

Materials & Methods

Oligonucleotide cDNA microarray analysis

The human cytokine/receptor Atlas nylon cDNA expression array containing duplicate cDNAs from 268 human cytokine related genes and 9 housekeeping genes immobilized onto a nylon membrane was used in accordance with the manufacturer's instructions. Briefly, radiolabeled cDNA probes were prepared from 5 µg of total RNA for each sample by a reverse transcription step in the presence of 3.5 µL (α -³³P) dATP, 1 µL CDS primer mix, 1 µL Superscript III (200 U), 2 µL dNTP (5 mM), 0.5 µL DTT (100 mM) and incubated at 42°C for 50 min. The probe was purified after synthesis using Micro Bio Spin columns. The incorporation of the radiolabel was checked using a scintillation counter and probes that had a count per minute (cpm) above 1×10^6 were deemed to have satisfactory signal strength for the array analysis. The array membranes were washed with deionized H₂O and pre-hybridized using 5 mL pre-warmed ExpressHyb containing 0.5 mg of heat-denatured sheared salmon testes DNA at 68°C for 4 h. Before hybridization, the cDNA probe was denatured by the addition of 5.5 µL of 10 x denaturing solution and was incubated at 68°C for 20 min. After denaturation of the probe, 5 µg of Cot-1 DNA and 55.5 µL of neutralization solution were added to the cDNA probe and incubated at 68°C for 10 min. Subsequently, the cDNA probe was added to 5 mL pre-warmed ExpressHyb and the membranes were hybridized with the cDNA probes at 68°C for 16 h with continuous rotation in a hybridization oven. After hybridization, these membranes were washed four times with 2x standard saline citrate (SSC) and 1% (w/v) sodium dodecyl sulphate (SDS) at 68°C for 30 min, once in 0.1 X SSC-0.5% (w/v) SDS solution for 30 min at 68°C and finally with 2 x SSC at 68 C for 10 min. The membranes were exposed to X-Ray film and developed. Quantification and normalization was performed using AtlasImage 2.01 software. This software automatically averaged

the two hybridization signals for a particular gene and compared the experimental intensity to the same gene in the control array. Genes showing a two-fold difference in signal intensity between experimental and control membranes were selected for confirmation of differential expression by qPCR.

Table 1: Sequences of primers used for quantitative PCR analysis of cytokines

Target cDNA	Primer Name	Sequence (5'-3')	Product Size (bp)
GAPDH	GAPDH_FP	CAATGCCTCCTGCACCAC	128
	GAPDH_RP	GGCCATCCACAGTCTTCTG	
IL-1 β	IL-1 β _FP	GCTGAGGAAGATGCTGGTTC	126
	IL-1 β _RP	TGCACATAAGCCTCGTTATCC	
IL-10	IL-10_FP	CCAAGACCCAGACATCAAGG	138
	IL-10_RP	AAGGCATTCTTCACCTGCTC	
GM-CSF	GM-CSF_FP	AGCCGACCTGCCTACAGAC	149
	GM-CSF_RP	AATCTGGGTTGCACAGGAAG	
INF- γ	INF- γ _FP	TGTGGAGACCATCAAGGAAG	158
	INF- γ _RP	GCGACAGTTCAGCCATCAC	
IRF1	IRF1_FP	AGACCAGAGCAGGAACAAGG	113
	IRF1_RP	TCTTAGCATCTCGGCTGGAC	

Table 2: Genes on ATLAS cytokine array differentially regulated by active HRgpA

Name	Differentially regulated genes	GenBank accession number	Relative fold change*
IL4R	interleukin-4 receptor alpha subunit precursor	X52425	2.00
IFN- γ	interferon gamma precursor	X01992	7.72
IFN- γ R	interferon-gamma receptor	J03143	2.31
IRF1	interferon regulatory factor 1	X14454	2.34
CDW40	CDW40 antigen; CD40L receptor precursor	X60592	4.62
trk-T3	trk-T3; P68 TRK-T3 oncoprotein	X85960	2.13
FGF8	fibroblast growth factor 8; androgen-induced growth factor precursor ; HBGF8	U36223	2.14
EGF	kidney epidermal growth factor; urogastrone	X04571	2.37

*Each value is the mean of duplicate hybridisations for one representative microarray.

Table 3: Genes on ATLAS cytokine array differentially regulated by inactive HRgpA

Name	Differentially regulated genes	GenBank accession number	Relative fold change*
IL-1 β	interleukin-1 beta precursor	K02770	4.51
IL-10	interleukin-10 precursor	M57627	2.07
IL-1RA	interleukin-1 receptor antagonist protein precursor	M63099	2.66
IL-1R1	interleukin-1 receptor type I precursor	M27492	112.34
IL-5RA	interleukin-5 receptor alpha subunit precursor	M75914	4.66
IL-6RA	interleukin-6 receptor alpha subunit precursor	M20566;	17.72
IFN-alpha-RA	interferon-alpha/beta receptor beta subunit precursor	X77722	172.35
IFN- γ antagonist	interferon gamma antagonist	A25270	2.46
IFNR- γ	interferon-gamma receptor	J03143	3.16
IRF1	interferon regulatory factor 1	X14454	2.54
BCGF1	B-cell growth factor 1 precursor	M15530	8.93
GM-CSF	granulocyte-macrophage colony stimulating factor	M11220	7.92
MIP1-alpha	macrophage inflammatory protein 1 alpha precursor	M23452	4.37
C5AR	C5a anaphylatoxin receptor	M62505	2.94
CDW40	CDW40 antigen; CD40L receptor precursor	X60592	4.22
CC CKR1	CC chemokine receptor type 1; macrophage inflammatory protein 1 alpha receptor; RANTES R; LD78 receptor; CMKBR1	D10925	3.72
CSF-1-R	macrophage colony stimulating factor I receptor precursor; fms proto-oncogene; CD115	X03663	2.57
trk-T3	trk-T3; P68 TRK-T3 oncoprotein	X85960	4.22
TFAR15	apoptosis-related protein TFAR15	AF022385	2.36
TNF-alpha	tumor necrosis factor precursor; cachectin	X01394	2.11
PDGF AP	PDGF associated protein	U41745	3.07
GAF	glia-activating factor precursor; fibroblast growth factor 9	D14838	2.42
FGFR1	fibroblast growth factor receptor1 precursor; basic fibroblast growth factor receptor;	X66945;	4.31
VEGFB	vascular endothelial growth factor B precursor + VEGF-related factor isoform VRF186	U48801; U43369	2.72
Wnt-5A	Wnt-5A	L20861	2.74
NOTCH1	neurogenic locus notch protein homolog 1 precursor; translocation-associated notch protein	M73980	3.29
BSG	basigin precursor; leukocyte activation antigen M6; collagenase stimulatory factor; extracellular matrix metalloproteinase inducer; CD147 antigen	L20471	3.80

* Each value is the mean of duplicate hybridisations for one representative microarray.

Table 4: Genes on ATLAS cytokine array differentially regulated by active Kgp

Name	Differentially regulated genes	GenBank accession number	Relative fold change*
IL-1R1	interleukin-1 receptor type I precursor	M27492	184.9
IL4R	interleukin-4 receptor alpha subunit precursor	X52425	4.52
IL5RA	interleukin-5 receptor alpha subunit precursor	M75914	2.43
IL6R-alpha	interleukin-6 receptor alpha subunit precursor	M20566	5.40
IRF1	interferon regulatory factor 1	X14454	2.92
BCGF1	B-cell growth factor 1 precursor	M15530	4.15
EPH-3	tyrosine-protein kinase receptor EPH-3	L41939	9.13
NT4	neurotrophin-4	M86528;	3.78
ERBB2	ERBB2 receptor protein-tyrosine kinase	M95667	10.95
VEGFB	vascular endothelial growth factor B precursor + VEGF-related factor isoform VRF186	U48801; U43369	2.92
Wnt-5A	Wnt-5A	L20861	2.41
JAG2	jagged2	AF003521	3.41
NOTCH1	neurogenic locus notch protein homolog 1 precursor	M73980	2.31
HGF activator	hepatocyte growth factor activator	D14012	3.15
PHB	prohibitin	S85655	3.57

*Each value is the mean of duplicate hybridisations for one representative microarray.