Construction of a microarray specific to the chicken immune system: profiling gene expression in B cells after lipopolysaccharide stimulation

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Abstract

The objective of this study was to profile gene expression in cells of the chicken immune system. A low-density immune-specific microarray was constructed that contained genes with known functions in the chicken immune system, in addition to chicken-expressed sequence tags (ESTs) homologous with mammalian immune system genes, which were systematically characterized by bioinformatic analyses. Genes and ESTs that met the annotation criteria were amplified and placed on a microarray. The microarray contained 84 immune system gene elements. As a means of calibration, the microarray was then used to examine gene expression in chicken B cells after lipopolysaccharide stimulation. Differential gene expression was observed at 6, 12, and 24 h but not at 48 h after stimulation. The results were validated by semiquantitative polymerase chain reaction. The microarray showed a high degree of reproducibility, as demonstrated by intra- and interassay correlation coefficients of 0.97 and 0.95, respectively. Thus, the low-density microarray developed in this study may be used as a tool for monitoring gene expression in the chicken immune system.

Résumé

La présente étude avait comme objectif d'étudier l'expression génique dans les cellules du système immunitaire du poulet. Une biopuce de faible densité, spécifique du système immunitaire, a été construite et contenait des gènes avec des fonctions connues dans le système immunitaire du poulet, de même que des séquences étiquettes exprimées chez le poulet (ESTs) mais homologues à des gènes du système immunitaire des mammifères, caractérisés systématiquement par analyses bio-informatiques. Les gènes et ESTs qui rencontraient les critères d'annotation ont été amplifiés et placés sur une biopuce. La biopuce contenait 84 éléments génétiques du système immunitaire. Comme méthode de calibration, la biopuce a été utilisée pour examiner l'expression génique des cellules B de poulet après stimulation par le lipopolysaccharide. Une expression génique différentielle a été observée 6, 12, et 24 h après la stimulation mais pas après 48 h. Les résultats ont été validés par réaction d'amplification en chaîne par la polymérase semi-quantitative. La biopuce avait une excellente reproductibilité tel que démontré par les coefficients de corrélation intra- et inter-essai qui étaient respectivement de 0,97 et 0,95. Donc, la biopuce de faible densité développée au cours de cette étude peut être utilisée comme outil pour surveiller l'expression génique du système immunitaire du poulet.

(Traduit par Docteur Serge Messier)

Introduction

Functional genomic techniques such as gene sequencing, sequence annotation, and gene expression profiling have led to the discovery of genes and genetic networks that regulate physiological pathways in various organisms. The establishment of expressed sequence tag (EST) databases and genome sequencing have expedited gene discovery in recent years. In the chicken, the latest estimate of available ESTs in public databases is well over 500 000. These ESTs are from a wide range of tissues and cell types, including embryonic and adult brain, ovary, chondrocytes, small intestine, pancreas, liver, kidney, adrenal gland, heart, adipose tissue, the DT40 cell line, and T cell-enriched activated splenocytes (1). Recently, several tissue-specific chicken microarrays have been constructed with these ESTs, including those derived from chicken lymphoid tissues, and have been used to examine gene expression profiles (2–5). For example, gene expression in chicken fibroblasts after infection with herpesvirus of turkey has been investigated (5). Gene expression in peripheral blood lymphocytes from birds with or without Marek's disease was assessed among inbred lines of birds that display susceptibility or resistance to Marek's disease (6). In addition, genetic networks involved in B cell development were identified by profiling gene expression in chicken B cells with the use of a bursal EST-based microarray (4).

In many cases, microarrays developed for the chicken have been constructed with the use of EST libraries. However, a large number of the chicken ESTs available in various databases are not annotated or may have been erroneously annotated. Annotating chicken immune system genes is especially important because of the significant divergence of many of these genes from their mammalian orthologs (7). Moreover, the chicken genome is smaller and less diverse than mammalian genomes (8); thus, it is likely that not every mammalian

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orthologous gene will be identified in the chicken genome. In addition, paralogous genes belonging to the same molecular family may be erroneously annotated in the sequence of common molecular motifs and domains in databases, impairing searches conducted with BLAST, GenBank's automated alignment-search program (www.ncbi.nlm.nih.gov/BLAST). These problems have resulted in the lack of annotation for several gene elements present in current chicken microarrays. Furthermore, these microarrays have a degree of redundancy, because each gene may be represented by more than 1 EST in the array.

To address such issues, we sought in the present study to annotate a subset of ESTs related to the chicken immune system that are stored in several DNA databases, with the goal of developing a low-density immune system microarray to profile gene expression in chicken lymphoid tissues. Low-density microarrays for studying immune system genes have previously been constructed and successfully used to profile gene expression in the immune system compartment (9,10). These low-density arrays are less costly than global microarrays, are focused on pathways of interest, and may be used to complement global profiling.

To achieve the objectives of this study, we selected several genes whose products are associated with immune and inflammatory responses, as well as housekeeping functions, for an annotation process involving BLASTn and tBLASTn. Subsequently, we assembled a low-density microarray with gene elements representing these families and used it to monitor temporal gene expression in chicken B cells stimulated with bacterial lipopolysaccharide (LPS).

Materials and methods

Bioinformatics approach

We compiled an extensive list of genes whose products are associated with immune and inflammatory responses, as well as housekeeping functions, and classified them functionally as follows: chemokines and chemokine receptors, cytokines and cytokine receptors, innate immunity molecules, adhesion molecules, cluster of differentiation molecules, immunoglobulins and T cell receptors, antigen presentation and processing molecules, apoptosis molecules, transcription and signal transduction molecules, and housekeeping and other molecules. The list was subjected to a bioinformatics approach: first, BLASTn was applied to screen GenBank for previously characterized chicken gene sequences; second, we annotated chicken EST sequences with possible functions in the immune system by using tBLASTn to compare chicken ESTs from various databases (Delaware [www.chickest.udel.edu/]; DT40 [pheasant.gsf.de/DEPARTMENT/dt40.html]; UMIST [www.chick.umist.ac.uk/]; and TIGR [www.tigr.org/tigr-scripts/tgi/]) with known human or mouse protein sequences. The EST selection criteria were based on score values and expectation (E) values. If the score values were higher than 250, the ESTs were considered to have high homology; if the score values were 100 to 250, the ESTs were considered to have medium homology (11). Sequences that did not meet the criteria (having a score value less than 100 and an E-value approaching zero) were discarded (11).

Primer design

Primers (Table I) were designed for amplification, by means of polymerase chain reaction (PCR), of the sequences identified through the bioinformatics approach. We used Vector NTI Software (Informax, Fredrick, Maryland, USA) and Primer 3 software (12) (www.genome.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) for primer design under the following parameters: amplicon length, 200 to 800 base pairs (bp); primer length, 20 to 24 nucleotides; primer melting temperature, 58°C to 65°C; guanine and cytosine content of the primer and amplicon, 40% to 60%; and difference in melting temperature between forward and reverse primers, 1°C to 2°C. All primers were designed under the same parameters to facilitate batch amplification and BLAST searches against chicken DNA sequences available in GenBank to ensure amplification specificity. Primers that met all the criteria were subsequently synthesized (Sigma-Genosys, Oakville, Ontario).

Reverse transcription (RT) and amplification of PCR fragments

Total RNA was extracted with the use of TRIzol reagent (Life Technologies, Gaithersburg, Maryland, USA) from the spleen, bursa of Fabricius, and thymus of mature White Leghorn hens euthanized by cervical dislocation at the Arkell Poultry Research Station, University of Guelph, Guelph, Ontario, according to the university's Animal Care Committee guidelines. After treatment with 2 units (1 μL) of DNase I and 1 μL of 10X DNase I buffer, 10 μg of total RNA was incubated at 37°C for 30 min and then DNase inactivated with 5 μL of DNase Inactivation Reagent (DNA-free; Ambion, Austin, Texas, USA) to remove contaminating DNA. The RNA quality was verified on a 1% 3-morpholinopropanesulfonic acid, 3-(*N*-morpholino)propanesulfonic acid (MOPS)-formaldehyde gel. Subsequently, RT was carried out with the use of 1 μg of template RNA, the cDNA synthesis conditions being 23°C for 10 min, 42°C for 15 min, 99°C for 5 min, and 5°C for 5 min with the use of random hexamers (GeneAmp RNA PCR Kit; Applied Biosystems Canada, Streetsville, Ontario). The conditions for RT-PCR amplification of genes and ESTs were as follows: 45 cycles at 94°C for 1 min, annealing of gene-specific primers at temperatures described below for 30 s, and extension for 2 min at 72°C, followed by a final extension at 72°C for 10 min. Two protocols were used to optimize the PCR conditions. The 1st used a range of annealing temperatures (50°C to 65°C) and $MgCl_2$ concentrations of 1 to 3 mM. Not all genes were amplified under these conditions; therefore, in a 2nd protocol, splenocytes cultured in 24-well plates in Roswell Park Memorial Institute tissue culture medium containing 10% fetal bovine serum, 2% chicken serum, 0.146 g of L-glutamine, 1.6 mM of 2-mercaptoethanol, 200 U/mL of penicillin, 80 μg/mL of streptomycin, 25 mg of gentamicin, and 250 μg of amphotericin B were stimulated with concanavalin A (Sigma-Aldrich Canada, Oakville, Ontario), 10 μg/mL, for 2, 4, 6, 24, and 48 h. Stimulation was followed by RNA extraction and cDNA synthesis. For a subset of genes that could not be amplified by either method, clones (indicated in Table I) were purchased from the Delaware Biotechnology Institute, University of Delaware (Newark, Delaware, USA).

Table I. Genes incorporated into the low-density chicken microarray

Gene ID	Gene/EST classification	GenBank accession number	Forward primer	Reverse primer	Amplicon length (bp)
Chemokines and chemokine receptors					
CXCR1	Chicken CXCR1	AF227961	ATGTGTGGGGATGGTGTCCAGG	TGAGGGCAAAGAGCAGGTCGTC	427
CXCR4	Chicken CXCR4	AF294794	GACGGTTTGGATCTGTCCTCTGGC	CTTCTCAGCCAACAGCTTTCGGG	477
CRL1	Chicken chemokine receptor CRL1	AF029369	GGGTTTGGGGGTGATTGGGTTC	TACACGATGGCCAAGTAGCGGTCC	494
SDF-1	Chicken stromal cell derived factor-1	AY451855	GATAGATCTCACCGTCGCCAGAATG	GTCGATATCTTTGTCTCTTGCCTTACTTG	296
C-orph-R-1	Putative chemokine orphan receptor-1	AJ444418	CTGGATGTGCAACAACAGCGACTG	AACCGACAGAGATGAGCTCCATGC	596
Cytokines and cytokine receptors					
gp130	Chicken gp130	AJ011688	ATGTTTTCTGGGTGGAGCTGGGC	AGTCAGGAAAGGTTTCCCGTGGC	535
TRAF6	Putative IL-1 signal transducer (TRAF6)	BU362046	TGGAGACGCAAAACACTCACATGG	GGATTGCGGTGAATTGTTGGTCTC	445
IL-1β	Chicken IL-1β	Y15006	CAGCGAAGAGACCTTCTACGG	TAGAGCTTGTAGCCCTTGATGC	501
IL-2	Chicken IL-2	AJ224516	TCTTTGGCTGTATTTCGGTAGC	CACAAAGTTGGTCAGTTCATGG	266
IL-2aR	Chicken IL-2α-receptor (CD25)	AF143806	CCTTTTGATGTGGCTCTTGCTTGG	CATCCACATTCTTGCACGTGATGG	491
IL-15	Chicken IL-15	AF139097	AGACTGGACTAACCATCTTCTTCC	GCTGTTGTGGAATTCAACTGG	296
IFN-g	Chicken IFN-g	Y07922	ACACTGACAAGTCAAAGCCGCAC	TTTTGAAACTCGGAGGATCCACC	204
IFNAR1	Chicken IFNαβ receptor-1	AF082664	CTAGCGGCTGTGCTGCTTTGTGT	GGCTCCATTTATGGACTGCAACG	414
IFNAR2	Chicken IFNαβ receptor-2	AF082665	TGGAAACACTGATGGGTGGACC	TGAGTGGGTGGCAGCTTTATGG	460
c-maf	Chicken c-maf	D28598	GAAGAGGTGATCCGGCTGAAGC	GGTTGTCGCTGCTGGATCCG	247
GATA3	Chicken GATA3	X56931	CCTCAGCCCTTTTTCCAAGACCTC	GCTTTCGGTCGTGATTTGCACC	426
Osteo[a]	Putative osteoprotegerin	pgm2n.pk007.b12	TTGTGATGTGCAACCAGTGCCC	CAGCCAGTTGGGTGTGAAACGAG	551
Gam-R	Chicken common γ-chain receptor	AJ419896	TTCGCTCGTGCCCATCCTTCTC	ACCTCCTGATTCGTCCAGCTGGTG	495
TGFβR1	Chicken TGFβ receptor 1	pgl1n.pk002.b4	GATTTAGGTGACACTATAG	TAATACGACTCACTATAGGG	1–1.5 kb
Innate immunity molecules					
NRAMP-1[a]	Chicken NRAMP1	pgm2n.pk014.h13	GATTTAGGTGACACTATAG	TAATACGACTCACTATAGGG	1–1.5 kb
TLR4	Chicken TLR4	AY064697	GAGGTCATCCCCAGCACAGCTTTC	GGAGGAAAAGCTCAGGTGCCTGAG	462
Ficol-2	Putative Ficolin 2	BU387979	TGCTCAGTGCATCAGCCACCAC	CAACGCGGAGTTCACAGGTTCC	408
Adhesion molecules					
ICAM-1	Putative ICAM-1	BX277938	CGCTATGGCGGCCAATGAAG	TGACGTCCACCCAGTTCCATCC	524
LFA-1	Putative LFA-1α	BQ038261	TGGGGCTTCAGTTTGTGCTGTGG	TTCTCAGCACCACAGCAGAATCGG	470
VCAM	Putative VCAM (CD106)	BU202635	AAGGTTCAGCCAGAGGATGC	TTGCTGTTACACAGGAGAGTGC	421
E-sel	Putative E-selectin	BG625680	CTGGATTCTATGGGCCGGGTTG	AGGAACGGGAGCAGTTCAGAGAGC	457
Cluster of differentiation molecules					
CD3	Chicken CD3	M59925	TGCGTGGCTGTGGCCAAGTT	AGTTGCCAGCTGGCTGTACTGTCC	468
CD4	Chicken CD4	Y12012	ATGCCAGCTGGAGATCAACGGTAG	TGCTTGTGCCATCCTTCTTGCC	451
CD5[a]	Chicken CD5	pgn1c.pk007.o18	CATCTGCCTTCCTCATCTGC	CTTGGAGATCCTCTTCATCAGC	449
Scav-R	CD6-like member of scavenger receptor family	BU126478	GGGACAGAAATACCTGAGCCAGGC	CCCCCAGACATTGTTGTGAAGCA	425
CD8a	Chicken CD8a	Z22726	CAGGGACAGAGGAACACGATGGAG	TCCTTGTTGACGTGGCTGCTCTG	419
CD8b	Chicken CD8b	Z26484	AACAGCACAGAGATTGTCTGCCCG	AGTCGATAGAAGCGGCGGATGG	482
CD11b	Putative CD11b (Mac-1α)	BU425066	CGGGTTATCAGACCTGCTGGTTGG	GCATGCGGTGACATTGAGGCAG	544
CD18	Chicken CD18	X71786	TCTGGCTGCCAGCAATGACCTG	CCAAAACCTATGCGGCGAGAGG	496
CD28	Chicken CD28	X67915	ATCCTCGTGGTGCTCTGCCTCATC	ACCAAGAAGTCCCGTCACTGCCAC	474
CD40-h	Chicken CD40 homologue	AJ293700	GCCTGGTGATGCTGTGAATTGCTC	AGCCCCTTTTCCTCACAGCTTGTC	462
CD44	Chicken CD44	AF153205	GGCAACAGCTGCTGATTTCCCCA	TCGTCACATGCTCCTGTTCGGTC	414

Table I. ***(continued)***

Gene ID	Gene/EST classification	GenBank accession number	Forward primer	Reverse primer	Amplicon length (bp)
CD45	Chicken CD45	L13285	CACATTCAGTTCACCAGCTGGCC	TTCGCCTCCAGCAGAGAAGGTTC	404
CD62L	Putative CD62L	BG625680	CTGGATTCTATGGGCCGGGTTG	AGGAACGGGAGCAGTTCAGAGAGC	457
CD63	Putative CD63	BU450169	GAGGGCGGAATGAAGTGCGTGAAG	TTGGCACCACAGCAGTGGAAGTCC	437
CD82	Putative CD82	AJ446108	CAGCGGGAAGGAGGATCCTGTAAG	GCACATAGTCCCACGCATCTTGC	469
CD80-h	Chicken CD80 homologue	Y08823	GAAGCGGCTCGGTTACGGATTTC	TGGCCCACTGAGTATTGGTTGGC	448
CD107	Chicken CD107 (LAMP-2)	U10547	TCCACTGTGACACACAACGGAAGC	TGGTTGGAGCAGGTGAAATGGTG	454
CD119	Putative CD119 (IFN-g receptor α)	BU465611	CGCAGTGCCTTCACCAACAGGA	TCTCTCTCATCCAAGGCCGAACC	404
CDw137	Putative CDw137	BU141439	GGAGTGCTGTGGATGCGAAGTGTG	TCTGGAGGTTCTTCCCTGGCACAG	502
CD164	Chicken CD164	AJ292037	CCTTTGCTTCGCTTCAGCGCTC	AGCCTGCAGACCCAGAACAAGGAC	556
Immunoglobulins and T cell receptors					
IgM[a]	Chicken IgM heavy chain	pgn1c.pk016.m15	GATTTAGGTGACACTATAG	TAATACGACTCACTATAGGG	1–1.5 kb
Antigen presentation and processing molecules					
Invariant	Chicken invariant chain	AJ292038	TGCAACCATGGCTGAGGAGCAG	GGTCTGATTTCAGCAGCAGGTGCC	422
TAP2[a]	Putative TAP-2	pat.pk0066.b6.f	GGTCTTTGATTACCTGGACTGG	TCCCGTAGGCAATGTTATCC	211
Rfp-Y	Chicken Rfp-y (class 1 α-chain)	AF218784	AAAGTGGAGGGTCTCACACG	AGCCGAAGTGTGGTAAGTGC	406
Calnexin	Putative calnexin	BU128302	ATGTCTCCTCCTGTGAATCCACCG	TGGGTTTGGGATCTTCCTGGG	421
Calreticulin	Putative calreticulin	AJ454899	TCTTCCGGGAGGAGTTCTTGGATG	GCGGATGTCCTTGTTGATGAGCAC	422
Apoptosis molecules					
Bcl-2-ov-R	Chicken Bcl-2-related ovarian killer protein	AF275944	GCTCGTCCGTCTTTGCTGCA	GGCGATGTTGCGGTAGACGTT	254
Bcl-x	Chicken Bcl-x	U26645	AGCGAGCTGGAGGAAGAGGATGAG	GACACAATGCGTCCCACCAGTACC	419
Caspase 1	Chicken caspase 1	AF031351	ATGAGCAGGGCAAGATCTTCGGG	CGCCCTGCAGTGCTTGTTGTTG	450
Caspase 3	Chicken caspase 3	AF083029	ATAAAAGATGGACCACGCTCAGGG	AAGTTTCCTGGCGTGTTCCTTCAG	699
Caspase 6	Chicken caspase 6	AF469049	AAGGCTGCCAGATAGACGTGGGAC	TGAACTCCAAGGAAGAGCCGTGC	557
Caspase 8	Chicken caspase 8	AY057939	ATGGAGTTCTCGCAGCTGCTCTTC	CGTCCGGCATTGTAGTTTCAGGAC	424
Caspase 9	Chicken caspase 9	AY057940	AAGGAGCAAGCACGACAGCTGG	AGCCAGCTCGAGTCGACAGATCAG	409
Fas	Chicken Fas	AF296874	AGTTTCAGTGGTCAGTGCTGCACG	TCTGCTGCAGCTGTGTTACCTTGG	476
Assoc-apop	Chicken association with apoptosis	U93865	GCCCTGACAGCTGTGAACACTGTG	ATGACCTCACATCTCCCACCCTCC	219
BAK	Putative BAK	BU422799	TCCGGAGCTACACCTTCTACC	AACATTGTCCAGATCGAGTGC	402
Granz	Putative granzyme-like molecule	BU409623	TGGGTGTTAACAGCTGCTCATTGC	CACCTGAATCCCCTCGACATGAGT	454
FLIP	Putative FLIP	AJ392248	CCTTACTAGGAATCCCAGACTCG	CCAGATTCTTGAATGGACACG	253
Transcription and signal transduction molecules					
erbB2	Chicken erbB2	AF306720	AACAGCTTTAACCCAGAGGCCCAG	CACCAGGAAATATGCTACCGGTGC	433
c-myc	Chicken c-myc	J00889	CCAGCAGCGACTCGGAAGAAGAAC	TGACAACCTTGGGCGCCTTCTC	441
c-fringe-1	Chicken c-fringe-1	U97157	ATCGCCGTCAAAACCACCAAGAAG	CGTGGCAAACCAGAAATGCACAG	414
Bu-1	Chicken Bu-1	X92865	TTGAGCCGATCATTGATGCCCG	AGCCTCCACATGGTCTCCATTGG	476
c-kit	Chicken c-kit	D13225	AATGCTCGTCTCCCTGTGAAGTGG	CAAACATCTTCGCGTACCAGGAGG	446
BASH	Chicken BASH	AB015289	ATGCAGACAATCGCACCAGTCACC	TGTGCATGTGCGAGTGCTCTGC	441
Grb2	Chicken Grb2	L19258	GGAAAGATTCCCCGAGCAAAGGC	AAACATGCCCGTCTGTCCGTGG	429
Cbl	Chicken cbl	AF318895	ATGTCGGCTCCGCTGAAGAAGG	CCAGCATGTGGCTGAATATCAGGG	439
ETS2	Chicken ETS2	X07202	TGTACAGAGGAATGCTCAAGCGGC	GCAAGTTCCAGGAAGCGTTCCTTG	409
JAK2/3	Putative JAK2/ JAK3 homologue	BU428135	TCCTGCTCTGCCAGTGTCTCACAG	TCGCCCACTGGTATTGCAATGG	580
STAT5	Chicken STAT5	AF074248	AGGAGATGCTGTCGGAGCTGAATG	TCACCTGGAAGACCAACTCGTTGC	450
NF-kB50[a]	Chicken NF-kB p50	pgn1c.pk003.j13	GATTTAGGTGACACTATAG	TAATACGACTCACTATAGGG	1–1.5 kb

Table I. ***(concluded)***

Gene ID	Gene/EST classification	GenBank accession number	Forward primer	Reverse primer	Amplicon length (bp)
Housekeeping and other molecules					
Grow-h[a]	Chicken growth hormone	pgp1n.pk001.l5	GGCTCGTGGTTTTCTCCTCTCCTC	TTGTCGTAGGTGGGTCTGAGGAGC	497
HSP70	Chicken HSP 70	J02579	CATCGATCTGGGCACCACGTATTC	AGTCGTTGAAGTAAGCGGGCACTG	434
SCA-2	Chicken stem cell antigen-2	L34554	CATCTGCTTTTCGTGCTCGGATG	TGATGTTGCAGAGGAAGGAGTCGC	230
VAV3	Chicken VAV3	AY046915	ATGGAACCGTGGAAGCAGTGCG	ACACTTTCTTCTGTGGGGAAGGGC	404
β_2m	Chicken β_2-microglobulin	Z48921	ACCAAGAACGTCCTCAACTGC	CGGGATCCCACTTGTAGACC	238
β-actin	Chicken β-actin	L08165	GCTGCGCTCGTTGTTGACAATG	AGAGGCATACAGGGACAGCACAGC	419
GAPDH	Chicken GAPDH	K01458	AAAGTCGGAGTCAACGGATTTGGC	TTCTGTGTGGCTGTGATGGCATG	545

EST — expressed sequence tag; bp — base pairs.

[a] Clones purchased from the Delaware Biotechnology Institute, University of Delaware, Newark, Delaware, USA.

Cloning of PCR fragments

Amplified PCR products were cloned into a uracil adenine vector (pDrive cloning vector; Qiagen, Mississauga, Ontario). The procedure included overnight ligation (at 10°C) of 13 to 65 ng of the PCR product into 50 ng of the vector, followed by electroporation into the bacterial host DH5α. Plasmid DNA from bacterial isolates was screened for correct size inserts by overnight digestion with *Eco*RI at 37°C. Plasmid DNA from positive clones was then column-purified (QIAprep Miniprep Plasmid Purification Kit; Qiagen), and the insert was amplified with the use of gene-specific primers, as above, and a 1:200 dilution of plasmid DNA. The PCR products were purified with use of the MinElute PCR Purification Kit (Qiagen); amplicon quality and size were confirmed on agarose gel before microarray spotting. The amplicon concentration was measured spectrophotometrically at a 260/280 nm ratio.

Spotting the microarray

All spot elements were printed in duplicate and replicated with 3 subgrids, each containing 186 spots in a 16 × 12 pattern. In total, 576 spot elements were contained within each microarray. Each array contained spots for PCR products, positive controls (housekeeping genes and β-actin serial dilutions [1/2, 1/4, and 1/8] of the original spotting concentration of 100 to 150 ng/μL), and negative controls (*Rhodococcus equi* VapA plasmid and dimethyl sulfoxide [DMSO]). The spot diameter was 90 to 100 μM and the center-to-center spot distance 250 and 300 μm between columns and rows, respectively. Each element contained 0.06 to 0.09 ng of PCR product at a concentration of 100 to 150 ng/μL in spotting buffer (100% DMSO). The PCR products were spotted on aminosilane-coated slides (GAPS II; Corning Life Sciences, Corning, Maine, USA) by means of the Virtek ChipWriter Professional Arrayer (Virtek Vision International, Waterloo, Ontario). Slides were printed at the Microarray Facility, University of Guelph.

Microarray hybridization and data analysis

With the use of TRIzol reagent, total RNA was extracted from a *Reticuloendotheliosis virus* (REV)-transformed *B21* chicken B cell line established by Haeri et al (13) 6, 12, 24, and 48 h after stimulation with a bacterial LPS cocktail (10 μg/mL: 1 part *Escherichia coli* O55:B5 and *Salmonella* Enteritidis and 2 parts *S.* Typhimurium SL1181, Re mutant [Sigma-Aldrich Canada]), as well as from unstimulated B cells at each time point. With 20 μg of total RNA as a template, we generated cyanine-labeled cDNA probes (Cy3 and Cy5) using a Micromax Direct Labelling Kit (PerkinElmer, Woodbridge, Ontario). Four independent cell-culture experiments were carried out to compare the stimulated and unstimulated B cells at each time point, with the use of 16 microarrays in total. In 2 experiments, unstimulated samples of B cells were labeled with Cy3 and stimulated samples with Cy5, and in the remaining experiments the opposite labeling was performed to account for any bias inherent to the fluorescent dyes. The labeled probes were hybridized to the microarrays for 16 h at 65°C. The slides were washed in sodium citrate–sodium chloride buffer (SSC) diluted from 20X (3 M sodium chloride and 0.3 M sodium citrate, pH 7.0) and dried by centrifugation (500 × *g* for 2 min). The washes were performed sequentially in 0.5X SSC (with 0.1% sodium dodecyl sulfate [SDS]), 0.06% SSC (with 0.1% SDS), and 0.06% SSC alone at room temperature. Images were acquired with a ScanArray Express instrument (PerkinElmer) and analyzed with the ScanArray Express software, version 3.0.

Mean spot intensity and median background intensity were normalized by means of locally weighted regression and smoothing scatter plots (LOWESS) (14) by R (www.r-project.org/). The efficiency of LOWESS normalization was evaluated by checking the Cy5 intensity — Cy3 intensity plot for data from each array before and after LOWESS normalization. The normalized natural log intensities were then analyzed with a mixed-model approach by SAS (SAS 9.1.3, Windows Pro; SAS Institute, Cary, North Carolina, USA). The mixed model used to identify significantly differentially expressed genes was as follows: $Y_{ijklmn} = \mu + L_i + T_j + D_k + R_l + S_m + L^*T_{ij} + e_{ijklmn}$, where Y_{ijklmn} represents each normalized signal intensity, μ is an overall mean value, L_i is the main effect of treatment i, T_j is the main effect of time point j, D_k is the main effect of dye k, R_l is the random effect of replicate l, S_m is the random effect of slide m, L^*T_{ij} is the interaction between treatment and time point, and e_{ijklmn} is a stochastic error (assumed to be normally distributed with mean 0 and variance σ^2). The criteria for differential expression

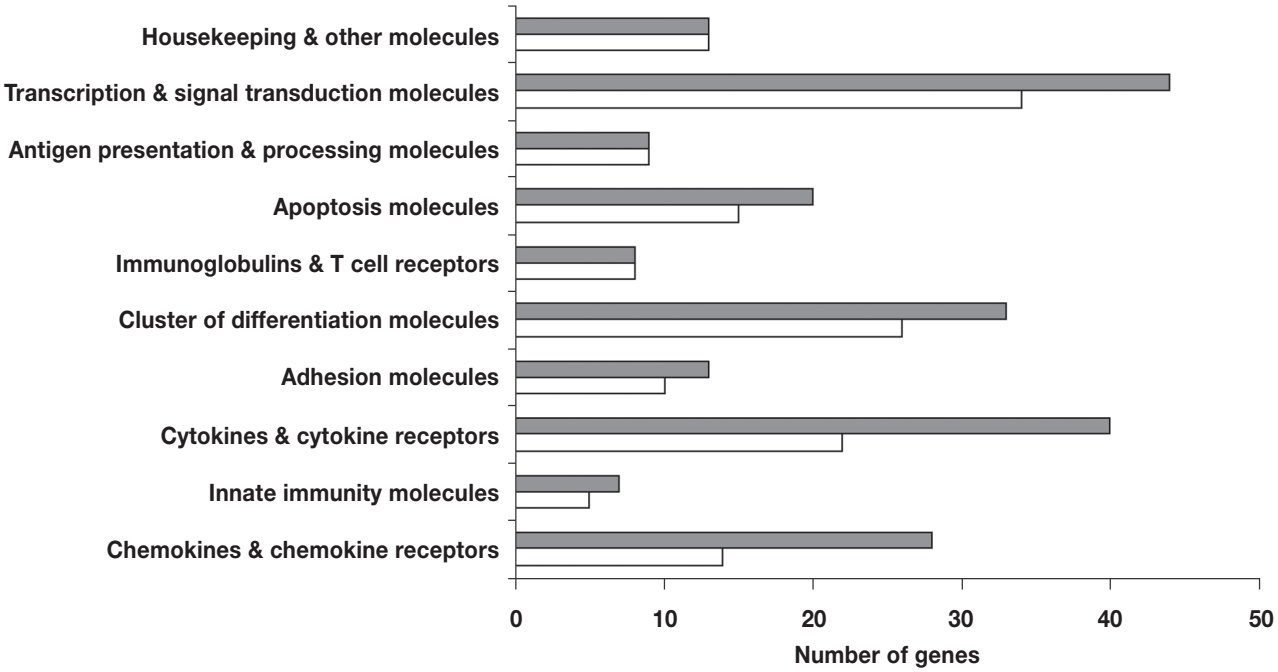

Figure 1. Number of genes and expressed sequence tags (ESTs) investigated before (grey bars) and remaining after (white bars) the bioinformatics annotation approach, those remaining being considered acceptable for the microarray. Genes and ESTs assigned to functional categories may be interpreted as a part of more than 1 family.

were established to include statistical significance reported at $P \leq 0.05$ and a signal/noise ratio ≥ 2.

We assessed intra- and interassay reproducibility by plotting LOWESS normalized values of signal intensity for each gene and then calculating the correlation coefficient between data sets. Intra-assay variability was assessed by dividing 1 source of RNA, labeling 1 portion with Cy3 and the 2nd with Cy5, and hybridizing both to the same microarray. The LOWESS normalized median intensity of the Cy3 channel was plotted against that of the Cy5 channel. The correlation coefficient of the 2 median intensities was calculated to evaluate the degree of linear relatedness. The interassay variability was determined by dividing 1 source of RNA, labeling each portion with Cy3, and hybridizing the 2 samples onto different arrays. The LOWESS normalized median intensity of the Cy3 channel of 1 slide was plotted against that of the other slide, and the correlation between the 2 median intensities was calculated.

Semiquantitative PCR

A subset of genes showing differential expression during microarray analysis was selected for validation by semiquantitative PCR. The expression of genes for leukocyte-function-associated antigen 1 (LFA-1), heat-shock protein 70 (HSP70), CD164, caspase 3, Toll-like receptor 4 (TLR4), and invariant chain was compared with that for β-actin as follows. Total RNA was extracted from unstimulated and LPS-stimulated B cells at the 6-h point and reverse-transcribed into cDNA, as described above. Using gene-specific primers, we conducted RT-PCR amplification under the following conditions: 35 cycles at 94°C for 1 min, annealing temperature of 55°C for 30 s, and extension for 2 min at 72°C, followed by a final extension at 72°C for 10 min. The number of cycles was determined by examining the dynamic range of PCR reactions from 25 to 40 cycles (data not shown). The PCR products from the stimulated cells were analysed by agarose gel electrophoresis, and the relative band density of the LPS-stimulated and unstimulated cells at 6 h was compared with that of β-actin with the use of GeneTools (version 3.00.22; Synoptics, Cambridge, England).

Results

To annotate chicken ESTs that had some sequence homology with mammalian immune system genes, we used a bioinformatics approach. Members of certain chicken gene families, such as transcription and signal transduction molecules, had the highest acceptance rates owing to sequence conservation. In contrast, some gene families, including chemokines, chemokine receptors, cytokines, and cytokine receptors, diverged greatly from their mammalian counterparts and, as a result, had the highest rejection rates owing to failure to achieve the minimum annotation requirements (score values less than 100 and E-values approaching zero). For example, 50% of chemokine and chemokine receptor sequences were rejected, whereas 100% of the sequences related to antigen presentation and processing molecules were accepted for microarray production (Figure 1). In total, 84 gene elements, including 12 EST clones from the University of Delaware, were PCR-amplified and purified for microarray spotting.

To determine intra-assay variability in gene expression profiles, we divided RNA, labeling 1 portion with Cy3 and the other with Cy5, and then hybridized both portions to a single array. The LOWESS normalized median intensity of the Cy3 channel was plotted against that of the Cy5 channel for all genes on the microarray (Figure 2A). The correlation coefficient was 0.97, indicating

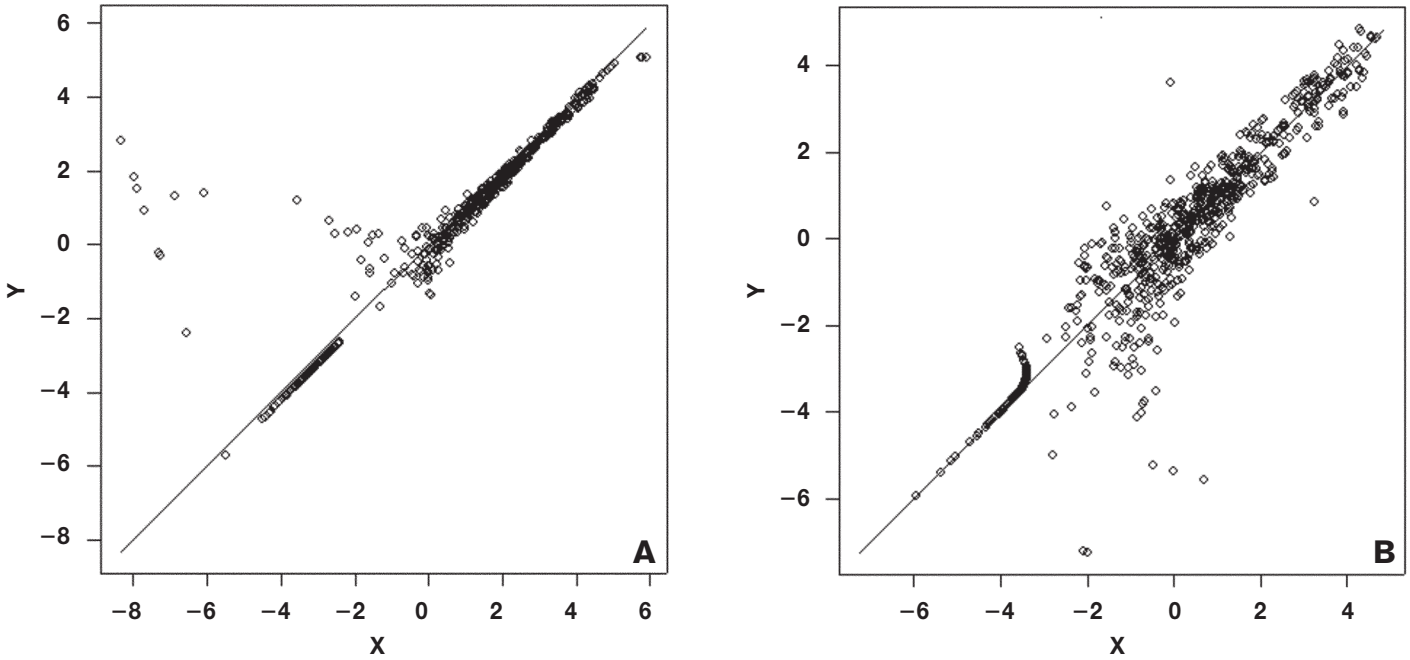

Figure 2. Variability of gene expression profiles, based on LOWESS normalized median signal intensities. (A) Within-array variation plot, where the X and Y axes represent the median intensity of cyanine-labeled cDNA probes from the same source of RNA but labeled with Cy3 (X) or Cy5 (Y) and hybridized to the same microarray. (B) Between-array variation plot, where the X and Y axes represent the median intensity of Cy3-labeled probes from the same RNA source but hybridized to a different array.

good reproducibility between labeling with Cy3 or Cy5 across the microarray. To determine interassay variability, we divided RNA into 2 aliquots, labeled both with Cy3, and hybridized them onto different microarrays. The LOWESS normalized median intensities of the Cy3 channel of 1 slide were plotted against those of the Cy3 channel of another slide (Figure 2B). The correlation coefficient was 0.95, indicating good reproducibility between slides.

We used a signal/noise ratio of 2 or greater to distinguish fluorescence due to hybridization from background fluorescence. Spots that did not meet this criterion were excluded from analysis. Between 27% and 59% of the genes were turned on at any given time, regardless of whether the B cells were treated with LPS. For each time point aside from 48 h, we observed statistically significant differences ($P \leq 0.05$) between the gene expression profile of LPS-stimulated and unstimulated cells: at 6 h, 6 (7%) of 84 genes were significantly differentially regulated, and at both 12 and 24 h, 2 (2%) of 84 genes were differentially expressed (Table II). Expression of housekeeping genes, including those encoding β-actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) did not change over time or after treatment with LPS. The negative control spots, VapA DNA, pDrive vector, and DMSO, did not hybridize. Importantly, spots representing genes known not to be expressed in B cells, such as CD3, CD8α, and CD8β, did not have a detectable signal (signal/noise ratio less than 2).

From the microarray results, we selected 6 genes (LFA-1, HSP70, CD164, caspase 3, TLR4, and invariant chain) that displayed significant differential expression 6 h after stimulation. In a representative experiment, upregulation of all 6 genes in B cells after stimulation with LPS was confirmed by semiquantitative RT-PCR (Figures 3A and 3B).

Discussion

We developed a chicken immune-specific microarray containing 84 gene elements associated with immune and inflammatory responses in the chicken and used the microarray to profile gene expression in chicken B cells in response to LPS. We validated the results by RT-PCR and assessed their reproducibility.

As a 1st step in constructing the microarray, we identified chicken genes that encode immune molecules in sequence databases. The chicken genome has recently been sequenced, but many of the genes have yet to be annotated (8). However, cross-species annotation has provided opportunities for gene discovery. For example, one-third of human genes were matched to chicken ESTs by means of BLAST, confirming previous comparative mapping studies that had noted some conservation between chicken and human genomes (15). Similarly, Tirunagaru et al (11), screening 5251 chicken EST clones for homology with known sequences, found that 25% of these clones matched previously characterized chicken genes and that 39% were homologous to genes in other species; only 11% did not have homologous hits. A recent analysis of chicken EST databases revealed that an in silico approach may serve as a useful discovery tool for immune system genes in the chicken (7), and we adapted this approach to annotate several previously unannotated chicken ESTs for inclusion in our microarray. Our criteria for annotating genes and ESTs were similar to those used by Tirunagaru et al (11) but more stringent than those used for chicken gene annotation in other studies (1,7). More stringent criteria exclude or minimize the possibility of inaccurate annotation. Although chicken genes that bear low homology with mammalian orthologs may be overlooked by a stringent approach, we wanted to increase confidence in the accuracy of our process.

Table II. Statistically significant changes in gene expression in B cells in response to stimulation with lipopolysaccharide for various times

Gene category and ID	Time (h)	*P*-value[a]	Average ratio	Fold change[b]
Adhesion molecules				
LFA-1	6	0.018402	2.040038	2.04
ICAM-1	24	0.030809	1.336307	1.33
Invariant	6	0.049729	1.134045	1.13
TAP2	12	0.013891	0.792727	−1.26
Apoptosis molecules				
Caspase 3	6	0.04422	1.232248	1.23
Cluster of differentiation molecules				
CD164	6	0.00000126	1.957402	1.95
Cytokines and cytokine receptors				
TGF-βR1	12	0.019326	0.828287	−1.20
HSP70	6	0.014048	1.299446	1.29
β_2m	24	0.01294	0.67667	−1.47
Innate immunity molecules				
TLR4	6	0.004582	1.609084	1.60

[a] Calculated with use of a mixed model, as described in the text.

[b] The minus signs indicate downregulation; the remaining genes were upregulated.

Commonly with microarray data, differential gene expression is determined by the relative fold-change of fluorescence intensity between treated and untreated groups. The arbitrary criterion in the microarray field for considering up- or downregulation of a gene is 2-fold or greater (5,6,16). Setting arbitrary values for relative fold-change in gene expression may result in false discovery rates, because slight expression changes that might be biologically important are sometimes overlooked (17). Mixed models have been used for analysis of gene expression data (18), the main advantage being the ability to control various parameters that could affect gene expression, such as variation in quality of microarray slides, probes, and labelling reactions between replicates. It is more statistically powerful to view these factors as random variables among other fixed effects such as treatment, time point, and dose (18,19). Therefore, we used a mixed model to assess the statistical significance of temporal gene-expression changes in LPS-stimulated B cells compared with unstimulated cells.

Reportedly, there is substantial variability in microarray data; as a result, replicating experiments is critical for minimizing false-discovery rates (20). Although there is no prescribed replicate number, at least for in vitro experiments, misclassification can be avoided by using 3 replicates (20). We conducted 4 independent cell-culture experiments. Assessing the quality of gene expression data from microarrays can be difficult owing to the multifactorial nature of the assay (21). Although biologic variation can be dealt with by increasing the number of replicates, technical error should be minimized to ensure that the data are not confounded by unnecessary variation. For example, within-array and between-array technical variation can be larger than individual-to-individual variation (21). Microarray data may be evaluated for variability by correlating signal intensity ratios between and within slides (1,22). Therefore, to further substantiate data from the current study, we determined both within-array and between-array reproducibility. The correlation of signal intensity from hybridized RNA was 0.97 in the "self-versus-self" test and 0.95 in the between-array test, agreeing with other examples of reproducible microarray data (correlation coefficients of 0.88, 0.93, and 0.972) (1,22).

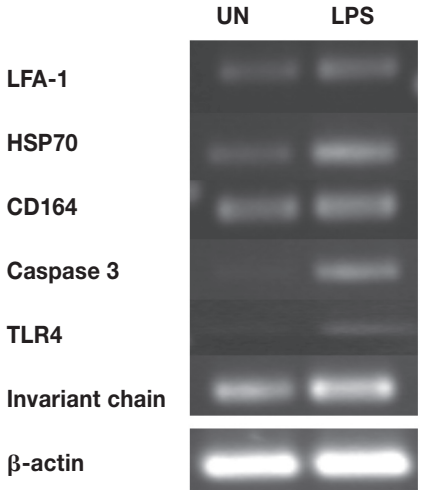

Figure 3A. Validation of microarray data by semiquantitative reverse transcription polymerase chain reaction (RT-PCR) of RNA extracted from unstimulated B cells cultured for 6 h (UN) and B cells stimulated with lipopolysaccharide (LPS) for 6 h, followed by agarose gel electrophoresis, for comparison of the amplified gene for β-actin with those for leukocyte-function-associated antigen 1 (LFA-1), heat-shock protein 70 (HSP70), CD164, caspase 3, Toll-like receptor 4 (TLR4), and invariant chain.

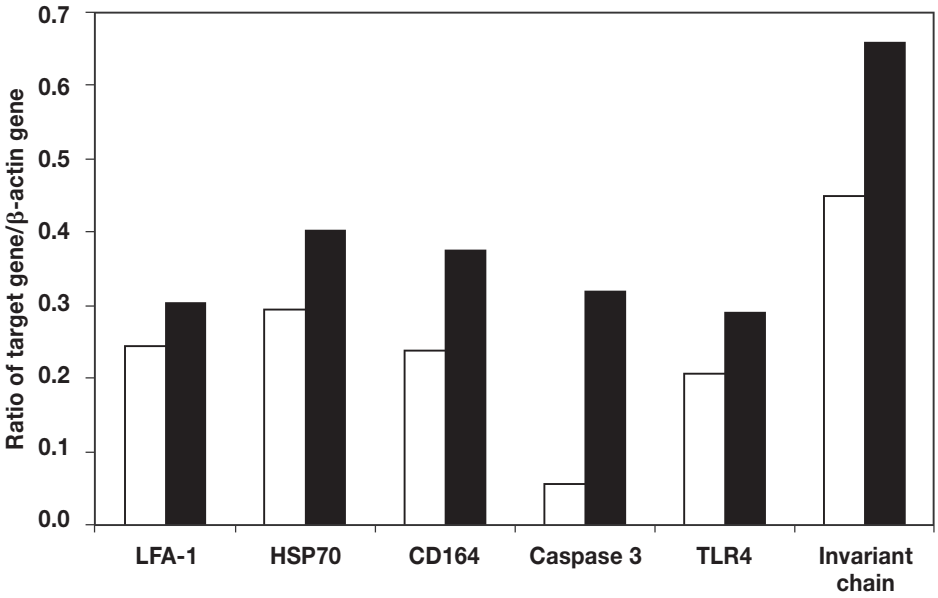

Figure 3B. Ratio of the raw volume fluorescence of the β-actin gene and each target gene in the unstimulated (white bars) and the LPS-stimulated (black bars) B cells, determined from the relative band density on the gel.

The chicken immune microarray was used to assess temporal gene expression in B cells after stimulation with LPS. To enhance the likelihood of stimulation, we used a cocktail of LPS from 3 strains, namely *E. coli* O55:B5, *S.* Enteritidis, and *S.* Typhimurium SL1181, Re Mutant, all of which have previously shown stimulatory effects on chicken cells (23–25). Microarray technology has been successfully used for expression profiling of LPS-responsive genes in several cell types, including B cells, hepatocytes, macrophages, neutrophils, and endothelial cells (26,27), coinciding with the results in our study. The LPS stimulation of chicken B cells resulted in changes in gene expression across time, most being observed after 6 h of stimulation. The observed time-dependency of gene expression in response to LPS is in agreement with previous findings (27).

Genes belonging to 7 out of 10 families described in this study were represented in the differential gene expression data. However, there was not 1 family in particular whose members were predominantly regulated in response to LPS. Of the 10 genes displaying differential expression after LPS stimulation, 7 were induced and 3 were repressed. In agreement with our findings, previous studies examining gene expression in human and chicken macrophages after stimulation with LPS have reported induction and repression of approximately 70% and 30%, respectively, of the genes represented on high-density microarrays (28,29). For technical validation of the microarray data, we selected a subset of genes that displayed enhanced expression 6 h after stimulation and confirmed the data by semiquantitative RT-PCR.

Expression of the TLR4 gene was induced after stimulation with LPS, which is known to exert its functions via binding to a complex of molecules, including LPS binding protein (LBP), CD14, and TLR4 (30). Chicken heterophils respond to LPS even in the absence of LBP (31). However, the response is significantly increased when the cell culture medium is supplemented with chicken serum, which contains LBP (31). Since the B cells used in the present study constitutively expressed TLR4 and were kept in a chicken serum-supplemented medium, these cells should have been optimally stimulated by LPS. Induction of the TLR4 gene in response to LPS, peaking 2 to 8 h after stimulation, has previously been observed (32), in association with PU.1, a transcription factor that belongs to the Ets family (32).

We also found induction of HSP70 after B cell stimulation with LPS. Previously it was shown that members of the HSP family are induced after LPS stimulation (33) and that heat shock proteins may act as TLR ligands (34). Through biochemical analyses, HSP70 has been shown to form a complex receptor in conjunction with HSP90, chemokine receptor CXCR4, and growth differentiation factor 5 (GDF5) that could bind LPS (35).

Stimulation with LPS also induced upregulation of adhesion molecule LFA-1 and its ligand, intercellular adhesion molecule (ICAM)-1, 6 h after stimulation. Both molecules have been implicated in B cell activation, as indicated by B cell aggregation in culture. In murine B cells, LPS stimulation caused an increase in cell aggregation that was largely facilitated by LFA-1. Activation of B cells with LPS induced a stronger avidity between LFA-1 and ICAM-1 in vitro than was identified without stimulation (36). More recently, the interaction between LFA-1 and ICAM-1 has been associated with the formation of mature B cell synapses after cellular activation (37). Thus, the upregulation of these adhesion molecules in the current study may be an indication of B cell activation induced by LPS.

In our study, transforming growth factor (TGF)-β receptor(R)-1 was downregulated 1.2 fold in response to LPS at 12 h. We suggest that this is related to a lack of TGF-β1 regulation in the culture. This growth factor has a regulatory effect, inhibiting B and T cell function. In chickens, TGF-β1 has been shown to reduce secondary antibody production and B cell proliferation (induced by LPS) by more than 90% (38). In order for this substance to have such substantial effects on cell proliferation, the receptor must be tightly regulated. The downregulation of TGF-βR1 observed in the present study indicates

a cellular process biased towards cell activation and proliferation, preventing the immunosuppressive effects of TGF-β1 by down-regulating the necessary receptor.

In accordance with the data obtained in other species (39), we detected significant induction of the invariant chain gene after LPS stimulation, which was confirmed by RT-PCR. Invariant chain is a monomorphic protein that is involved in antigen processing and presentation by binding to the newly synthesized major histocompatibility complex (MHC) class II molecules to protect them from binding to low-affinity peptides in the endoplasmic reticulum (40). In addition, it has been suggested that invariant chain may play a role in differentiation of B cells (40). Therefore, it is plausible that LPS stimulation results in activation of the chicken MHC class II antigen presentation pathway, as marked by enhanced expression of the invariant chain gene.

According to the RT-PCR data, the caspase-3 gene was induced more than 4-fold in LPS-stimulated B cells. Members of the caspase family are involved in the induction of apoptosis. Caspase-3 activation in the chicken correlates with apoptosis of B cells (41). Although LPS is a known activating ligand for B cells, it is possible that stimulation by LPS results in induction of apoptosis due to activation-induced cell death or via other mechanisms. For example, LPS has been shown to induce apoptosis in lymphocytes by activation of caspase-11 in a caspase-3- and caspase-7-dependent manner (42).

Another gene whose differential expression was confirmed by RT-PCR in this study was CD164, or endolyn. The expression pattern of this molecule in the lymphoid tissues of the chicken has not been studied. Furthermore, little is known about the potential role of this molecule in response to LPS.

These examples of gene regulation and function are merely a glimpse into the cellular regulation affected by LPS stimulation. By inferring function to the genes showing differential expression, one can further validate the microarray results obtained from gene profiling of chicken B cells. The ability to analyse and profile gene expression in the immune system of the chicken will provide opportunities for future studies in chicken immunology.

Acknowledgments

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