed using Boyden chambers.

2.2. Cell Culture

The T-HESC cell line [12] was used for all experiments. T-HESC were obtained from American Type Cell Culture (ATCC, Manassas, VA) (CRL-4003). No ethical permissions were required for this study since the study was carried out in a commercially available cell line. The cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma, St. Louis, MO) as described [6]. When the cells reached 80% confluence they were starved for 24 hours in starvation medium (DMEM+ ITS+ puromycin+ penicillin/streptomycin) before treatment with cytokines or growth factors.

2.3. Concentration-Response Curves for Cytokines

Three concentrations were tested for each cytokine and growth factor: TNFα (0.05, 0.5, and 5 ng/ml), IL1β (0.01, 0.1, and 1 ng/ml), IL8 (25, 150, and 500 ng/ml), IL6 (1, 5, and 10 ng/ml) and TGFβ (0.6, 1.2 and 10 ng/ml). All cytokines and TGFβ were obtained from Cell Sciences (Canton, MA). T-HESC cells were treated with individual cytokines and combinations of cytokines for 48 hours or 70 hours in the absence of FBS. After treatment, RNA was isolated from T-HESC and utilized for real time RT-PCR and DNA microarray analysis.

2.4. RNA Isolation and Quantification

For RNA isolation, T-HESC cells were washed twice with 2 ml phosphate buffered saline (PBS), and 1 ml Tri reagent (Molecular Research Center, Cincinnati, OH) was added to each culture flask. RNA was purified using RNeasy mini kit columns (Qiagen, Valencia, CA) and quantified using the RNA 6000 Nano LabChip in an Agi-

lent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) as described [14].

2.5. Microarray Analysis

Codelink Whole Human Genome Bioarrays (Applied Microarrays, Tempe, AZ) were used to perform microarray analysis of gene expression as described [14]. Statistical analysis of the microarray data was carried out using Gene Spring 7.0 software (Agilent, Santa Clara, CA).

2.6. Real Time Reverse Transcription-Polymerase Chain Reaction (Real Time RT-PCR)

Real time RT-PCR was used to measure the expression levels of IL8, *MMP*3, IL1*B*, and IL6 for the analyses of concentration-response curves when T-HESC cells were treated with TNFα ± IL1β. These genes were chosen based on their fold expression in the microarray analysis. Primers and TaqMan probes were obtained as Assays on Demand from Applied Biosystems/Life Technologies (Foster City, CA) (Hs00174097_m1 for IL1*B*, Hs01567913_g1 for IL8, Hs00174131_m1 for IL6, and Hs00968308_m1 for *MMP*3). The expression of target genes was normalized to the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). The results were analyzed using qBase software by the delta CT relative quantification method [15].

2.7. Invasion and Migration in Response to Cytokines

Boyden chambers (BD Biosciences, Bedford, MA) were used to analyze the migratory and invasive activities of T-HESC in response to TNFα ± IL1β in two sets of experiments. The first experiment consisted of treating T-HESC cells with TNFα (5 ng/ml) ± IL1β (1 ng/ml) for 48 hours before plating on the Boyden chambers. The treated cells were dyed using a fluorescent CellTracker Probe (Molecular Probes, Eugene, OR) for cell counting after migration/invasion. The treated and dyed cells were placed on the control (migration) and invasion inserts of the Boyden chambers in medium containing cytokines. Each chamber was seeded with 2.5×10^4 cells. Treatment of the cells with TNFα (5 ng/ml) ± IL1β (1 ng/ml) continued throughout the incubation of the cells in the Boyden chamber. The cells were incubated in the chambers for 22 hours; they migrated or invaded in response to the chemoattractant, fibronectin (25 µg/ml, BD Biosciences, Bedford, MA) in the bottom chamber. After 22 hours of incubation the membranes were dissected free from the chambers, fixed in 4% paraformaldehyde for 2 minutes at room temperature and mounted on microscopy slides using immersion oil. The migration and invasion of T-HESC cells treated with TNFα ± IL1β was compared to vehicle-treated control cells. This experiment was repeated 5 times with different passages of T-HESC cells. In the second experiment, TNFα ± IL1β were used as chemoattractants; that is, TNFα ± IL1β were placed at the bottom of the Boyden chambers in place of fibronectin. The untreated cells were dyed with CellTracker dye, then seeded in the Boyden chambers at 2.5×10^4 cells/chamber. The chambers were incubated and processed as in the first experiment. In both experiments fluorescent cells that migrated/invaded were counted using ImageJ software (National Institutes of Health, Bethesda, MD).

2.8. Statistical Analyses

Data from the real-time RT-PCR experiments were analyzed using qBase software [15], and Graph Pad Prism 4.0 (San Diego, CA) software was used to perform analysis of variance (ANOVA) on real time RT-PCR data. Newman Keuls was used as the post hoc test. Gene Spring 7.0 software (Agilent) was used to perform ANOVA on the microarray data. ANOVA was also used to analyze on the data from cell invasion and migration experiments using the GraphPad Prism 4.0 software (San Diego, CA).

3. Results

Concentration-response curves identified 5 ng/ml TNFα and 1 ng/ml IL1β as the optimal concentrations of these cytokines for response in T-HESC cells after 48 hours of treatment (data not shown). In contrast, T-HESC cells did not respond to TGFβ, IL8, or IL6, either singly or in combination (data not shown).

DNA microarray identified 249 genes to be differentially expressed in the analysis of T-HESC cells treated with TNFα ± IL1β (**Table 1**). The gene ontologies that featured in this microarray data were peptidases, cell ad-

hesion, cell death/apoptosis, cell cycle, growth factors, cytoskeletal organization, channels/carriers, enzymes/metabolism, defense/immune system, receptors and ligands, signal transduction, transcriptional regulation, cancer related, vesicle trafficking, chaperonins and other. The genes with a two-fold or greater change in expression and p value of 0.05 or less when compared to control were considered significant. As recommended by minimum information about microarray experiment (MIAME) standards [16], the entire data set for these microarrays has been deposited in National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO; www.ncbi.nlm.nih.gov/geo). The data can be accessed through GEO Series accession number GSE40007.

Four of the differentially expressed genes were chosen for confirmation by real time RT-PCR. IL1*B* was significantly up-regulated in response to TNFα + IL1β (**Figure 1(a)**, **Figure 1(b)**). The expression of IL8 showed

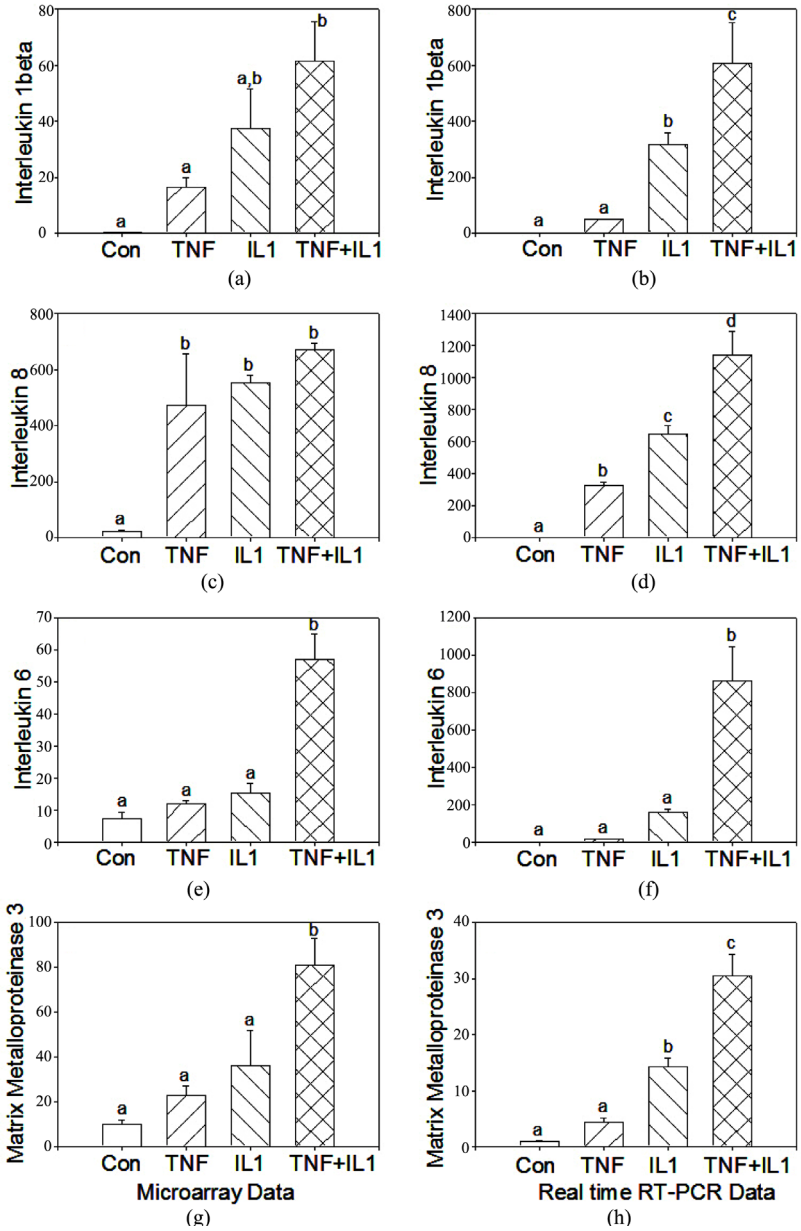

Figure 1. Differential expression of interleukin 1β (a, b), IL8 (c, d), IL6 (e, f), and matrix metalloproteinase 3 (g, h) in human telomerase-immortalized endometrial stromal cells (T-HESC) in response to treatment with TNFα (TNF) ± IL1β (IL1) for 48 hours. Data are shown from DNA microarray analysis (a, c, e, and g), and from real time RT-PCR analysis (b, d, f, and h). Data are the mean ± S.E., experimental n = 3. Bars with different letter superscripts denote that the data for those groups are significantly different from each other (ANOVA, Newman Keuls post hoc test, $p < 0.05$).

Table 1. Differentially expressed genes in human telomerase-immortalized endometrial stromal cells (T-HESC) treated with the cytokines TNFα ± IL1β.[1]

ACCN#[2]	Gene symbol	Con	TNF	IL1β	TNF + IL1β
	Peptidases/related				
NM_003817	ADAM7	1.17	1.58	1.33	2.45
NM_004390	CTSH	3.51	7.22	2.62	5.86
NM_004079	CTSS	2.53	27.38	11.63	27.64
NM_002422	MMP3	10.00	22.59	93.66	81.07
NM_007289	MME	1.80	3.29	2.96	6.78
NM_006587	CORIN	2.12	0.77	0.95	0.65
NM_002575	SERPINB2	1.40	18.72	9.97	17.18
NM_002974	SERPINB4	0.13	0.07	0.49	1.65
NM_005025	SERPINI1	1.76	1.02	1.30	0.89
NM_001899	CST4	0.64	1.34	0.86	0.89
NM_002801	PSMB10	13.72	32.52	21.99	27.56
NM_020903	USP29	0.56	1.02	0.92	1.14
NM_002771	PRSS3	2.78	2.31	2.49	1.16
	Cell adhesion				
NM_181847	AMIGO2	11.28	15.73	15.01	27.73
AB002377	ANKRD28	19.84	11.82	10.64	9.65
NM_001332	CTNND2	4.47	3.30	2.33	2.23
NM_138455	CTHRC1	163.71	302.87	205.68	222.93
NM_080680	COL11A2	0.79	1.62	0.87	1.87
NM_001856	COL16A1	1.99	1.90	3.44	7.51
NM_019035	PCDH18	14.10	6.30	6.41	4.56
NM_000885	ITGA4	12.98	7.09	8.80	2.86
NM_004791	ITGBL1	2.16	1.10	1.68	1.16
NM_000873	ICAM2	0.46	1.63	0.64	1.51
BG032839	PCOTH	6.12	5.05	4.76	3.09
NM_015429	ABI3BP	1.17	3.41	1.02	4.29
NM_012198	GCA	0.35	1.10	0.49	0.92
NM_004148	NINJ1	7.08	17.86	12.91	18.19
NM_004196	CDKL1	2.04	1.28	1.39	1.00
	Cell cycle				
NM_022909	CENPH	11.95	8.06	10.15	3.83
NM_001240	CCNT1	3.89	2.62	2.81	1.79
NM_174942	GAS2L3	8.18	4.94	5.25	3.52
NM_144658	DOCK11	3.92	2.46	2.35	1.61

Continued

NM_015714	G0S2	3.35	56.22	26.31	71.78
NM_007069	HRASLS3	8.35	22.60	11.22	15.94
NM_178428	LCE2A	12.49	5.85	7.09	1.43
NM_030915	LBH	66.32	35.75	35.43	17.75
NM_002358	MAD2L1	10.76	8.01	8.35	3.84
NM_032793	MFSD2	0.34	0.42	0.61	1.14
NM_015946	PELO	17.36	16.53	12.80	7.36
NM_001165	BIRC3	0.36	3.74	1.08	2.46
NM_001225	CASP4	14.64	32.62	21.14	26.54
	Growth factors				
NM_001953	ECGF1	1.37	7.04	2.45	4.63
NM_002006	FGF2	20.82	39.09	28.51	53.87
NM_000618	IGF1	0.66	0.80	0.80	1.58
NM_000618	IGF-IB	0.86	1.14	1.06	1.63
NM_030968	C1QTNF1	2.74	10.70	5.58	17.75
	Cytoskeletal				
NM_005159	ACTC	93.67	25.60	45.09	12.87
NM_001069	TUBB2	21.37	20.63	16.15	9.36
NM_003380	VIM	681.53	478.57	440.76	328.93
NM_002276	KRT19	76.84	59.23	36.94	30.65
NM_002273	KRT8	3.31	1.69	1.67	1.18
NM_031957	KRTAP1-5	22.18	7.82	9.75	3.33
AK128036	KRT18	17.73	15.22	8.71	6.09
NM_001747	CAPG	10.17	5.69	6.60	2.84
NM_002381	MATN3	4.25	1.83	2.70	1.45
NM_005379	MYO1A	1.06	1.98	1.61	4.26
NM_031956	NYD-SP14	1.54	6.17	3.93	6.73
NM_153267	MAMDC2	17.89	20.61	15.54	8.96
	Immune system				
NM_001710	BF	0.50	2.34	1.95	7.73
NM_004335	BST2	0.63	5.49	1.50	10.51
NM_031890	CECR6	2.63	4.58	3.16	7.51
NM_001250	CD40	0.34	1.23	0.64	1.03
NM_001779	CD58	4.91	13.38	5.74	8.17
NM_004355	CD74	0.21	1.40	0.22	1.09
NM_002231	CD82	7.35	21.47	17.49	22.45
NM_004233	CD83	0.38	1.07	0.39	0.77

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NM_002985	CCL5	1.23	98.81	2.54	52.84
NM_006273	CCL7	2.17	14.34	15.83	56.62
NM_016557	CCRL1	2.51	5.98	2.74	4.44
NM_001565	CXCL10	0.31	3.68	2.41	4.22
NM_002089	CXCL2	1.28	4.64	47.90	103.75
NM_002090	CXCL3	5.05	9.62	15.48	31.98
NM_000063	C2	2.55	4.57	3.36	6.88
NM_000064	C3	0.15	0.60	0.84	5.66
NM_001312	CRIP2	2.51	1.68	2.15	1.26
NM_031476	CRISPLD2	0.78	0.69	0.90	4.30
NM_018659	CYTL1	0.40	2.48	1.61	2.83
NM_033255	EPSTI1	3.21	11.00	5.63	18.67
NM_005755	EBI3	1.62	11.10	2.32	9.26
NM_024503	HIVEP3	0.50	0.65	0.88	1.93
NM_207585	IFNAR2	4.20	10.06	5.39	9.26
AA463818	IFITM5	1.44	2.24	2.89	4.13
NM_022168	IFIH1	2.56	7.03	2.88	7.36
NM_004031	IRF7	4.35	10.81	6.03	14.29
NM_002201	ISG20	1.25	3.00	2.90	4.70
NM_005101	G1P2	22.54	46.03	30.04	55.91
NM_022873	G1P3	2.59	4.64	2.54	5.88
NM_005532	IFI27	8.08	67.13	17.37	82.11
NM_006332	IFI30	8.81	36.07	17.09	29.23
NM_005533	IFI35	15.02	35.59	20.08	33.11
NM_006417	IFI44	2.79	6.99	3.57	9.81
NM_006820	IFI44L	0.79	3.96	1.18	8.68
NM_000576	IL1B	0.45	11.85	37.22	61.25
NM_004221	IL32	11.52	193.11	38.77	115.47
NM_000600	IL6	7.43	11.84	15.26	57.08
NM_000880	IL7	0.31	0.83	0.95	1.53
NM_000584	IL8	23.31	472.44	550.94	670.07
NM_002189	IL15RA	4.43	10.04	8.12	15.60
NM_181079	IL21R	0.50	0.91	0.57	1.99
NM_002462	MX1	5.53	17.16	5.53	23.43
NM_002463	MX2	1.80	2.71	1.93	4.20
NM_005746	PBEF1	4.75	7.95	13.02	24.14
NM_014059	RGC32	21.18	32.92	19.81	6.53

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NM_030754	SAA2	2.30	4.05	3.48	5.15
AK023341	NAMPT	1.02	1.90	3.61	6.39
NM_012449	STEAP1	0.97	2.11	2.31	3.77
NM_145006	SUSD3	5.36	9.93	6.54	5.28
NM_006307	SRPX	27.45	14.69	19.06	13.67
NM_006573	TNFSF13B	0.74	3.93	0.99	3.71
NM_002546	TNFRSF11B	4.30	14.52	8.98	18.19
NM_006291	TNFAIP2	1.91	3.05	6.21	13.26
NM_006290	TNFAIP3	1.18	5.37	3.02	10.03
NM_007115	TNFAIP6	47.67	62.33	69.42	157.64
	Receptors/ligands				
NM_000675	ADORA2A	1.07	1.51	2.57	7.14
NM_001146	ANGPT1	10.93	4.20	4.81	8.33
NM_020350	AGTRAP	13.86	28.25	17.23	29.54
NM_001621	AHR	1.48	3.82	4.05	5.43
NM_001878	CRABP2	35.59	6.20	16.08	4.08
NM_000739	CHRM2	2.86	1.57	1.27	1.12
NM_001957	EDNRA	11.15	4.51	8.84	10.56
NM_198569	GPR126	0.70	1.58	0.54	1.83
NM_001505	GPR30	1.86	1.25	1.66	0.80
NM_000815	GABRD	1.94	4.40	2.64	4.79
NM_005264	GFRA1	4.81	15.75	4.25	10.28
NM_152430	OR51E1	1.08	2.30	2.82	2.75
NM_005037	PPARG	7.59	3.96	3.46	3.16
NM_000955	PTGER1	1.88	0.77	0.82	0.47
NM_005854	RAMP2	1.92	1.49	1.11	0.93
NM_206963	RARRES1	1.31	2.01	1.96	4.02
BX648828	ROBO2	1.17	6.74	2.38	7.88
NM_003268	TLR5	0.49	0.85	0.93	1.03
	Signaling				
AK055561	ERN1	1.59	3.15	2.02	3.28
NM_017729	EPS8L1	1.57	5.05	1.95	2.90
BC040073	H19	2.82	1.80	1.57	1.41
NM_006558	KHDRBS3	15.22	13.64	10.60	7.06
NM_005949	MT1F	0.41	0.58	0.64	2.55
NM_005204	MAP3K8	1.15	2.13	4.20	6.50
NM_198270	NHS	7.07	10.27	8.96	18.16

Continued

NM_013956	NRG1	1.70	5.07	5.29	8.27
NM_002502	NFKB2	1.34	2.12	1.90	4.18
NM_003738	PTCH2	1.51	2.49	1.99	5.21
NM_018492	PBK	1.81	0.95	1.22	0.49
NM_002667	PLN	2.70	15.38	6.40	8.46
NM_002668	PLP2	76.69	52.09	54.19	27.74
BX647593	RABL3	18.94	14.52	12.39	9.19
NM_002928	RGS16	0.46	3.83	6.18	1.83
NM_003702	RGS20	13.19	14.61	9.23	5.72
NM_005613	RGS4	4.22	1.95	2.65	1.34
NM_002924	RGS7	3.40	3.24	1.49	0.97
NM_031286	SH3BGRL3	27.48	21.54	19.37	12.19
NM_017720	STAP2	1.78	6.79	2.04	3.24
NM_031244	SIRT5	2.39	1.78	1.32	1.14
NM_005905	SMAD9	1.47	0.43	0.60	0.41
NM_021967	SERF1A	6.09	11.14	8.27	9.34
U81001	SNRPN	7.40	4.43	6.43	3.18
NM_198538	SBSN	1.61	2.55	3.14	6.50
NM_006528	TFPI2	59.73	196.06	114.78	270.54
NM_024873	TNIP3	0.39	2.32	0.97	3.26
NM_052864	TIFA	1.53	3.52	2.01	3.05
NM_006398	UBD	0.22	1.73	0.27	1.85
NM_021116	ADCY1	1.00	1.01	1.62	2.95
	Transcription				
NM_004024	ATF3	6.45	8.28	14.12	16.94
NM_024812	BAALC	3.13	5.66	3.64	3.61
NM_005195	CEBPD	4.79	8.43	14.27	26.51
NM_001822	CHN1	91.85	70.70	81.88	41.69
NM_012242	DKK1	76.96	77.46	39.54	43.95
NM_016323	HERC5	1.49	5.11	2.18	6.65
NM_017912	HERC6	7.24	19.79	9.99	32.31
NM_021063	HIST1H2BD	1.99	3.51	2.07	4.07
NM_003516	HIST2H2AA	7.32	24.50	12.07	30.15
NM_003528	HIST2H2BE	5.77	16.37	8.30	22.28
NM_002165	ID1	1.68	0.34	1.05	0.21
NM_014583	LMCD1	2.62	5.57	2.64	5.61
NM_001290	LDB2	3.71	2.55	2.21	1.84

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NM_002402	MEST	10.88	1.49	4.91	0.95
NM_002449	MSX2	0.71	1.38	1.14	1.50
NM_006167	NKX3-1	2.36	5.11	6.71	12.12
NM_002824	PTMS	65.65	47.54	37.46	30.93
AK095843	ZNF883	1.09	0.70	0.43	0.52
NM_003222	TFAP2C	7.27	7.80	13.62	22.43
NM_025079	ZC3H12A	5.76	9.67	14.77	22.79
NM_002583	PAWR	2.35	2.02	1.46	1.17
	Cancer related				
NM_002350	LYN	1.73	2.50	1.95	3.59
NM_014751	MTSS1	5.12	9.45	5.68	7.91
NM_152858	WTAP	19.85	47.39	38.36	69.56
NM_012258	HEY1	1.29	0.90	1.35	2.74
NM_002129	HMGB2	132.06	85.51	81.03	61.72
NM_198389	PDPN	0.44	1.35	1.52	2.48
NM_203401	STMN1	8.08	8.28	7.18	3.66
T92525	TM4SF1	1.94	3.83	1.77	3.02
	Vesicle traffic				
NM_004209	SYNGR3	0.70	1.60	1.09	1.67
NM_003764	STX11	1.29	3.95	2.20	5.03
NM_001233	CAV2	37.41	24.89	28.18	17.15
NM_177478	FTMT	13.60	37.36	23.54	43.41
N47412	FTH1	96.62	185.95	122.52	180.14
NM_005723	TSPAN5	29.76	24.58	23.11	14.73
	Channels/carriers				
NM_002977	SCN9A	34.40	19.34	17.66	12.17
NM_017585	SLC2A6	0.88	1.64	1.14	2.28
NM_199329	SLC43A3	2.35	4.54	6.64	14.16
NM_182936	SLC8A3	19.70	41.98	23.74	43.99
NM_005630	SLCO2A1	0.18	1.11	0.38	2.48
NM_007332	TRPA1	0.55	5.45	3.54	15.81
NM_017680	ASPN	2.04	0.16	0.76	0.30
NM_005603	ATP8B1	3.99	4.03	2.63	1.91
NM_130463	ATP6V1G2	2.55	1.52	1.83	1.19
NM_018672	ABCA5	2.32	4.71	2.28	5.70
	Enzymes/Metab				
NM_016816	OAS1	1.97	4.33	2.22	6.06

Continued

NM_003733	OASL	0.21	0.91	0.27	1.65
NM_033068	ACPT	1.86	3.02	2.84	3.72
NM_000693	ALDH1A3	1.59	9.74	6.50	25.74
NM_001628	AKR1B1	21.52	103.85	46.04	137.31
NM_001732	BTN1A1	21.64	15.49	13.32	9.76
BC052289	CPA4	1.94	0.59	0.90	0.42
NM_003851	CREG1	7.58	11.64	7.89	14.67
NM_018371	CSGALNACHT1	0.55	0.36	0.91	2.28
NM_016246	DHRS10	6.88	12.81	7.58	8.38
NM_013989	DIO2	2.38	3.51	4.94	8.04
NM_001935	DPP4	1.24	1.62	2.53	4.60
NM_005803	FLOT1	45.35	19.03	23.97	15.37
NM_000167	GK	2.93	3.70	2.93	5.69
NM_031302	GLT8D2	15.55	7.90	10.25	10.28
NM_025193	HSD3B7	1.56	1.18	1.04	0.70
NM_005525	HSD11B1	0.75	39.11	22.01	97.54
NM_016048	ISOC1	2.82	6.06	4.14	5.38
NM_003937	KYNU	0.48	3.73	1.45	4.40
NM_032857	LACTB	31.98	57.66	40.03	40.52
NM_194436	LDHD	2.19	3.47	3.36	4.44
NM_015907	LAP3	9.60	29.99	14.72	33.31
NM_002543	OLR1	3.23	10.12	3.24	5.65
NM_175886	PRPS1L1	9.75	5.67	5.78	3.98
NM_002573	PAFAH1B3	20.92	19.58	16.95	10.21
NM_017554	PARP14	3.41	5.16	3.72	6.56
NM_004878	PTGES	1.66	3.87	4.24	13.26
NM_016147	PME-1	84.44	79.05	57.95	42.37
NM_032957	RTEL1	5.59	23.26	15.09	10.62
NM_014509	SERHL2	1.96	1.56	1.25	0.87
W01427	NR2F2	10.63	7.36	7.78	4.56
NM_003896	ST3GAL5	3.70	2.90	3.38	1.41
NM_006645	STARD10	7.31	13.29	7.21	12.01
NM_021199	SQRDL	7.49	20.23	10.97	16.50
NM_000636	SOD2	4.43	69.46	49.27	118.66
NM_013251	TAC3	0.34	6.33	1.02	4.26
NM_016381	TREX1	12.84	25.77	13.25	19.95

Continued

NM_001071	TYMS	5.72	4.39	4.28	2.49
	Other				
NM_025004	CCDC15	3.02	2.68	2.25	1.16
NM_004764	PIWIL1	1.61	2.57	2.61	3.43
NM_138419	FAM54A	1.32	0.87	0.89	0.47
NM_144617	HSPB6	9.70	3.07	4.10	2.67
W60905	PPIA-like	0.83	0.98	1.15	1.65

[1]Values were determined by DNA microarray and are the mean of an experimental n of 3 passages of cells. GeneSpring 7.0 software was used to perform ANOVA on microarray data. [2]Abbreviations used: Genbank accession number (ACCN#), control (Con), tumor necrosis factor α (TNF), interleukin 1β (IL1β), transcriptional regulation (transcription), enzymes/metabolism (enzymes/metab).

a robust up-regulation in response to TNFα and IL1β (**Figure 1(c)**, **Figure 1(d)**). IL6 was significantly up-regulated in response to TNFα + IL1β compared to TNFα or IL1β alone (**Figure 1(e)**, **Figure 1(f)**). The expression of *MMP*3 was significantly increased in T-HESC cells treated with TNFα + IL1β (**Figure 1(g)**, **Figure 1(h)**) as demonstrated by microarray analysis and real time RT-PCR.

Since T-HESC cells are under the influence of TNFα + IL1β for a total of 70 hours in the Boyden chambers during migration and invasion experiments, gene expression was measured in T-HESC cells at 70 hours. **Figure 2** shows the significant up-regulation of IL8, IL6 and IL1*B* in T-HESC cells in response to IL1β alone and to TNFα + IL1β compared to control and TNFα alone at 70 hours as measured by real time RT-PCR.

The migration and invasion of T-HESC cells as measured with Boyden chambers were not significantly changed in response to treatment with TNFα or IL1β alone or in combination (**Figure 3(a)-(b)**) (n = 5). Similarly, TNFα and IL1β did not act as chemoattractants to stimulate the migration or invasion of T-HESC cells (**Figure 3(c)-(d)**).

4. Discussion

In this project we investigated the effects of five macrophage-secreted factors on gene expression in a human endometrial stromal cell line (T-HESC). TNFα and IL1β stimulated differential gene expression in T-HESC cells as shown by DNA microarray and real time RT-PCR. Thus, the most important finding of this study is that TNFα ± IL1β partially reproduced the effect of factors secreted by macrophages (macrophage conditioned medium) on T-HESC cells. In contrast, T-HESC cells did not respond to TGFβ, IL6, or IL8. This finding suggests that endometrial stromal cells may not be the primary target for these macrophage-secreted factors.

Cellular processes involved in the development of endometriosis include cell migration, invasion, survival, adhesion, proliferation, and angiogenesis [9]. Many of the gene ontologies identified in this study are clearly associated with cellular functions that are necessary for the establishment of endometriosis. For example, peptidases and their regulators are important to cellular invasion through the extracellular matrix, and cytoskeletal organization is associated with the ability of cells to migrate and invade. Genes in the ontology of cell cycle are involved in proliferation, and the ontology of cell adhesion is important to the ability of endometrial cells to adhere to the peritoneal organs or the peritoneal wall. The ontologies of growth factors, receptors and ligands, signal transduction, and transcriptional regulation are all related to the regulation of cellular functions involved in the development of endometriosis. Moreover, the ontology of defense/immune system is relevant to the inflammatory response that has been implicated in endometriosis [3] [8] [17].

The four genes chosen for confirmation by real time RT-PCR following microarray analysis, IL8, IL6, *MMP*3 and IL1*B*, can be designated inflammatory markers as they are all implicated in inflammation and are up-regulated in endometriosis [6], as well as in T-HESC treated with TNFα + IL1β. These genes have been extensively studied in endometriosis [3] [17]-[21]. Increased numbers of macrophages are found in the peritoneal fluid of women with endometriosis compared to that of women without endometriosis [17]. Macrophages secrete IL8 which has been shown to be increased in women with endometriosis [22]. These data associate IL8 with the pathogenesis of endometriosis. IL1β is known to induce the expression of IL6 and IL8 in T-HESC cells [23]-[25]. This study confirms the literature reports. Ponce and coworkers [26] have demonstrated that the expression of

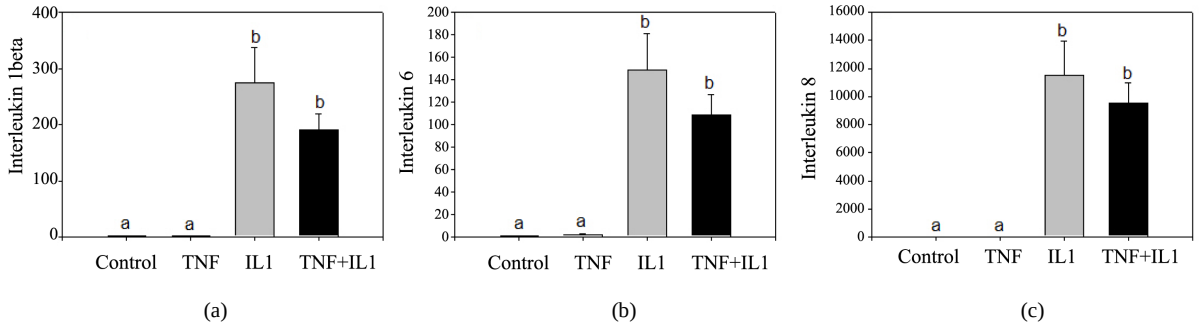

Figure 2. Differential gene expression of IL1β (a); IL6 (b); and IL8 (c) in human telomerase-immortalized endometrial stromal cells in response to treatment with TNFα (TNF) ± IL1β (IL1) for 70 hours as measured by real time RT-PCR. This time period corresponds to the time at which the cell invasion and migration assays were carried out. Data are the mean ± S.E., experimental n = 3. Bars with different letter superscripts denote that the data for those groups are significantly different from each other (ANOVA, Newman Keuls post hoc test, $p < 0.05$).

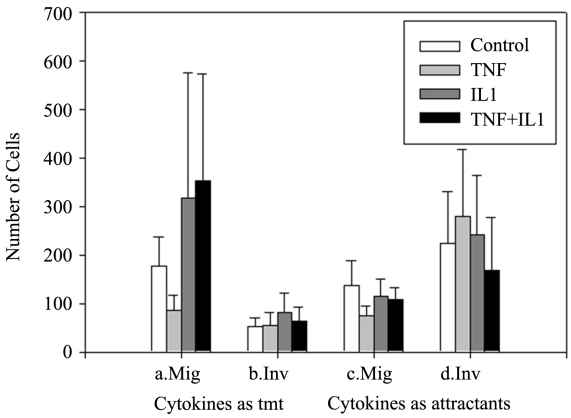

Figure 3. Migration and invasion of human telomerase-immortalized endometrial stromal cells (T-HESC) under control conditions or treated with TNFα, IL1β, or TNFα + IL1β in Boyden chambers. Groups a and b illustrate migration (mig) and invasion (inv) in response to treatment (tmt) with cytokines during the migration or invasion process. Groups c and d show migration and invasion when cytokines were placed in the bottom chamber as chemoattractants. Data are the mean ± S.E., experimental n = 5. No significant differences were identified among the groups (ANOVA, $p < 0.05$).

IL6 mRNA is down-regulated in endometriosis tissue compared to normal endometrium during the late-secretory phase of the menstrual cycle. On the other hand, Fassbender and coworkers [27] identified increased expression of IL6 in macroscopically normal endometrium from patients with endometriosis. The reports in the literature and our study demonstrate the dynamic expression of IL6 in endometrial stromal cells and endometriosis. MMP3 was up-regulated in ectopic endometrial tissue compared to eutopic endometrium in women with endometriosis [5] [28]. The up-regulation of MMP3 in our study indicates that MMP3 expression is regulated by TNFα and IL1β in endometrial stromal cells. These cytokines may also be responsible for the up-regulation of MMP3 in endometriosis.

The treatment of T-HESC cells with the combination of TNFα + IL1β for 48 hours substantially increased the expression of IL1*B*, IL8, IL6 and *MMP*3. The effect of the combined cytokines on the expression of IL1*B*, IL6, and *MMP*3 appeared to be synergistic, whereas the effect on IL8 expression appeared to be additive. In contrast, treatment of the cells with the combination of TNFα + IL1β for 70 hours did not result in a further increase in expression of the genes tested. Rather the combination of cytokines showed a small, albeit statistically insignificant, decline in expression levels compared to IL1β alone. It is unclear whether the change in response between 48 hours and 70 hours was due to loss of activity of TNFα or due to the attenuation of the response of the cells to the treatment.

The expression of the IL8 and IL6 genes was up-regulated in response to TNFα + IL1β in T-HESC cells, but the cells did not respond to treatment with IL8 or IL6. We tested higher concentrations of IL8 (25, 150, 500

ng/ml) compared to the concentrations reported in the literature [29] and still did not observe a response by T-HESC cells. It is possible that cultured T-HESC cells do not have receptors for IL8 or IL6. It is also possible that a different set of genes is affected in T-HESC cells in response to IL8 and IL6 than the ones tested in this study (IL8 and *MMP*3).

We had hypothesized that factors secreted by macrophages would increase the migratory and invasive properties of T-HESC cells [1]. Two separate experiments were designed to examine the effects of TNFα and IL1β on migration and invasion when endometrial stromal cells are bathed in the pool of cytokines (as the treatment of T-HESC cells) or when endometrial stromal cells are attracted towards a source of cytokines (TNFα ± IL1β as chemoattractants) in endometriosis. However, TNFα and IL1β did not affect the migration or invasion of T-HESC cells in this study in either experimental paradigm. TNFα and IL1β are not specifically known as chemokines so their inability to act as chemoattractants was not surprising. The lack of an effect of these two cytokines on migration and invasion of cultured T-HESC cells does not negate their effect on other cellular functions needed for the establishment of endometriosis.

5. Conclusion

In conclusion, TNFα and IL1β partially reproduced the effect of macrophage conditioned medium on gene expression in T-HESC cells. However, TNFα and IL1β failed to demonstrate an effect on the migration and invasion of T-HESC cells. Thus other cytokines, in combination with TNFα and IL1β, chemokines, and other growth factors, are expected to fully duplicate the effect of factors secreted by macrophages on endometrial stromal cells.

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