Expression of genes encoding antimicrobial proteins and members of the toll-like receptor/nuclear factor-κB pathways in engineered human skin

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ABSTRACT

Skin functions as a first line of defense against microbial invasion. Tissue-engineered cultured skin substitutes (CSS) are used to aid wound closure in massively burned patients, and have been used to facilitate safe and effective wound closure in adult patients with chronic wounds. Although they contain only two cell types at grafting, they can potentially contribute to innate defense against pathogens and stimulation of adaptive immunity. Gene microarrays were used to identify expression in cultured skin of genes involved in innate and adaptive immune responses, and to evaluate the effects of cytokine stimulation on expression levels. Cultured skin expressed multiple antimicrobial protein genes, including human β defensins 1 and 2 and S100A12. In addition, the antiviral gene APOBEC3G, which was not previously identified in skin, was expressed in CSS and up-regulated by interleukin-1α and tumor necrosis factor α. Cathelicidin was not expressed in unstimulated CSS, but was induced by cytokine treatment. Further, genes encoding several proinflammatory cytokines and members of the toll-like receptor and nuclear factor κ B pathways were expressed in CSS, suggesting that cells in CSS can mediate activation of inflammatory responses. The observed expression patterns indicate that engineered human skin utilizes innate defense mechanisms similar to those reported for native skin. Therefore, regulation of these pathways by cytokine stimulation may offer a mechanism for increasing innate immunity in CSS to combat wound infection after grafting onto patients.

Skin functions as a first line of defense against microbial invasion. In addition to its role as a physical barrier, cells in the skin can express antimicrobial peptides, such as β defensins, that act as mediators of innate immunity.[1–3] Rather than relying on specific antigen recognition, as in adaptive immunity, defensins and related antimicrobial peptides take advantage of shared structural and functional characteristics of microorganisms.[1,4] This makes antimicrobial peptides effective at killing a wide variety of pathogens. In addition to their roles in innate immunity, human defensins have been shown to be chemotactic to monocytes, dendritic cells, and T cells.[5] Thus, defensins might represent one link between innate and adaptive immunity.

The toll-like receptors (TLRs) are also important in innate immunity. TLRs are a family of transmembrane proteins that recognize conserved microbial features called pathogen-associated molecular patterns (PAMPs).[6,7] PAMPs include carbohydrates, lipids, peptides, and nucleic-acid structures that are specifically found in microorganisms. TLRs are expressed in immune cells, including monocytes, macrophages, and dendritic cells, and have recently been identified in epithelial cells, including keratinocytes.[6–9] At least 12 different TLRs have been identified in mammals, with each responding to different sets of PAMPs to stimulate innate immune responses through nuclear factor-κB (NF-κB) and interferon-regulatory factor (IRF) dependent signaling pathways.[6,10] Upon ligand binding to a specific TLR, the intracellular domain of the receptor can interact with the adapter protein myeloid differentiation marker 88 (MyD88) (reviewed in McInturff and colleagues[7,11]). MyD88 and the toll-interacting protein (TOLLIP) associate with the serine-threonine kinase interleukin (IL)-1 receptor-associated kinase (IRAK). This interaction activates downstream tumor necrosis factor (TNF) receptor-activated factor 6 (TRAF6), which leads to degradation of the NF-κB inhibitor IκB (inhibitor of κ light polypeptide gene enhancer in B cells). This in turn permits migration of NF-κB to the nucleus, where it activates transcription of genes that initiate an inflammatory response. TLRs may also be either directly or indirectly involved in regulating expression of defensin antimicrobial peptides. For example, signaling via the TLR2 receptor was shown to be important for expression of murine β defensin 3, the homolog of human β defensin 2[9] and for expression of cathelicidin in human keratinocytes in response to vitamin D.[12]

We recently reported expression of human β defensins in cultured skin substitutes (CSS), a tissue-engineered skin substitute model composed of primary keratinocytes, fibroblasts, and biopolymers.[13] CSS have been used clinically to aid wound closure in massively burned patients,[14–16] giant nevus patients,[17] and patients with chronic ulcers.[18] Wound infection is a serious and frequent complication in

burns and chronic wounds, which can reduce engraftment of CSS. Because CSS are avascular they are highly susceptible to microbial contamination, particularly from the time of grafting until vascularization is achieved. This has been managed clinically through the use of topical dressing fluids containing multiple antimicrobial drugs during the early healing period, but excessive antibiotic usage may contribute to the emergence of resistant bacterial strains.[19,20] Although CSS contain only two cell types at grafting, they can potentially contribute to innate defense against pathogens and stimulation of adaptive immunity. Hypothetically, enhancement of innate immunity in CSS could facilitate graft healing and decrease the need for exogenous antimicrobial agents. We utilized a microarray-based approach to identify genes expressed in CSS that are involved in innate and adaptive immune responses, and to determine if these genes could be up-regulated in CSS by exposure to inflammatory cytokines.

In keratinocytes, defensin gene expression is increased in response to cytokines including IL-1α.[21] Several antimicrobial peptides have been identified in psoriatic plaques or are elevated in psoriasis, a T cell-mediated inflammatory skin disease characterized by elevated cytokine levels.[22,23] IL-17 is a T cell-derived cytokine that is elevated in psoriasis,[24] and was shown to induce HBD-2 in primary keratinocytes and human airway epithelial cells.[25,26] Compared with other cytokines, including IL-1 and TNF-α, IL-17 was the most potent, resulting in a greater than 75-fold increase in HBD-2 mRNA in airway epithelial cells.[26] Based on these reports, we sought to determine whether IL-17 stimulation, compared with IL-1α and TNF-α, could up-regulate expression of antimicrobial peptides in CSS.

MATERIALS AND METHODS

Preparation of CSS

Primary human fibroblasts and keratinocytes were isolated from fresh skin obtained, with Institutional Review Board approval and in compliance with the 1975 Declaration of Helsinki, from breast tissue of a healthy adult female. Fibroblasts and keratinocytes were cultured separately in growth medium selective for each cell type,[27,28] and were expanded for a maximum of two passages before harvest for preparation of CSS. Fibroblasts were grown to confluence (95–100%) and were harvested and inoculated onto collagen-glycosaminoglycan polymer substrates at a density of $5\times10^5/cm^2$. Keratinocytes were harvested at subconfluence (85–90%) and inoculated onto the dermal substrates 1 day following fibroblast addition, at a density of $1\times10^6/cm^2$. CSS were cultured at the air–liquid interface for 2 weeks *in vitro*, using specific CSS maturation medium described in detail elsewhere.[29,30] Epidermal barrier development *in vitro* was evaluated by measuring surface electrical capacitance (SEC) using a dermal phase meter (NOVA DPM 9003; NOVA Technology Corp., Gloucester, MA); a decrease in SEC during *in vitro* incubation was observed, indicating reduced surface hydration and increased epidermal barrier function.[30,31] CSS were incubated (2.0×2.0 cm; $N=3$ per group) for 24 hours in control media, or media supplemented with 100 ng/mL IL-1α, TNF-α, or IL-17 (Peprotech, Inc., Rocky Hill, NJ). At the end of this incubation, biopsies of CSS were collected for RNA isolation and histological analysis.

Northern blot hybridization for HBD-2

RNA was isolated from biopsies of CSS using the RNeasy Mini Kit (Qiagen, Inc., Santa Clarita, CA). To use as a hybridization probe, the coding sequence for HBD-2 was amplified and cloned using the following primers: forward 5′-CATGAGGGTCTTGTATCTCCTCTT-3′; reverse 5′-CAGCTTCTTGGCCTCCTCAT-3′. RNA isolated from CSS was electrophoresed on a 1% agarose gel using NorthernMax buffers and reagents (Ambion, Inc., Austin, TX). The gel was transferred to a BrightStar-Plus membrane (Ambion, Inc.) and was hybridized to the HBD-2 cDNA probe using the AlkPhos Direct Labeling and Detection System with CDPStar (GE Healthcare, Piscataway, NJ).

Hybridization to microarrays

For each sample, 2 μg total RNA was converted to biotin-labeled cRNA using the TrueLabeling-AMP 2.0™ Kit (SuperArray Bioscience Corp., Frederick, MD) with biotin-UTP (PerkinElmer, Boston, MA). The labeled cRNA was used for hybridization to the Human Innate and Adaptive Immunity Oligo Array (SuperArray Bioscience Corp.), which includes 113 genes involved in the host immune response to infection. A table listing gene symbols and their location on the microarray grid is shown in Figure 2. Details can be obtained from the following website: http://www.superarray.com/gene_array_product/HTML/OHS-052.html. Ten micrograms of each cRNA sample was hybridized overnight at 60°C in separate tubes containing the array membranes, following the manufacturer's instructions. Washes and chemiluminescent detection were also performed according to the product manual. Multiple film exposures were collected over the next 24 hours. An exposure time of 3 seconds was determined to provide the best resolution of hybridization signal intensity for the largest number of oligos contained on the array. A high-resolution image was obtained by scanning the film at 1200 dpi using an Epson Expression 1680 scanner (Epson America, Inc., Long Beach, CA).

The microarray images were analyzed using GEArray Expression Analysis Suite software (SuperArray Bioscience Corp.). The expression levels were normalized based on the signal intensity of control oligonucleotides spotted on the membranes. Genes that were considered to be present in at least one sample are listed in Table 1. A twofold increase or decrease in expression level was used as a threshold for determining whether a gene was up- or down-regulated by cytokine treatment.

Reverse transcription polymerase chain reaction (RT-PCR)

To confirm the results of the microarray analysis, RT-PCR was used to analyze expression in newly prepared CSS. CSS were prepared, and cytokine treatment was performed, as described above. RNA was isolated from control or cytokine-treated CSS, and RT-PCR was performed

Table 1. Innate and acquired immunity genes expressed in CSS

		Normalized expression level				Ratio of treated / control		
Spot	Gene name	Control	IL-1α	TNFα	IL-17	IL-1α/control	TNFα/control	IL-17/control
3	APOBEC3G	0.024	0.095	0.152	*0.023*	**3.915**	**6.284**	0.968
9	CAMP	*0.004*	*0.025*	0.052	*0.023*	**5.613***	**11.710**	5.084*
12	CASP4	0.734	0.586	0.664	0.593	0.799	0.905	0.808
13	CCL2	0.363	0.450	0.722	0.429	1.240	1.991	1.183
15	CD14	0.057	0.048	*0.042*	*0.023*	0.842	0.745	**0.400**
23	CSF3	0.714	0.563	0.879	0.788	0.788	1.230	1.103
24	CXCR4	*0.007*	0.049	0.071	*0.010*	**7.079**	**10.128**	1.465
26	DAF	0.482	0.351	0.508	0.442	0.729	1.055	0.918
31	DEFB1	0.760	0.466	0.735	0.815	0.613	0.968	1.073
33	DEFB127	0.026	*0.015*	0.087	*0.033*	0.577	**3.387**	1.288
34	DEFB4	0.973	0.825	0.851	1.112	0.848	0.875	1.143
36	FN1	1.099	0.777	0.934	0.970	0.708	0.850	0.883
41	HMOX1	0.661	0.777	0.720	0.742	1.175	1.089	1.122
44	IFNGR1	0.167	0.249	0.226	0.114	1.486	1.348	0.680
45	IFNGR2	0.496	0.434	0.572	0.466	0.875	1.153	0.940
47	IKBKB	0.137	0.087	0.136	0.055	0.631	0.991	**0.396**
49	IL12RB2	*0.006*	*0.012*	0.067	*0.019*	1.882	**10.626**	**2.918***
50	IL1A	0.123	0.176	0.433	0.397	1.428	**3.508**	**3.216**
51	IL1B	0.252	0.378	0.396	0.452	1.500	1.573	1.797
52	IL1F10	0.172	0.225	0.321	0.243	1.308	1.868	1.413
53	IL1F5	0.530	0.522	0.667	0.864	0.984	1.257	1.628
55	IL1F7	0.372	0.370	0.245	0.148	0.993	0.658	**0.398**
56	IL1F8	0.029	0.063	0.062	0.052	**2.166**	**2.145**	1.792
57	IL1F9	0.570	0.707	0.764	0.779	1.239	1.339	1.366
58	IL1R1	0.327	0.466	0.381	0.333	1.424	1.164	1.017
59	IL1R2	0.291	0.332	0.356	0.467	1.142	1.227	1.608
63	IL1RN	0.523	0.692	0.694	0.890	1.322	1.325	1.701
64	IL6	0.671	0.786	0.748	1.090	1.172	1.115	1.626
65	IRAK1	0.720	0.682	0.681	0.722	0.948	0.946	1.003
67	IRF1	0.193	0.347	0.520	0.321	1.797	**2.690**	1.663
72	LTF	*0.004*	0.044	*0.025*	*0.019*	**11.471**	**6.509***	**5.111***
73	LY96	0.061	0.066	0.160	0.057	1.066	**2.608**	0.926
76	MAPK8	*0.014*	0.042	*0.050*	0.084	**2.929**	**3.480***	**5.870**
77	MIF	0.643	0.910	0.871	0.958	1.415	1.355	1.490
78	MYD88	0.323	0.574	0.437	0.399	1.777	1.351	1.235
80	NFKB1	0.526	0.798	0.656	0.698	1.516	1.247	1.325
81	NFKB2	0.082	0.204	0.295	0.102	**2.491**	**3.609**	1.246
82	NFKBIA	0.805	0.700	0.870	0.919	0.869	1.080	1.141
94	RNASE7	0.426	0.618	0.583	0.696	1.451	1.371	1.636
95	S100A12	0.305	0.555	0.604	0.756	1.819	1.978	**2.477**
97	SERPINE1	0.084	0.097	0.183	0.064	1.159	**2.177**	0.766
98	SFTPD	0.022	*0.017*	*0.052*	*0.041*	0.777	**2.410**	1.877
99	SLC11A1	*0.012*	*0.032*	*0.047*	0.059	**2.690***	**3.927***	**4.906**
104	TLR2	0.142	0.532	0.499	0.156	**3.756**	**3.518**	1.103
107	TLR6	0.208	0.067	0.579	0.142	**0.320**	**2.782**	0.684
110	TNF	*0.006*	0.077	0.083	*0.043*	**12.167**	**13.061**	**6.754***
111	TNFRSF1A	0.382	0.539	0.544	0.415	1.413	1.424	1.087
112	TOLLIP	0.225	0.349	0.339	0.213	1.552	1.509	0.946
113	TRAF6	0.016	*0.021*	0.066	*0.044*	1.323	**4.096**	**2.713**
114	TREM1	*0.014*	*0.011*	*0.049*	0.071	0.769	**3.493***	**5.086**

Values shown in italics were determined to be ''absent'' using GEArray Expression Analysis Suite software. The lower limit for ''absent'' calls is different for each membrane because they were determined based on background levels and control signal intensities. Ratios of treated/controls that are shown in bold represent ≥twofold expression *decreases* compared with controls. Ratios of treated/controls that are shown in bold underline represent ≥twofold expression *increases* compared with controls.

*Indicates that the treated value was an ''absent'' call; therefore, the ratio may not be a valid representation of the expression increase.

using the Titan One Tube RT-PCR System (Roche Applied Science, Indianapolis, IN). The primer sequences were as follows: APOBEC3G: forward 5′-GAATACCGT CTGGCTGTGCT-3′, reverse 5′-CAGGGGCTCCAGG ATATGTA-3′; TLR2: forward 5′-TCGGAGTTCTCCC AGTGTTT-3′, reverse 5′-CTGCCCTTGCAGATACCA TT-3′; NF-κB2: forward 5′-ACACGCCTCTTGACCTC ACT-3′, reverse 5′-CTTGTCTCGGGTTTCTGGAC-3′. Conditions for amplification reactions, including starting RNA amounts, number of cycles, and annealing temperatures, were optimized in preliminary experiments to assure that products analyzed were in the linear amplification phase. The specific conditions (cycle number and annealing temperature) for each primer pair are as follows: APOBEC3G: 45 cycles at 59 °C; TRL2: 38 cycles at 56 °C; NF-κB2: 35 cycles at 60 °C. Control primers for amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Clontech (Mountain View, CA). For each reaction, master mixes were prepared, divided equally between test primers and control primers, and the same annealing temperature and PCR profiles were used. For negative control reactions, RNA was omitted and water was used. Products were analyzed by electrophoresis on 1.5% agarose gels.

Real-time PCR

RNA prepared from newly prepared control and cytokine-treated CSS was used for real-time PCR analysis.

Total RNA concentration was determined using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). RNA with a $A_{260/280}$ ratio of 2.0 or greater, and a $A_{260/230}$ ratio of 1.7 or greater, was further assessed for quality and integrity by electrophoresis in a 1% agarose gel using NorthernMax buffers (Ambion). First-strand cDNA was synthesized using 2 μg of total RNA using the RT^2 First Strand Kit (SuperArray Bioscience Corp.) in 20 μL total reaction volume. Real-time PCR reactions contained: 1 μL volume of cDNA preparation, 1 μL volume of gene-specific RT^2 qPCR Primers (SuperArray Bioscience Corp.), and 12.5 μL volume of RT^2 SYBR Green/Fluorescein PCR Master Mix (SuperArray Bioscience Corp.) in a total volume of 25 μL. Real-time PCR amplification was performed using the BIO-RAD iCycler iQ system (BioRad, Hercules, CA) with the following profile: 10 minute denaturation at 95 °C, followed by 45 cycles of amplification (95 °C for 15 seconds then 60 °C for 1 minute). After the real-time PCR reactions were completed, a melt curve was performed from 50 to 95 °C with temperatures increasing at 0.5 °C every 10 seconds. Completed PCR reactions were run on 2% agarose Tris-Borate-EDTA gels to confirm appropriate target sizes, absence of artifacts, and confirmation of the melt curve. The C_T and ΔC_T values were determined using the iCycle iQ system software (BioRad).[32] The comparative $\Delta\Delta C_T$ method was used to calculate the fold differences between the housekeeping gene (GAPDH) and the genes of interest (DEFB4, CD14, and cathelicidin antimicrobial peptide [CAMP]).[32] Three separate experiments (biological triplicates) were performed for each target gene and GAPDH, and the results are presented as mean ± SE.

RESULTS

IL-17 increases HBD-2 expression in CSS

CSS were prepared with primary human fibroblasts, keratinocytes, and biopolymers, and were cultured for 2 weeks at the air–liquid interface to promote barrier formation. CSS were incubated for 24 hours in control medium, or medium containing 100 ng/mL IL-1α, TNF-α, or IL-17. Northern blot analysis indicated that IL-17 exposure increased expression of HBD-2, to a greater degree than either IL-1α or TNF-α (Figure 1). This suggests that IL-17 may be a potent stimulator of antimicrobial peptide gene expression in skin. Examination of hematoxylin and eosin-stained cross-sections of CSS revealed that 24-hour cytokine incubation did not negatively impact tissue morphology (data not shown).

Expression of innate and adaptive immunity genes in CSS

To further assess the effects of IL-1α, TNF-α, and IL-17 on antimicrobial gene expression in CSS, a membrane-based microarray approach was used to simultaneously analyze expression of 113 genes involved in innate and adaptive immunity. Interestingly, although expression of HBD-2 (gene symbol DEFB4) was observed using microarrays, the dramatic increase in HBD-2 expression in response to cytokine stimulation that was found by Northern blot analysis (Figure 1) was not duplicated (Figure 2). The signal level for HBD-2 is relatively intense compared with other genes represented on the microarray membrane, and appears to be saturated even in the control condition (Figure 2), making a fold-change determination using the microarray difficult for this gene.

Despite potential limitations to this approach, the microarray analysis was useful for identification of expression of genes involved in innate and adaptive immunity, including several that were not previously identified in CSS (Figure 2 and Table 1). In addition to HBD-2 and HBD-1 (DEFB4 and DEFB1 genes), which were previously shown to be expressed in CSS,[13] β defensin 127 (DEFB127) was expressed in CSS and was increased by TNF-α exposure. S100A12 was expressed in unstimulated CSS and expression was increased by IL-17. S100A12, also known as calgranulin C, has direct antimicrobial activity and, via binding to the receptor for advanced glycation end products (RAGE), has proinflammatory activity. The antiviral gene APOBEC3G, which has not been previously identified in skin, was expressed in CSS and was up-regulated by TNF-α. IRF-1, a transcription factor that mediates interferon-responsive gene expression in response to viral stimulation, is expressed in CSS and is up-regulated by TNF-α; similar regulation by TNF-α has been observed in rat thyroid cells.[33]

Eight genes were identified that were not expressed in unstimulated CSS, but were up-regulated by IL-1α, TNF-α, and/or IL-17 (Table 1). These include CAMP, chemokines receptor CXCR4, and lactoferrin (LTF). CAMP, which encodes the antimicrobial peptide LL-37, was not expressed in CSS, but was up-regulated 11.7-fold by TNF-α. The mRNA for CXCR4, the receptor for CXCL12, was

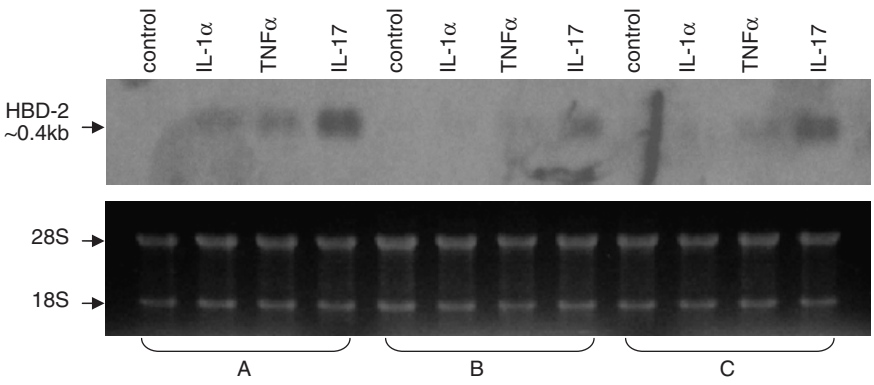
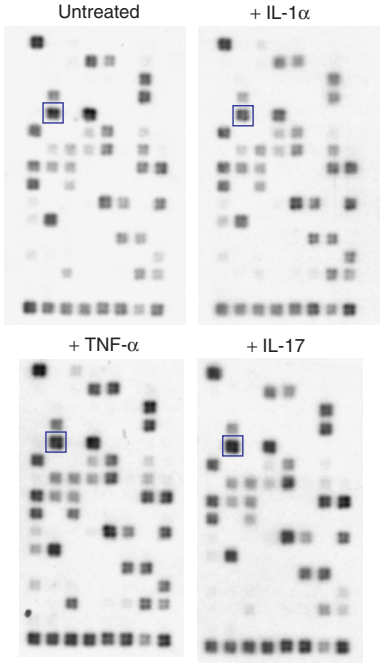


Figure 1. Expression of HBD-2 in CSS is increased by cytokine stimulation. Northern blot analysis of HBD-2 expression (top) in RNA isolated from triplicate cultured skin grafts (labeled A–C) grown in control media, or media supplemented with 100 ng/mL IL-1α, TNFα, or IL-17. The absolute levels vary among grafts A, B, and C, but all show increased expression of HBD-2, with the greatest increase observed after stimulation with IL-17. Ethidium bromide-stained gel photo (bottom), showing 28S and 18S ribosomal RNA bands, demonstrates equivalence of RNA loading. CSS, cultured skin substitutes; TNF, tumor necrosis factor; IL, interleukin.

strongly induced in CSS by IL-1α and TNF-α. This receptor is involved in cancer metastasis, HIV infection, and inflammatory diseases such as rheumatoid arthritis.[34] LTF, an abundant component of exocrine secretions including milk, tears, saliva, and bronchial mucus, is an iron-binding protein with antibacterial and anti-inflammatory activity.[35,36] LTF is released from specific granules in circulating polymorphonuclear neutrophils (PMNs), but its expression has not been previously reported in dermal fibroblasts or epidermal keratinocytes.[37] In CSS, LTF expression was increased by cytokine treatment; in particular, IL-1α treatment caused an 11.4-fold increase in LTF expression. Solute carrier family 11 A1 (SLC11A1), also called natural resistance-associated macrophage protein (NRAMP), was elevated nearly fivefold by IL-17. SLC11A1/NRAMP expression was identified in peripheral blood leukocytes, lungs, and spleen, but has not been previously identified in skin.[38] In mice, NRAMP is involved in resistance to infection with *Mycobacteria, Salmonella*, and *Leishmania*.[38]

Several proinflammatory cytokines were expressed in unstimulated CSS, including IL-6, and the genes encoding IL-1α (IL1A) and IL-1β (IL1B); IL1A expression was increased by TNF-α and IL-17. In addition, the IL-1 family members IL1F5, IL1F7, IL1F8, IL1F9, and IL1F10, were all expressed in CSS. IL1F7 was decreased by IL-17, and IL1F8 was increased by IL-1α and TNF-α. The IL-1 family genes are present in a cluster on human chromosome 2 that is genetically linked to ankylosing spondylitis, a heritable disease that causes chronic inflammation of the spine, and psoriasis, a chronic inflammatory skin and joint disease.[39,40] ILR1 and IL1R2, which encode type I and type II receptors for IL-1, respectively, were expressed in unstimulated CSS, as was the IL-1 receptor antagonist IL1RN. The TNF gene, which encodes TNF-α, was not expressed in unstimulated CSS but was significantly increased by cytokine treatment.

Several members of the TLR/NF-κB signaling pathway were expressed in CSS. TLR2 and TLR6 were both



1	2	3	4	5	6	7	8
RPS27A	ADORA2A	APOBEC3G	ARTS-1	AZU1	BPI	C5	C8A
9	10	11	12	13	14	15	16
CAMP	NLRC4	CASP1	CASP4	CCL2	CCR3	CD14	CD1D
17	18	19	20	21	22	23	24
CHIT1	CHUK	CLEC7A	COLEC12	CRISP3	CRP	CSF3	CXCR4
25	26	27	28	29	30	31	32
CYBB	CD55	DCD	DEFA4	DEFA5	DEFA6	DEFB1	DEFB118
33	34	35	36	37	38	39	40
DEFB127	DEFB4	DMBT1	FN1	GNLY	GPR4	HAMP	TMPRSS11D
41	42	43	44	45	46	47	48
HMOX1	IFNA1	IFNB1	IFNGR1	IFNGR2	IFNK	IKBKB	IL10
49	50	51	52	53	54	55	56
IL12RB2	IL1A	IL1B	IL1F10	IL1F5	IL1F6	IL1F7	IL1F8
57	58	59	60	61	62	63	64
IL1F9	IL1R1	IL1R2	IL1RAP	IL1RAPL2	IL1RL2	IL1RN	IL6
65	66	67	68	69	70	71	72
IRAK1	IRAK2	IRF1	KIR3DL1	LALBA	LBP	LEAP2	LTF
73	74	75	76	77	78	79	80
LY96	LYZ	MAPK14	MAPK8	MIF	MYD88	NCF4	NFKB1
81	82	83	84	85	86	87	88
NFKB2	NFKBIA	NOS2A	CFP	PGLYRP1	PGLYRP2	PGLYRP3	PLUNC
89	90	91	92	93	94	95	96
PPBP	PRG2	PROC	PTAFR	RNASE3	RNASE7	S100A12	SERPINA1
97	98	99	100	101	102	103	104
SERPINE1	SFTPD	SLC11A1	STAB1	TGFB1	TLR1	TLR10	TLR2
105	106	107	108	109	110	111	112
TLR3	TLR4	TLR6	TLR8	TLR9	TNF	TNFRSF1A	TOLLIP
113	114	115	116	117	118	119	120
TRAF6	TREM1	PUC18	Blank	Blank	AS1R2	AS1R1	AS1
121	122	123	124	125	126	127	128
GAPDH	B2M	HSP90AB1	HSP90AB1	ACTB	ACTB	BAS2C	BAS2C

Figure 2. Membrane-based microarray analysis of relative expression of innate and adaptive immunity pathway genes. Images shown (left) depict 3-second film exposures. The grid (right) lists the genes represented on the membranes and their location. Each ''spot'' is actually a cluster of 4 individual spots; the total cluster intensity for each spot on the array was quantified. Spots 1 and 121–128 represent control oligonucleotides used for normalization. Hybridization to HBD-2 (gene symbol DEFB4) appeared saturated on all 4 membranes (boxes), suggesting that this analysis technique is useful for genes expressed at low levels (i.e., below the level of detection by Northern blotting), but not for genes expressed at higher levels.

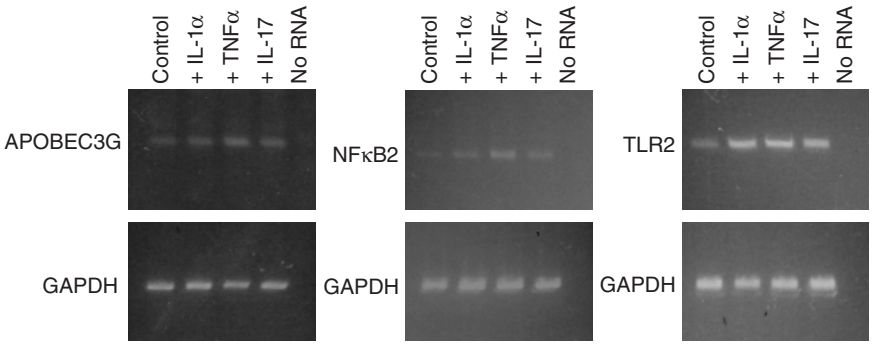


Figure 3. Validation of expression of APOBEC3G, NF-κB2, and TLR2 by RT-PCR analysis. Gene-specific primers were used as indicated; GAPDH primers were used as positive controls. Reactions with no RNA added were used as negative controls. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, reverse transcription polymerase chain reaction; TLR, toll-like receptors; NF-κB, nuclear factor-κB.

expressed in unstimulated CSS. TLR2 expression was increased by both IL-1α and TNF-α, but not IL-17. Interestingly, CD14, a receptor for bacterial LPS that interacts with TLR2, was down-regulated by IL-17. TLR6 expression was decreased over threefold by IL-1α, but increased nearly threefold by TNF-α. Other members of the NF-κB signaling pathway expressed in CSS include: MyD88; TOLLIP; IRAK; TRAF6, which is increased by TNF-α; IRF1; IKBKB (IκB kinase β), which is down-regulated by IL-17; NFKB1, which encodes NF-κB (p105); and NFKB2, which encodes NF-κB2 (p49/p100) and is increased by IL-1α and TNF-α.

Validation of microarray expression data

RT-PCR was used to confirm expression of selected genes in CSS, using RNA isolated from newly prepared control or cytokine-treated CSS. APOBEC3G, NF-κB2, and TLR2 were analyzed using gene-specific primers. The results confirm expression of these genes in CSS, as seen in the microarray analysis (Figure 3). Additional confirmation of expression was provided using real-time PCR for quantitative analysis of HBD-2 (DEFB4 gene), CD14, and CAMP (Figure 4). Expression of DEFB4 was increased by approximately 1.7-fold by IL-1α treatment, sixfold by TNF-α, and 18-fold by IL-17. CD14 expression was reduced an average of twofold by IL-17 treatment, similar to the 2.5-fold reduction seen using the OligoArray method. The fold changes observed by real-time PCR for CAMP were slightly different than the results obtained using the OligoArray. However, this quantitative analysis must be viewed with caution; the control CAMP expression level was negligible (by real-time PCR) or not detected (OligoArray), and it is difficult to interpret a fold-increase from a very small or undetectable level of expression. The real-time PCR results confirmed the Northern blot hybridization data for HBD-2, and suggest that the OligoArray was too sensitive to detect differences in levels of relatively highly-expressed genes. This is supported by data obtained for the threshold cycle (C_T) values for the three genes quantified by real-time PCR.[32] The ΔC_T value, which represents the cycle number at which the amplified target reaches a fixed threshold level relative to the amplified control gene (GAPDH),[32] was 2.2 ± 0.52 for DEFB4, compared with 14.1 ± 0.67 for CD14 and 13.7 ± 0.38 for CAMP. This indicates that amplification of DEFB4 reached the threshold level at an earlier cycle than either CD14 or CAMP; thus DEFB4 transcripts were present at substantially higher levels per cell than either CD14 or CAMP.

DISCUSSION

Microarray analysis is a powerful technique for gene expression analysis that permits evaluation of multiple genes in a single experiment. However, the present study identified some limitations of this approach for simultaneous quantitative analysis of genes expressed at widely varying levels. In this study, Northern blot analysis showed that HBD-2 expression increased dramatically in response to cytokine stimulation, but this increase was not observed using the membrane-based microarray. The disparate results for HBD-2 appear to be due to differences in sensitivity between the two techniques. Northern blot hybridization is much less sensitive than microarray analysis, and is well suited for evaluation of genes expressed at relatively high levels, whereas microarray analysis is very sensitive and can detect even slight variations in genes expressed at very low levels. Analysis of the experimental

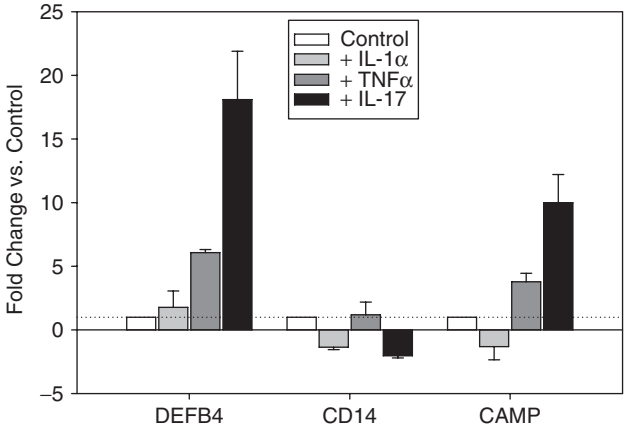


Figure 4. Quantitative examination of HBD-2 (DEFB4), CD14, and CAMP expression in CSS by real-time PCR. Fold changes compared with controls were determined using the comparative $\Delta\Delta C_T$ method.[32] The results are presented as means of triplicate experiments; error bars = ± SEM. The reference line (dotted line) represents control expression (ratio=1). CSS, cultured skin substitutes; PCR, polymerase chain reaction; CAMP, cathelicidin antimicrobial peptide.

data obtained in the present study suggests that the microarray signal for HBD-2 expression was saturated for each condition, so that the differences in signal level that were observed using Northern blot hybridization were not identified. Results of real-time PCR analysis, which were performed on RNA isolated from newly prepared control and cytokine-treated CSS, confirmed the increase in HBD-2 expression in response to cytokine stimulation that was observed in CSS by Northern blotting. This further suggests that the OligoArray was too sensitive to detect differences in levels of relatively highly-expressed genes. These experiments highlight the benefits and limitations of the different methods for gene expression analysis. Caution must be used in interpretation of data when either a small fold-change is seen in a gene with high basal expression, or a very large fold-change is seen in a gene with negligible or absent basal expression.

Despite the potential limitations of the approach, pathway-specific microarray analysis did identify several antimicrobial pathway genes expressed in CSS, including some that are regulated by IL-1α, TNF-α, and IL-17 (Figure 2 and Table 1). These results extend the findings of previous studies that demonstrated expression of antimicrobial proteins, including human β defensins 1 and 2, APOBEC3G, and S100A12, in CSS.[13,41] Other antimicrobial peptide genes, such as LL-37, were not expressed in unstimulated CSS but were up-regulated by cytokine treatment. The observed expression patterns indicate that engineered human skin utilizes many of the same innate defense mechanisms seen in native skin.[42,43] Further, expression of members of the TLR and NF-κB signaling pathways suggests that cells in CSS can mediate activation of inflammatory responses.[44]

Interestingly, of the cytokines examined, TNF-α appeared to be the most effective at increasing innate and adaptive immunity gene expression levels in CSS. Substantial changes in expression of cytokine-responsive genes were observed without any deleterious effects on the morphology of the skin grafts. The results suggest that cytokine stimulation of CSS before grafting may be an effective method to increase innate immunity of CSS, which may lead to increased resistance to infection and improved healing of wounds grafted with engineered skin. In future studies, the antimicrobial activity of cytokine-stimulated CSS will be evaluated to determine whether increased expression of specific genes in the TLR/NF-κB improves killing of wound microorganisms.

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