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Gene expression profiling of bovine mammary gland epithelial cells stimulated with lipoteichoic acid plus peptidoglycan from *Staphylococcus aureus*

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ABSTRACT

A Gram-positive bacterium, *Staphylococcus aureus* is known to be one of the major pathogenic bacteria responsible for causing bovine mastitis. Among the various cell wall components of *S. aureus*, lipoteichoic acid (LTA) and peptidoglycan (PGN) are closely associated with inflammatory responses. However, the role of LTA and PGN derived from *S. aureus* in bovine mastitis has not been clearly elucidated. In this study, we characterized the gene expression profile of a bovine mammary gland epithelial cell line, MAC-T cells, in the presence of LTA and PGN from *S. aureus*. LTA plus PGN, but not LTA or PGN alone, activated MAC-T cells. The analysis of transcriptional profiles using an Affymetrix genechip microarray showed that stimulation with LTA plus PGN produced a total of 2019 (fold change >1.2) differentially expressed genes (DEGs), with 801 up-regulated genes and 1218 down-regulated genes. Of the up-regulated genes, 14 inflammatory mediator-related DEGs, 22 intra-cellular signaling molecule-related DEGs, and 15 transcription factor-related DEGs were observed, whereas among the down-regulated DEGs 17 inflammation-related DEGs were found in MAC-T cells. The microarray results were confirmed using real-time RT-PCR of 18 genes with substantial changes in expression (9 each from the up-regulated and down-regulated DEGs). These results provide a comprehensive analysis of gene-expression profiles elicited by *S. aureus* LTA and PGN in MAC-T cells, contributing to an understanding of the pathogenesis for *S. aureus*-induced bovine mastitis.

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1. Introduction

Bovine mastitis is an inflammatory disease caused by intramammary microbial infections in dairy cattle [1], resulting in economic losses through reduced milk yield and quality, along with increased veterinary costs [2]. Once pathogens pass the teat canal, they multiply in the gland cistern and glandular tissue, causing bovine mastitis [3]. Clinical mastitis is mainly caused by *Escherichia coli* infections, and is often accompanied by severe clinical symptoms including hot and swollen udders, fever, and loss of appetite [4]. Subclinical mastitis, characterized by non-visible clinical signs of illness, is more common than clinical mastitis and also leads to a huge economic loss [1]. *Staphylococcus aureus* is well recognized as a major pathogen responsible for causing subclinical mastitis in cattle [5]. It colonizes the mammary gland tissues by internalizing epithelial and endothelial cells [6]. Subsequently, *S. aureus* enhances the expression of various pro-inflammatory mediators in the mammary gland epithelial cells [7]. Although the expression of pro-inflammatory mediators is important for eliminating an invasive pathogen, excessive production of these cytokines often leads to a continuous inflammatory response in the mammary glands, possibly resulting in subclinical mastitis [7].

Among the virulence factors of *S. aureus*, lipoteichoic acid (LTA) and peptidoglycan (PGN) have manifested their pathologic roles in infectious diseases [8]. LTA is regarded as a counterpart of lipopolysaccharide (LPS) of Gram-negative bacteria due to its structural and functional similarities. LTA is an amphiphilic molecule composed of hydrophilic polysaccharides and hydrophobic glycolipids [9]. It is involved in biofilm formation, bacterial adherence to the host, and stimulates the production of various inflammatory mediators [10,11]. Nevertheless, LTA differs from LPS since (i) LTA alone cannot cause sepsis, while LPS alone is sufficient to do so [12]; (ii) LTA is recognized by Toll-like receptor 2 (TLR2), while LPS is sensed mostly by TLR4 [13,14]; and (iii) LTA is secreted from the cell wall during cell growth, while LPS is not [15]. On the other hand, PGN is a common cell wall constituent of both Gram-

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negative and Gram-positive bacteria. Remarkably, Gram-positive bacteria possess much more PGN than Gram-negative bacteria, it comprises approximately 90% of the Gram-positive bacterial cell wall [16]. PGN is mainly recognized by intracellular pattern recognition receptors, nucleotide-binding oligomerization domains (NODs). NOD1 exclusively recognizes PGN of Gram-negative bacteria, whereas NOD2 recognizes PGN of both Gram-positive and Gram-negative bacteria [17]. Once PGN is recognized by NOD, PGN also triggers signaling pathways for the production of various inflammatory mediators, including cytokines, chemokines, and lipid metabolites [18,19].

LTA alone does not strongly elicit inflammatory responses, whereas the combination of LTA and PGN synergistically induces inflammatory responses and leads to systemic inflammation [8]. Although *S. aureus* has been considered to be a major pathogenic Gram-positive bacterium, responsible for causing bovine mastitis, the precise role of both cell wall components of *S. aureus* in bovine mastitis has not been clearly elucidated. Therefore, in order to gain insights into *S. aureus*-induced mastitis, we investigated the gene-expression profiles in a bovine epithelial cell line, MAC-T cells, in response to LTA and PGN using a DNA microarray analysis.

2. Materials and methods

2.1. Bacteria, reagents and chemicals

S. aureus ATCC 29213 was purchased from the American Type Culture Collection (Manassas, VA, USA) and was grown in tryptic soy broth (BD Biosciences, Franklin, NJ, USA). LPS from *E. coli* O111:B4 and polymyxin B (PMB) were purchased from Sigma-Aldrich (St. Louis, MO, USA). *S. aureus* PGN and a synthetic lipopeptide, Pam2CSK4, were obtained from InvivoGen (San Diego, CA, USA).

2.2. Preparation of S. aureus LTA

Highly-pure and structurally-intact LTA from *S. aureus* was prepared as previously described [20]. The structural intactness of LTA was confirmed by performing high-field nuclear magnetic resonance spectroscopy and matrix-assisted laser desorption ionization-time of flight mass spectrometry as previously described [21]. Any biological contaminants in the purified LTA, including endotoxins, proteins and nucleic

Table 1
Primer sequences of genes used for PCR analysis.

Genes	GenBank accession no.	Primer orientation	Primer sequence (5′ → 3′)	Product size
Bovine NOD2	NM_001002889	Forward	CCTTGCCGGGTGAGGCCAAG	487
		Reverse	CCCGCAGCCGTGATGTGGTT	
Bovine TLR2	NM_174197	Forward	AGGTGGCAGGCAGCTACGGAC	415
		Reverse	CCTCTGCAGGTCTCTGTTGCCGA	
Bovine β-actin	NM_173979.3	Forward	CCGGTCGACACCGCAACCAG	503
		Reverse	GACCCCGTCACCGGAGTCCA	
Mouse NOD2	NM_145857	Forward	GAGGAGTCGTGATGGTTGGT	404
		Reverse	CAGTGGAGAGGCAGAGAACC	
Mouse TLR2	NM_011905	Forward	GACTCACAGCAGCCATGAAA	451
		Reverse	TCGCGGATCGACTTTAGACTT	
Mouse β-actin	NM_001101.3	Forward	GTGGGGCGCCCCAGGCACCA	540
		Reverse	CTCCTTAATGTCACGCACGATTTC	
CTGF	NM_174030	Forward	GTGGGAGGAGGCCAGTAGAAAGCC	148
		Reverse	GATGGCTGGAGAACGCACATCCG	
TGF-β2	NM_001113252	Forward	CCCCTCCATCTCGTCGCTCCAA	103
		Reverse	GCAACGTCGTTCCCCAAGTGGAAA	
PTGS-1	NM_001105323	Forward	CTGGGTGGCCCTCCAGAATGTTGA	90
		Reverse	GCCAGCCACTGTTCTGGATCAGC	
CCDC-80	NM_001098982	Forward	GATGGCCACCTGAAACCCGCAAA	88
		Reverse	TTCGTTTGCCTTCAAAGGGCCCC	
CSRP-2	NM_001038183	Forward	CTGCGACCTGTTCTCGAACGCTCA	110
		Reverse	TCGGCGTGGTACACGGTCCTC	
WISP-2	NM_001102176	Forward	TCTGTGTCAGCCGCTCTGCAGG	104
		Reverse	GACCAGCTGGCTTGGGAATACGC	
PC-TP	NM_174835	Forward	ATGCTGCTCCTTGAAGTGCGACG	115
		Reverse	GATGTCAGCCACACACGCTCGG	
Transcription factor 4	NM_001034621	Forward	TCCGAGGCCATGTACTGCGCAT	137
		Reverse	GAAGGGTAGCCTGGCGAGTCCC	
LMO-4	NM_001034751	Forward	TTGAGAGGAGCTCGTGGCCCC	140
		Reverse	GGTCCGCAATCTTGCCCCCG	
GPNMB	NM_001038065	Forward	AGATGCCAAGGGTGAGTGAGTCAGA	110
		Reverse	TGAGCCTCGGGGTGGATCATGT	
MMP-1	NM_174112	Forward	GAGACCAACATGCCCAGACTGCC	80
		Reverse	GAAGTTGCTGCTGGGAAGCCGT	
CD36	NM_174010	Forward	TCCTGGACCCTGAACACTAGCCTTC	82
		Reverse	TGGGTCTGTGTTTTGCAGGGACAC	
CCR7	NM_001024930	Forward	TCTCCTCAGGCTCTCCACGCTG	80
		Reverse	CCTGGCTGGGAACATGGCTTAGG	
RENBP	NM_001046223	Forward	GCAGCGGACCATCTTCAGCGA	91
		Reverse	GGCTTCGTTCTGGTACCGTGCA	
MMP-9	NM_174744	Forward	CCTTCGACCTCCTGAAGTGCCCT	94
		Reverse	TTCCCTATTGGCAGGGTCCCCC	
CEACAM1	NM_205788	Forward	ACCCTGAATGTCCTCTACCCAGTGG	83
		Reverse	ACCACGGGGCCCTCATGTTCT	
IL-13RA	XM_583913	Forward	TGGAACCTTCATCCCCTCCAGCA	80
		Reverse	CTGTAGTCACAGCTGGCTGACACG	
LTBP2	NM_174385	Forward	CTTCCCGGTGCCAAAGTGGGT	80
		Reverse	CTCGGAGGGATAGTTCAGCCCCC	
GAPDH	U85042.1	Forward	ATGATTCCACCCACGGCAA	122
		Reverse	ATCACCCCACTTGATGTTGGC	

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