Genes Immun. Author manuscript; available in PMC 2011 September 01.

Published in final edited form as:
Genes Immun. 2011 March ; 12(2): 78–89. doi:10.1038/gene.2010.61.

Characterisation of the macrophage transcriptome in glomerulonephritis-susceptible and -resistant rat strains

Klio Maratou[1], **Jacques Behmoaras**[2], **Chris Fewings**[1], **Prashant Srivastava**[1], **Zelpha D'Souza**[1], **Jennifer Smith**[3], **Laurence Game**[4], **Terence Cook**[2], and **Tim Aitman**[1]

[1]Physiological Genomics and Medicine Group, MRC Clinical Sciences Centre, Hammersmith Hospital, Du Cane Road, London, W12 0NN, UK

[2]Centre for Complement and Inflammation Research, Department of Medicine, Imperial College, Hammersmith Hospital, Du Cane Road, London, W12 0NN, UK

[3]Renal Section, Department of Medicine, Imperial College, Hammersmith Hospital, Du Cane Road, London, W12 0NN, UK

[4]Genomics Laboratory, MRC Clinical Sciences Centre, Hammersmith Hospital, Du Cane Road, London, W12 0NN, UK

Abstract

Crescentic glomerulonephritis (CRGN) is a major cause of rapidly progressive renal failure for which the underlying genetic basis is unknown. WKY rats show marked susceptibility to CRGN, while Lewis rats are resistant. Glomerular injury and crescent formation are macrophage-dependent and mainly explained by seven quantitative trait loci (*Crgn1-7*). Here, we used microarray analysis in basal and lipopolysaccharide (LPS)-stimulated macrophages to identify genes that reside on pathways predisposing WKY rats to CRGN. We detected 97 novel positional candidates for the uncharacterised *Crgn3-7*. We identified 10 additional secondary effector genes with profound differences in expression between the two strains (>5-fold change, <1% False Discovery Rate) for basal and LPS-stimulated macrophages. Moreover, we identified 8 genes with differentially expressed alternatively spliced isoforms, by using an in depth analysis at probe-level that allowed us to discard false positives due to polymorphisms between the two rat strains. Pathway analysis identified several common linked pathways, enriched for differentially expressed genes, which affect macrophage activation. In summary, our results identify distinct macrophage transcriptome profiles between two rat strains that differ in susceptibility to glomerulonephritis, provide novel positional candidates for *Crgn3-7*, and define groups of genes that play a significant role in differential regulation of macrophage activity.

Keywords

Crescentic glomerulonephritis; macrophage; microarray; rat

Corresponding author: Tim Aitman, Tel: +44-2083834253, Fax: +442083838577, t.aitman@csc.mrc.ac.uk.

Conflict of interest

The authors declare no conflict of interest.

Supplementary Information accompanies the paper on Genes and Immunity website

Europe PMC Funders Author Manuscripts

Introduction

Crescentic glomerulonephritis (CRGN) is a major cause of rapidly progressive renal failure for which the underlying genetic basis is largely unknown [1]. The Wistar-Kyoto (WKY) rat shows marked susceptibility to CRGN, as administration of a small dose of nephrotoxic serum (NTS), that is subnephritogenic in other strains, leads to rapid onset of nephrotoxic nephritis (NTN). In WKY rats, NTN is characterised by the development of albuminuria by day 4, crescent formation in the majority of glomeruli by day 11 and progression to severe scarring, that is characteristic of CRGN, with renal failure by 6 weeks [2]. Monocytes/macrophages and CD8+ cells are the major cell types in the glomerular infiltrate, detected as early as 2.5 hours after injection of NTS, and reaching a maximal number between days 4 and 8 [2]. The model is highly reproducible and the histology closely resembles that seen in human glomerulonephritis. In contrast Lewis (LEW) rats, which share the same MHC haplotype (RT1-l), develop only mild glomerular hypercellularity with no crescents after administration of the same dose of NTS [3].

In WKY rats, macrophage depletion studies [4] have shown that glomerular injury in CRGN is macrophage dependent. This suggests at least in part, shared pathophysiology with human CRGN, where macrophage accumulation correlates with the degree of histological and functional injury [5-7]. Furthermore, bone marrow-derived macrophages (BMDMs) from WKY rats show a number of phenotypic differences compared with those from LEW rats including enhanced antibody-dependent cytotoxicity, Fc receptor-mediated phagocytosis and Fc-receptor-dependent oxidative burst, as well as increased inducible nitric oxide synthase gene (*Nos2*) expression upon lipopolysaccharide (LPS) stimulation [3,8]. However, bone marrow and kidney transplantation studies between WKY and LEW rats show that, in addition to circulating cells, genetic susceptibility to CRGN is also partly dependent on intrinsic factors within the kidney itself [9,10].

In previous studies, we carried out a genome-wide linkage analysis of an F2 population derived from WKY and LEW rats and mapped seven CRGN quantitative trait loci (QTL) (*Crgn1-7*) linked to crescent formation, proteinuria or macrophage infiltration [3]. The two most significant linkage peaks, with lod score over 8, were on chromosomes 13 (*Crgn1*) and 16 (*Crgn2*). By a combination of expression studies, fine mapping and functional assays, we identified deletion of an Fc receptor gene, *Fcgr3-rs*, as a cause of macrophage over-activity and CRGN susceptibility at *Crgn1*. Subsequently, we identified over-activity of the AP-1 transcription factor JunD as a cause of enhanced macrophage oxygen burst activity as well as iNOS synthesis and WKY susceptibility to CRGN at *Crgn2* [8]. Recently we generated a double congenic rat strain where both *Crgn1* and *Crgn2* from NTN-resistant LEW rats were introgressed into the genetic background of the WKY rat [10]. Our results show that *Crgn1* and *Crgn2* have an additive protective effect against glomerular crescent formation [10]. However, this study also highlighted the effects of *Crgn3-7*, as bone marrow transplantation experiments showed that the double congenic line shows residual susceptibility to CRGN, which is mainly attributable to macrophage activation rather than macrophage number [10].

The aim of this study was to compare macrophage gene expression between the CRGN-susceptible WKY and CRGN-resistant LEW strains to identify genes that reside on effector pathways for macrophage-mediated damage to glomeruli in WKY rats. To achieve this aim we used rat exon arrays, in basal (unstimulated) and LPS-stimulated macrophages. Our results revealed extensive strain differences in macrophage gene expression, identified several differentially expressed genes that positionally map to *Crgn3-7* (primary effector candidates), as well as a subset of genes, that although they map outside the known CRGN QTL regions, showed highly significant differential expression between WKY and LEW macrophages, before and after LPS stimulation. We also identified a small number of

alternative splicing events that cause transcript isoform differences between macrophages from the two strains.

Results

Differential gene expression between WKY and LEW macrophages

Exon array analysis identified 800 transcripts that were differentially expressed between WKY and LEW bone marrow-derived macrophages, in the basal state, with a 5% false discovery rate (FDR) threshold (Supplementary Table S1). 52.9% of these transcripts showed higher expression in WKY compared to LEW. Notably, a subset of 19 genes showed very strong differential expression in basal conditions (>5 fold change, <1% FDR) (Table 1). Following LPS stimulation, 887 transcripts were differentially expressed (Supplementary Table S2), and 60.0% of these showed higher expression in WKY compared to LEW. There was an overlap of exactly 400 genes between the basal and LPS conditions, while 487 genes were differentially expressed between the two strains only after LPS stimulation. In addition, 15 genes showed very strong macrophage gene differential expression (>5 fold change, <1% FDR) between the two strains following LPS stimulation (Table 2). Eleven of these genes (*Arg1*, *Fcgr3-rs*, *Grit*, *Igf2bp1*, *IMAGE:5598800*, *Lilrb3l*, *Ly49si1*, *Ly49si2*, *Mmp7*, *Orl1684* and *Pirb*) showed similarly strong differential expression in both the basal and LPS-stimulated state.

Validation of differentially expressed genes

Sixty-one of the genes showing differential expression (<5% FDR) between WKY and LEW in basal macrophages and 68 genes showing differential expression in LPS-stimulated macrophages mapped within the 1.5-LOD support interval of our previously localised crescentic glomerulonephritis susceptibility QTLs (*Crgn3-7*) [3] (Table 3). Because these genes are differentially expressed and map to a known QTL, they can be considered as candidate genes for *Crgn3-7* that may confer primary susceptibility to CRGN. Thirty-two of these genes showed similarly strong differential expression in both the basal and LPS-stimulated state. To test the accuracy of the microarray results, we randomly selected six genes (*Aig1, Csf1, Epsti1, Mcoln3, Spata6* and *Stxbp5*) and carried out quantitative real-time PCR (qPCR) on these six differentially expressed transcripts (Figure 1A). With the exception of *Csf1*, the differences in gene expression shown by the microarray were confirmed for all of these genes.

In addition to the sixty-one genes that map to CRGN QTL regions, a large number of genes (739 transcripts) that were differentially expressed (<5% FDR) between WKY and LEW basal macrophages, do not map to previously localised CRGN QTLs. Although these genes are not primary positional candidates for the *Crgn3-7* QTLs, they may contribute indirectly to the effector pathways that lead to WKY susceptibility to CRGN. To separate these genes from the *Crgn3-7* positional candidates, we refer to these 739 transcripts as “secondary effector” genes. To validate these results, we selected a set of 18 of these genes for analysis by qPCR. Eight of the 18 genes (*Arg1*, *Ca5b*, *Ccl9*, *Igf2bp1*, *Lilrb3l*, *Mmp7*, *Pirb* and *Trpc6*) were selected because they were amongst the 19 genes that show profound differences in expression (>5 fold change, <1% FDR) between WKY and LEW basal macrophages, while the remaining ten (*Agmat*, *Ccl6*, *Ccrl2*, *Cd4*, *Cd55*, *Ifngr1*, *Il18*, *Il1rn*, *P2rx7*, and *Trf*) were selected based on literature searches, as having known or suspected links to glomerulonephritis. Consistent with the microarray results, qPCR showed that all eighteen genes were significantly differentially expressed between WKY and LEW macrophages and matched the directional change of the microarray data (Figure 1 B and C). Two of the genes *Agmat* and *Arg1* showed more than 50 fold greater expression by qPCR analysis in WKY compared to LEW. Conversely, three genes *Mmp7, Lilrb3l* and *Trpc6*

were expressed at over 20-fold higher levels in LEW compared to WKY macrophages. To ensure that the strong differential expression detected by qPCR for *Agmat, Arg1* and *Mmp7* (the three most strongly differentially expressed genes) is not an artefact due to genomic sequence variation, we sequenced the qPCR primer binding regions by capillary sequencing and found no SNPs in the WKY or LEW rat strains that were able to affect the binding properties of the primers used for qPCR amplification. Overall, therefore, we successfully validated 23 out of the 24 transcripts selected on the basis that they either i) map to *Crgn3-7*, ii) showed marked inter-strain differential expression, or iii) have previously reported links to glomerular inflammation.

Testing for influence of Crgn1 and Crgn2 on selected gene expression

To establish whether differential expression of these genes was under the genetic control of our previously identified QTL genes at *Crgn1* and Crgn2, we measured the expression of the 23 validated selected genes in a panel of congenic strains. The panel of congenic strains consisted of: congenic rats WKY.L*Crgn1* and WKY.L*Crgn2*, in which we previously introgressed the *Crgn1* or *Crgn2* QTL region from the resistant LEW strain into the WKY genetic background [8,10]; a reciprocal congenic strain on the Lewis background for *Crgn1* (LEW.W*Crgn1*); and a double congenic strain (WKY.L*Crgn1,2*) where both *Crgn* loci from the resistant LEW strain were introgressed into the WKY genetic background. The expression levels in the macrophages of the congenic animals matched those of the genetic background parental strain for all genes, indicating that they are not influenced in *trans* by genes encoded at *Crgn1* and/or *Crgn2* (Figure 2). Most significantly our data shows that none of the five validated positional candidate genes at *Crgn3-7* are influenced by *Crgn1* or *Crgn2*.

Pathway and functional annotation analysis

To characterise functionally the sets of genes that were differentially expressed between WKY and LEW macrophages, we performed pathway and functional annotation analysis using the DAVID Bioinformatics Resources [11,12]. We used the lists of 800 and 887 genes that were differentially expressed between WKY and LEW macrophages before and after LPS stimulation respectively (<5% FDR) and found five KEGG (Kyoto Encyclopedia of Genes and Genomes) or PANTHER (Protein ANalysis THrough Evolutionary Relationships) pathways that were enriched (EASE score < 0.01) in basal macrophages and four pathways that were enriched in LPS-stimulated macrophages (Table 4). These results suggest that differential gene expression between the two strains may be functionally related to altered endocytosis, regulation of the actin cytoskeleton, leukocyte transendothelial migration and Fc gamma receptor mediated phagocytosis. Furthermore, we identified 33 Biological Process annotation terms, that were significantly enriched (Benjamini correction < 0.05) in basal macrophages and five Biological Process annotation terms, that were significantly enriched in LPS-stimulated macrophages (Table 5). These results provide strong evidence that the differences in gene expression between WKY and LEW (before and after LPS stimulation) are likely to impact on regulation of macrophage activation, as the top functional annotation terms, in both basal and LPS-stimulated macrophages, are cell activation and leukocyte or lymphocyte activation.

Alternative splicing analysis between WKY and LEW macrophages

Affymetrix exon arrays allow the detection of splicing differences in two groups of data. We initially used the intersect of three commonly used alternative splicing detection algorithms (MiDAS, Rank Product of Slice Index and Alternative Splice ANOVA) and found 154 and 94 transcripts with isoform differences between WKY and LEW for basal or LPS-stimulated macrophages, respectively. However, capillary sequencing of the probe-set regions of 7 selected transcripts that showed evidence of alternative exon use (*Akr7a2, Coq10b, Cxcl10,*

Pa2g4, Pttg1ip, Reps2 and *Slfn3*), revealed that all 7 selected transcripts contained SNPs within the probe-binding sequence of the exon that was predicted to be alternatively spliced (Supplementary Table S3). This suggested that the set of transcripts showing apparent alternative splicing contained a high number of false positives, due to SNPs affecting the hybridisation properties of the corresponding probe-sets. We therefore carried out a more in depth analysis of the data at probe level, permitting us to remove SNP containing probe-sets, by using a filter that marks probe-sets as false positives when not all four probes within a probe-set are differentially expressed between the two rat strains. Our revised analysis identified a much smaller number of potentially alternatively spliced genes. Four transcripts (*Dnm3, Hes1, Ndrg4* and *Rtel1*) showed isoform differences between WKY and LEW macrophages in the basal state, and four transcripts (*Mical2, Polr1a, Sgms1* and *Ube3a*) showed isoform differences following LPS stimulation (Table 6). None of these transcripts show a single internal exon that is included or excluded in a strain specific pattern. Rather, the isoform differences that we detect concern differential expression of probe-sets mapping to the 5′ or 3′ UTR of a gene, or multiple consecutive exons of a transcript. To validate these data, we selected two transcripts (*Dmn3* and *Sgms1*), sequenced their differentially expressed exons and confirmed that they did not contain any SNPs. Next we used qPCR to test whether specific isoforms of the two selected transcripts are differentially expressed between WKY and LEW macrophages. We used pairs of primers to amplify the exons showing differential expression between strains (*Dnm3* exon 20, and *Sgms1* exon 10) and compared the results to a second control pair of primers designed to amplify exons showing similar expression levels between the two strains (*Dnm3* exons 15-16 and *Sgms1* exons 3-4). Our data confirms the microarray data predictions for i) *Dnm3* where differential expression between strains was found for exon 20, while no differential expression was detected for exons 15 and 16; and ii) *Sgms1* where differential expression between strains was found for exon 10, while no differential expression was detected for exons 3 and 4 (Figure 3). In summary, although our analysis has revealed a high number of polymorphisms between the two strains that account for the majority of transcripts showing apparent splicing differences, we also identified a small number of alternatively spliced transcripts that are differentially expressed between WKY and LEW macrophages.

Discussion

The aim of this study was to identify differentially expressed macrophage genes that reside on effector pathways for macrophage-mediated damage to glomeruli following CRGN induction in WKY rats. To achieve this aim, we compared gene expression in basal and LPS-stimulated bone marrow-derived macrophages between CRGN-susceptible WKY and CRGN-resistant LEW inbred rat strains using rat exon arrays that provide extensive coverage of the rat genome. We used LPS stimulation because LPS, an outer membrane component of Gram-negative bacteria, is widely used as an *in vitro* stimulus that mirrors pro-inflammatory macrophage activation. Our results reveal extensive strain differences in macrophage gene expression, with several genes that reside within *Crgn3-7* showing major expression differences, as well as a subset of genes that, although they map outside the known CRGN QTL regions, also show highly significant differential expression between WKY vs. LEW macrophages, before and after LPS stimulation.

We found 61 genes, differentially expressed between WKY and LEW in basal macrophages and 68 genes differentially expressed in LPS-stimulated macrophages, which map within our previously localised CRGN QTLs *Crgn3-7*. Most of these genes are novel candidates as they have not been previously identified to have a role in renal disease. We validated the microarray results by an independent assay, for five out of six selected genes (*Aig1, Epsti1, Mcoln3, Spata6* and *Stxbp5*). Analysis in congenic strains showed that none of these five genes appear to be influenced by *Crgn1* or *Crgn2*. Therefore, as these five genes both are

differentially expressed and map to a CRGN susceptibility locus, they can be considered as attractive differentially expressed positional candidate genes for glomerulonephritis susceptibility encoded at *Crgn3-7*. The potential role of these genes, *Aig1* (an androgen inducible gene), *Epsti1* (an epithelial-stromal interaction protein), *Mcoln3* (an inward rectifying cation channel), *Spata6* (a spermatogenesis associated gene) and *Stxbp5* (a syntaxin-binding protein implicated in the formation of complexes involved in neurotransmitter release), in macrophage function is currently unknown.

This study has identified a number of differentially expressed genes for *Crgn3-7*. However, it should be noted that one or more of these QTLs may account for genetic susceptibility mediated by intrinsic rather than circulating factors. In our recently published research aiming to dissect the contribution of *Crgn3-7* to susceptibility to CRGN in the WKY rat, we performed bone marrow transplantation experiments between congenic and parental rats [10]. Specifically, when Lewis bone marrow is transplanted to a WKY rat, this leads to an almost 90% protection in the glomerular crescent formation following glomerulonephritis induction. These results suggest that a residual 10 % of the CRGN susceptibility may be due to intrinsic cell activation in the glomerulus. As this current work focused on circulating cells, candidates for intrinsic factor CRGN susceptibility are not detectable via this screen.

We found 11 genes (*Arg1*, *Fcgr3-rs*, *Grit*, *Igf2bp1*, *IMAGE:5598800*, *Lilrb3l*, *Ly49si1*, *Ly49si2*, *Mmp7*, *Orl1684* and *Pirb*) that showed profound differences in expression between the two strains (>5-fold change, <1% FDR), for both basal and LPS-stimulated macrophages. Aside from *Fcgr3-rs*, the remaining 10 genes do not map to previously identified CRGN QTL regions, although they show significant differential expression both between WKY and LEW macrophages in the basal state and after LPS stimulation. These 10 genes may therefore play a significant role in differential regulation of macrophage activation between WKY and LEW rats and may have a secondary effector role in mediating CRGN susceptibility. Of these 10 genes, *Arg1, Mmp7* and *Pirb* have been identified in other studies as having potential roles in macrophage function or glomerulonephritis. Particularly, Arg1 competes for substrate with inducible nitric oxide synthase (iNOS) (encoded by *Nos2*) an enzyme that controls the production of nitric oxide (NO), and this can prevent NO-mediated apoptosis in activated macrophages [13],[14]. In addition to a primary role in macrophage function, *Arg1* has been implicated in having a role in glomerulonephritis, as up-regulation of this gene has previously been shown to occur in macrophages and glomeruli isolated from rats following induction of acute immune complex glomerulonephritis [15]-[18]. We found *Arg1* to be markedly over-expressed in WKY compared to LEW basal macrophages. *Mmp7* (also referred to as matrilysin) is a secreted protease, which is important for mediating proteolytic release of tumor nercrosis factor from macrophages [19] as well as the activation of anti-pathogenic and chemotactic proteins such as defensins (reviewed by Burke [20]). Macrophage-produced Mmp-7 has also been shown to be necessary for macrophage infiltration [21]. We found this gene to be highly under-expressed in WKY compared to LEW basal macrophages. Pirb (also known as Lilrb3) is a receptor expressed on immune cells where it binds to MHC class I molecules on antigen-presenting cells and transduces a negative signal that inhibits stimulation of an immune response (reviewed by Takai [22]). We found this gene to be down-regulated in WKY compared to LEW, and studies from Pirb-deficient mice have shown that its absence in macrophages causes increased macrophage infiltration and affects integrin signaling [23].

Both of our previously identified *Crgn1* and *Crgn2* genes [3];[8] were detected as significantly differentially expressed (<5% FDR) by this study. The molecular basis for *Crgn1* is a deletion within *Fcgr3-rs* which abolishes the expression of this gene in WKY macrophages. Such disruptions in gene expression are easily detected by microarrays and *Fcgr3-rs* was found to be amongst the 11 genes that showed profound differences in expression between

WKY and LEW (>5-fold change, <1% FDR), for both basal and LPS-stimulated macrophages. The molecular basis for *Crgn2* is over-expression of *JunD* in WKY rats caused at least in part by a promoter polymorphism. This gene was detected by the exon microarray as having modest (1.42 fold) yet highly significant ($p = 3.5e^{-4}$; < 5% FDR) differences in macrophages gene expression between the two strains. We find that the differences in expression for this gene are detected more easily when qPCR is used, where over 4 fold relative gene expression changes are detected between WKY and LEW basal macrophages [8]. Compared to quantitative PCR based methods microarrays may show a compression of measured fold changes [24], which could have led to missing subtle differences between transcripts if the data was filtered according to fold change. However, our data analysis is not based on fold change filtering, but based on use of statistical tests (ANOVA) together with false-discovery rate correction.

Our previous work has showed that WKY macrophages are primed for an inflammatory response and show a number of phenotypic differences relating to macrophage activation compared with those from LEW rats, including enhanced antibody-dependent cytotoxicity, Fc receptor-mediated phagocytosis and Fc-receptor-dependent oxidative burst, as well as increased inducible nitric oxide synthase gene (*Nos2*) expression upon lipopolysaccharide (LPS) stimulation [3,8]. Our functional annotation and pathway analysis supports these results, because the canonical pathways which associate with the inter-strain differential expression in basal and LPS-stimulated macrophages also relate to macrophage activation. For example, Fc receptor-mediated phagocytosis is widely used as one of the measures of macrophage activation. The canonical pathways which associate with the inter-strain differential expression in basal and LPS-stimulated macrophages are also connected. Specifically, phagocytosis is a specific form of endocytosis, while remodelling of the actin cytoskeleton is both an integral part of Fc gamma receptor-mediated phagocytosis [25], as well as being important for macrophage migration [26]. Dysregulation of Fc gamma receptors is known to associate with glomerulonephritis susceptibility both in WKY rats and in humans ([3,27,28]). Therefore, some of the differentially expressed genes that associate with the above connected pathways may represent novel targets for therapeutic intervention in nephritis.

RNA splicing is an important mechanism for generating transcriptional diversity and regulating gene expression. We used alternative splicing analysis to identify a total of 8 genes with alternatively spliced isoforms differentially expressed between WKY and LEW, in basal (*Dnm3, Hes1, Ndrg4* and *Rtel1*) or LPS-stimulated macrophages (*Mical2, Polr1a, Sgms1* and *Ube3a*). None of these genes map to our previously localised CRGN QTLs and their role in macrophage function is currently unknown. However, *Dnm3*, a dynamin GTPase, has been implicated in phagocytosis [29], while *Sgms1*, a key component of lipid rafts, is involved in T cell receptor signalling and T cell activation [30]. These genes may merit further investigation as regulators of activity in macrophages from the WKY or other rat strains. It is interesting that the inter-strain alternative splicing differences detected by this study were dependant on the activation state of the macrophages. Following our stringent alternative splicing detection analysis, the four differentially alternatively spliced transcripts in basal state did not show any inter-strain difference following the LPS treatment. This was also the case for the four differentially alternatively spliced transcripts in LPS-treated macrophages as the differences were no longer observed in basal conditions. Our results therefore suggest treatment-specific alternative splicing differences in rat BMDMs.

Our alternative splicing analysis also revealed that there are a high number of polymorphisms between the WKY and LEW strains, which affect the hybridisation properties of probe-sets and lead to the appearance in the microarray data of alternatively spliced transcripts. We resolved this difficulty by analysing the data at the probe, rather than

the conventionally used probe-set level, together with a filter that marks probe-sets as false positives when not all four probes within a probe-set are differentially expressed between the two rat strains. The problem of SNPs resulting in the appearance of false positive alternative splicing events has been noticed before [31,32]. In humans, data from the 1000 Genomes Project has allowed production of a list of all the SNP-containing probe-sets in the Affymetrix Gene Chip Human Exon 1.0 ST array, which can now be masked [33,33]. Similarly, full sequencing of the WKY and LEW genomes will be needed to identify definitively and exclude all SNP-containing probe-sets from the rat exon array.

In summary our findings reveal distinct macrophage transcriptome profiles between two rat strains that differ in susceptibility to glomerulonephritis. We have detected novel positional candidate genes for the CRGN susceptibility loci *Crgn3-7* and identified highly differentially expressed genes that may play a part in regulation of macrophage activity. Many of these genes have not previously been implicated in CRGN and represent novel candidate disease susceptibility genes and potential targets for therapeutic intervention in immunologically-mediated glomerulonephritis.

Materials and Methods

Animals

Wistar-Kyoto (WKY/NCrl, designated here as WKY) and Lewis (LEW/Crl, designated here as LEW) rats were purchased from Charles River (Margate, UK). All procedures were performed in accordance with the United Kingdom Animals (Scientific Procedures) Act.

Bone marrow collection and macrophage cell culture

Femurs from adult (8-10 weeks) WKY and Lewis rats were isolated and flushed with Hanks buffer (Invitrogen, Paisley, UK). Total BM-derived cells were plated and cultured for 6 days in DMEM (Invitrogen) containing 25 mM HEPES (Sigma, Dorset, UK), 25% L929 conditioned medium, 25% decomplemented FBS (Biosera, Ringmer, UK), penicillin (100 U/ml; Invitrogen), streptomycin (100 μg/ml; Invitrogen), and L-glutamine (2 mM; Invitrogen). These cells were characterized as macrophages by immunohistochemistry for CD68 (Supplementary Figure S1). BMDMs were cultured in serum/antibiotic-free HBSS (Invitrogen) for 16 hours. Next, cells were split into two groups of 1×10^6 cells each and cultured for a further 24 hours in the presence/absence of 10ng/ml lipopolysachharide (LPS) (Sigma). Cells were finally homogenized in TRIzol (Invitrogen) and stored at −70°C.

RNA Extraction, array hybridization and data analysis

Total RNA was extracted, labeled and hybridized to Gene Chip Rat Exon 1.0 ST Arrays (Affymetrix, Santa Clara, CA, USA) as previously described [34]. Four BMDM preparations, each generated from a single animal (biological replicates), were used for each condition. The microarray data is available in MIAME-compliant (minimum information about a microarray experiment) format at the ArrayExpress database (http://www.ebi.ac.uk/arrayexpress) [35] under accession code E-MEXP-2624. CEL intensity files were produced using GeneChip Operating Software version 1.4 (Affymetrix) and quality tested using the Affymetrix Expression Console. All 16 files were deemed suitable for further study. Probe-level data were normalized using Robust Multi-array Average (RMA) [36]. Detection of differential expression at the gene level was performed in Partek Genomics Suite 6.4 (Partek Incorporated, St.Louis, MO, USA) using transcripts with “extended” annotations. Data was summarized at the gene level using a One-Step Tukey’s Biweight Algorithm, which reduces the effect of outlier probe-sets (Statistical Algorithms Description Document; Affymetrix-White-Paper). An ANOVA model, using strain, condition and batch as co-factors, was used to generate raw p-values, while step-up FDR [37] was used for multiple test corrections.

Interval estimates of QTL location

To calculate 1.5-LOD support interval estimates, data from the original genome screen for NTN susceptibility loci in F2 rats [3] was re-analysed using the CRAN package R/qtlbim v1.9.4 [38].

Functional Association Analysis

Lists of differentially expressed genes were uploaded into The Database for Annotation, Visualization and Integrated Discovery v6.7 (DAVID; http://david.abcc.ncifcrf.gov/) [11,12] to determine differentially regulated pathways using the full rat genome as reference background. Data were analyzed with the "Functional Annotation Chart" tool using KEGG and PANTHER pathways or the Biological Process (BP) Gene Ontology (GO) FAT set term. Cut off criteria used were either a Benjamini multiple testing correction p-value less than 0.05, or an EASE score p-value of less than 0.01. EASE score is a modified Fisher's exact test [39], and it is the default test used by DAVID to identify statistical significance for functional association analysis.

Quantitative PCR

Primers used are listed in Supplementary Table S4. PCRs were performed with an ABI 7900 Sequence Detection System (Applied Biosystems, Warrington, UK). Macrophage quantitative real-time PCRs (qPCRs) were performed using a two-step protocol starting with cDNA synthesis using Cells-to-cDNA II (Applied Biosystems/Ambion, Austin, TX, USA), followed by PCR using SYBR Green Jumpstart Taq Ready mix (Sigma). A total of 10ng of cDNA per sample was used. After an initial denaturation step (94°C for 2min), samples were cycled 40 times at 94°C for 15 s, 60°C for 1 min and 72°C for 1min. All samples were amplified using an independent set of biological duplicates with three technical replicates per sample. The 7900 Fast system SDS software (Applied Biosystems) was used to obtain C_T values. Results were analysed using the comparative C_T method [40]. Each sample was normalized to the reference gene *Hprt*, to account for cDNA loading differences.

Alternative Splicing Analysis

The Robust Multi-array Analysis (RMA) [36] algorithm was used for probe-set (exon-level) intensity analysis. Detection of alternative splicing events was performed using three parallel approaches: 1) Microarray Detection of Alternative Splicing (MiDAS) (Alternative Transcript Analysis Methods for Exon Arrays; Affymetrix-White-Paper) was calculated using the Bioconductor v2.4 [41] package OneChannelGUI v1.4.5 [42]; 2) Rank Product of Splice Index [43] (100 permutations) was calculated also using OneChannelGUI v1.4.5 [42]; 3) An Alternative Splice ANOVA model was applied using Partek Genomics Suite v6.4 (Partek Incorporated) together with a filter to select for probe-sets showing significant alternative splicing score determined at a 5% False Discovery Rate (FDR) [37]. Next, transcripts predicted to be alternatively spliced using an intersect of the above three approaches, were filtered to remove data in which apparent alternative use of exons was most likely due to single nucleotide polymorphisms (SNPs) within the probe-binding regions. For this filter, data was analysed at the probe level, as follows: Affymetrix perfect match (pm) probe intensities were measured using Affymetrix Power Tools version apt-1.12.0 (http://www.affymetrix.com/partners_programs/programs/developer/tools/powertools.affx#1_1). The data obtained were imported on to R statistical tools version 2.11.0 and subjected to quantiles normalization using the Bioconductor package preprocessCore version 1.8 [44]. The normalized probe intensities were mapped to the Affymetrix transcript annotation (NetAffx release 28). Probes were median summarized for the four biological replicates and the respective medians from both strains were subtracted and squared. The values obtained were subjected to the empirical fluctuation test based on

moving sum estimate (MOSUM) implemented in R package strucchange version 1.4-0 [45]. P-values less than 0.1 suggested that a transcript contained SNPs and was a false positive for alternative splicing. Such transcripts were filtered out. Finally, all results were subjected to manual analysis using the Partek gene view plots as well as probe-level expression signal plots to remove false positives that had escaped the different analytical filtering steps.

Sequencing

WKY and LEW genomic DNA was isolated using a standard protocol. PCR was performed using KOD DNA polymerse (Merk Chemicals Ltd, Nottingham, UK) with primers listed in Supplementary Table S5. The amplified products were run on a 4% agarose gel. Bands were extracted and purified using a QIAquick Gel Extraction Kit (Qiagen, Crawley, UK). Samples were Cycle Sequenced using BigDye v3.1 (Applied Biosystems) chemistry with an ABI 3730 capillary- sequencer (Applied Biosystems). Alignment was performed in Sequencher v4.9 (Gene Codes Corporation, Ann Arbor, MI, USA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Kylie McDonald and Hetal Patel for technical support, and Dr Richard Hull for useful discussions. This work was supported by the 6th Framework Program of the European Union, EURATools (LSHG-CT-2005-019015), by an MRC intramural grant to Tim Aitman and by an Imperial College Junior Research Fellowship to Jacques Behmoaras.

Reference List

1. Segelmark M, Hellmark T. Autoimmune kidney diseases. Autoimmun Rev. 2010; 9:A366–A371. [PubMed: 19906361]
2. Tam FW, Smith J, Morel D, Karkar AM, Thompson EM, Cook HT, et al. Development of scarring and renal failure in a rat model of crescentic glomerulonephritis. Nephrol Dial Transplant. 1999; 14:1658–66. [PubMed: 10435873]
3. Aitman TJ, Dong R, Vyse TJ, Norsworthy PJ, Johnson MD, Smith J, et al. Copy number polymorphism in Fcgr3 predisposes to glomerulonephritis in rats and humans. Nature. 2006; 439:851–5. [PubMed: 16482158]
4. Isome M, Fujinaka H, Adhikary LP, Kovalenko P, El-Shemi AG, Yoshida Y, et al. Important role for macrophages in induction of crescentic anti-GBM glomerulonephritis in WKY rats. Nephrol Dial Transplant. 2004; 19:2997–3004. [PubMed: 15574997]
5. Hooke DH, Hancock WW, Gee DC, Kraft N, Atkins RC. Monoclonal antibody analysis of glomerular hypercellularity in human glomerulonephritis. Clin Nephrol. 1984; 22:163–8. [PubMed: 6391767]
6. Hooke DH, Gee DC, Atkins RC. Leukocyte analysis using monoclonal antibodies in human glomerulonephritis. Kidney Int. 1987; 31:964–72. [PubMed: 3495689]
7. Markovic-Lipkovski J, Muller CA, Risler T, Bohle A, Muller GA. Association of glomerular and interstitial mononuclear leukocytes with different forms of glomerulonephritis. Nephrol Dial Transplant. 1990; 5:10–7. [PubMed: 2109281]
8. Behmoaras J, Bhangal G, Smith J, McDonald K, Mutch B, Lai PC, et al. Jund is a determinant of macrophage activation and is associated with glomerulonephritis susceptibility. Nat Genet. 2008; 40:553–9. [PubMed: 18443593]
9. Smith J, Lai PC, Behmoaras J, Roufosse C, Bhangal G, McDaid JP, et al. Genes expressed by both mesangial cells and bone marrow-derived cells underlie genetic susceptibility to crescentic glomerulonephritis in the rat. J Am Soc Nephrol. 2007; 18:1816–23. [PubMed: 17475818]

10. Behmoaras J, Smith J, D'Souza Z, Bhangal G, Chawanasuntoropoj R, Tam FW, et al. Genetic Loci modulate macrophage activity and glomerular damage in experimental glomerulonephritis. J Am Soc Nephrol. 2010; 21:1136–44. [PubMed: 20488952]
11. Dennis G Jr. Sherman BT, Hosack DA, Yang J, Gao W, Lane HC, et al. DAVID: Database for Annotation, Visualization, and Integrated Discovery. Genome Biol. 2003; 4:3.
12. Huang, dW; Sherman, BT.; Lempicki, RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc. 2009; 4:44–57. [PubMed: 19131956]
13. Mori M. Regulation of nitric oxide synthesis and apoptosis by arginase and arginine recycling. J Nutr. 2007; 137:1616S–20S. [PubMed: 17513437]
14. Sonoki T, Nagasaki A, Gotoh T, Takiguchi M, Takeya M, Matsuzaki H, et al. Coinduction of nitric-oxide synthase and arginase I in cultured rat peritoneal macrophages and rat tissues in vivo by lipopolysaccharide. J Biol Chem. 1997; 272:3689–93. [PubMed: 9013624]
15. Jansen A, Lewis S, Cattell V, Cook HT. Arginase is a major pathway of L-arginine metabolism in nephritic glomeruli. Kidney Int. 1992; 42:1107–12. [PubMed: 1280702]
16. Cook HT, Jansen A, Lewis S, Largen P, O'Donnell M, Reaveley D, et al. Arginine metabolism in experimental glomerulonephritis: interaction between nitric oxide synthase and arginase. Am J Physiol. 1994; 267:F646–F653. [PubMed: 7524362]
17. Waddington SN, Mosley K, Cook HT, Tam FW, Cattell V. Arginase AI is upregulated in acute immune complex-induced inflammation. Biochem Biophys Res Commun. 1998; 247:84–7. [PubMed: 9636659]
18. Waddington SN, Tam FW, Cook HT, Cattell V. Arginase activity is modulated by IL-4 and HOArg in nephritic glomeruli and mesangial cells. Am J Physiol. 1998; 274:F473–F480. [PubMed: 9530263]
19. Gearing AJ, Beckett P, Christodoulou M, Churchill M, Clements JM, Crimmin M, et al. Matrix metalloproteinases and processing of pro-TNF-alpha. J Leukoc Biol. 1995; 57:774–7. [PubMed: 7759957]
20. Burke B. The role of matrix metalloproteinase 7 in innate immunity. Immunobiology. 2004; 209:51–6. [PubMed: 15481140]
21. Haro H, Crawford HC, Fingleton B, Shinomiya K, Spengler DM, Matrisian LM. Matrix metalloproteinase-7-dependent release of tumor necrosis factor-alpha in a model of herniated disc resorption. J Clin Invest. 2000; 105:143–50. [PubMed: 10642592]
22. Takai T. Paired immunoglobulin-like receptors and their MHC class I recognition. Immunology. 2005; 115:433–40. [PubMed: 16011512]
23. Pereira S, Zhang H, Takai T, Lowell CA. The inhibitory receptor PIR-B negatively regulates neutrophil and macrophage integrin signaling. J Immunol. 2004; 173:5757–65. [PubMed: 15494528]
24. Canales RD, Luo Y, Willey JC, Austermiller B, Barbacioru CC, Boysen C, et al. Evaluation of DNA microarray results with quantitative gene expression platforms. Nat Biotechnol. 2006; 24:1115–22. [PubMed: 16964225]
25. Coppolino MG, Krause M, Hagendorff P, Monner DA, Trimble W, Grinstein S, et al. Evidence for a molecular complex consisting of Fyb/SLAP, SLP-76, Nck, VASP and WASP that links the actin cytoskeleton to Fcgamma receptor signalling during phagocytosis. J Cell Sci. 2001; 114:4307–18. [PubMed: 11739662]
26. Jones GE. Cellular signaling in macrophage migration and chemotaxis. J Leukoc Biol. 2000; 68:593–602. [PubMed: 11073096]
27. Tarzi RM, Cook HT. Role of Fcgamma receptors in glomerulonephritis. Nephron Exp Nephrol. 2003; 95:e7–12. [PubMed: 14520009]
28. Kovalenko P, Fujinaka H, Yoshida Y, Kawamura H, Qu Z, El-Shemi AG, et al. Fc receptor-mediated accumulation of macrophages in crescentic glomerulonephritis induced by anti-glomerular basement membrane antibody administration in WKY rats. Int Immunol. 2004; 16:625–34. [PubMed: 15096492]
29. Otsuka A, Abe T, Watanabe M, Yagisawa H, Takei K, Yamada H. Dynamin 2 is required for actin assembly in phagocytosis in Sertoli cells. Biochem Biophys Res Commun. 2009; 378:478–82. [PubMed: 19032944]

30. Jin ZX, Huang CR, Dong L, Goda S, Kawanami T, Sawaki T, et al. Impaired TCR signaling through dysfunction of lipid rafts in sphingomyelin synthase 1 (SMS1)-knockdown T cells. Int Immunol. 2008; 20:1427–37. [PubMed: 18820264]
31. Benovoy D, Kwan T, Majewski J. Effect of polymorphisms within probe-target sequences on olignonucleotide microarray experiments. Nucleic Acids Res. 2008; 36:4417–23. [PubMed: 18596082]
32. Nurtdinov RN, Vasiliev MO, Ershova AS, Lossev IS, Karyagina AS. PLANdbAffy: probe-level annotation database for Affymetrix expression microarrays. Nucleic Acids Res. 2010; 38:D726–D730. [PubMed: 19906711]
33. Gamazon ER, Zhang W, Dolan ME, Cox NJ. Comprehensive survey of SNPs in the Affymetrix exon array using the 1000 Genomes dataset. PLoS One. 2010; 5:e9366. [PubMed: 20186275]
34. Gillett A, Maratou K, Fewings C, Harris RA, Jagodic M, Aitman T, et al. Alternative splicing and transcriptome profiling of experimental autoimmune encephalomyelitis using genome-wide exon arrays. PLoS One. 2009; 4:e7773. [PubMed: 19915720]
35. Parkinson H, Kapushesky M, Kolesnikov N, Rustici G, Shojatalab M, Abeygunawardena N, et al. ArrayExpress update--from an archive of functional genomics experiments to the atlas of gene expression. Nucleic Acids Res. 2009; 37:D868–D872. [PubMed: 19015125]
36. Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, et al. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics. 2003; 4:249–64. [PubMed: 12925520]
37. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful apprach to multiple testing. J Roy Statist Soc Ser B (Methodological). 1995; 57:289–300.
38. Yandell BS, Mehta T, Banerjee S, Shriner D, Venkataraman R, Moon JY, et al. R/qtlbim: QTL with Bayesian Interval Mapping in experimental crosses. Bioinformatics. 2007; 23:641–3. [PubMed: 17237038]
39. Hosack DA, Dennis G Jr. Sherman BT, Lane HC, Lempicki RA. Identifying biological themes within lists of genes with EASE. Genome Biol. 2003; 4:R70. [PubMed: 14519205]
40. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc. 2008; 3:1101–8. [PubMed: 18546601]
41. Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, et al. Bioconductor: open software development for computational biology and bioinformatics. Genome Biol. 2004; 5:R80. [PubMed: 15461798]
42. Sanges R, Cordero F, Calogero RA. oneChannelGUI: a graphical interface to Bioconductor tools, designed for life scientists who are not familiar with R language. Bioinformatics. 2007; 23:3406–8. [PubMed: 17875544]
43. Della BC, Cordero F, Calogero RA. Dissecting an alternative splicing analysis workflow for GeneChip Exon 1.0 ST Affymetrix arrays. BMC Genomics. 2008; 9:571. [PubMed: 19040723]
44. Bolstad BM, Irizarry RA, Astrand M, Speed TP. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics. 2003; 19:185–93. [PubMed: 12538238]
45. Zeileis A, Leisch F, Hornik K, Kleiber C. strucchange: An R Package for Testing for Structural Chang in Linear Regression Models. Journal of Statistical Software. 2002; 7:1–38.

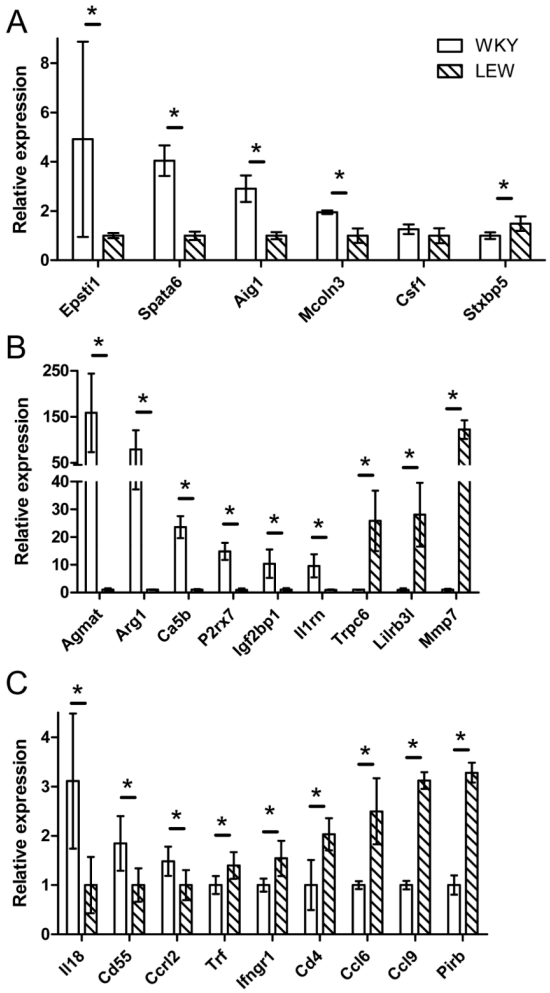

Figure 1. Validation of microarray data with quantitative PCR of selected differentially expressed genes

A) positional differentially expressed QTL candidate genes; B and C) secondary effector genes. Relative gene expression was compared to *Hprt* for WKY and LEW basal macrophages. The secondary effector genes are presented in two separate graphs (B, C) according to scale of relative gene expression. All samples were amplified using an independent set of biological duplicates with three technical replicates per sample. * = $p < 0.05$ using a Mann-Whitney non-parametric test (one-tailed). Error bars represent standard deviation.

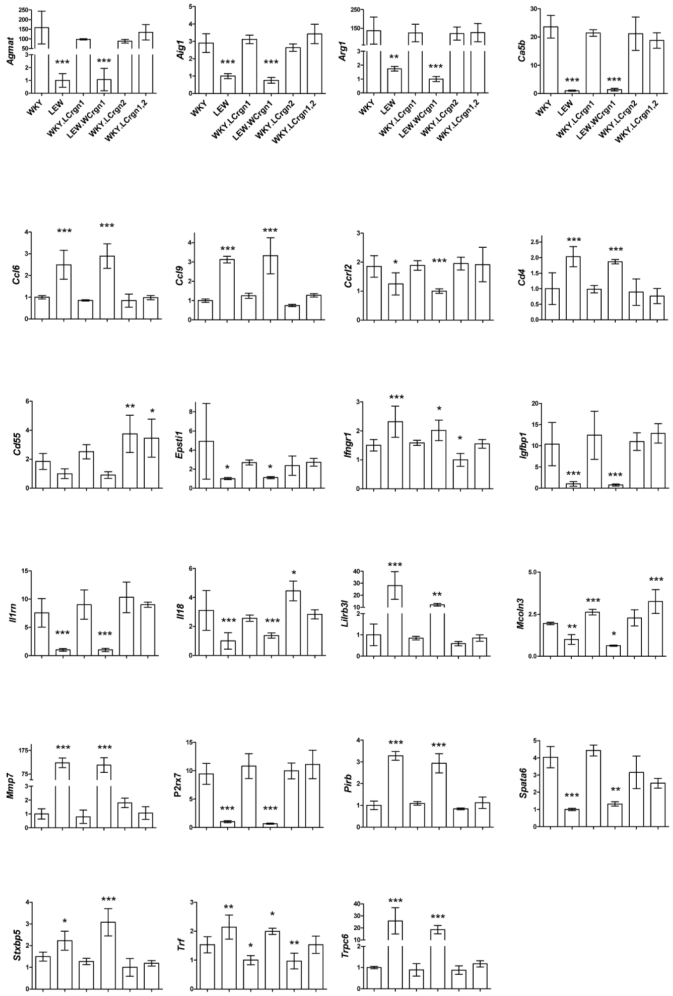

Figure 2. Selected gene expression in a panel of congenic strains

Relative gene expression compared to *Hprt* for WKY, LEW, WKY.L*Crgn1*, LEW.W*Crgn1*, WKY.L*Crgn2*, and WKY.L*Crgn1,2* in basal macrophages. The sample order shown in the top row of graphs is maintained for all subsequent graphs. All samples were amplified using an independent set of biological duplicates with three technical replicates per sample. * = $p < 0.05$ **P < 0.01, ***P < 0.001 statistically significantly different to WKY, using a one-way ANOVA with Bonferonni's correction. Error bars represent standard deviation.

Europe PMC Funders Author Manuscripts

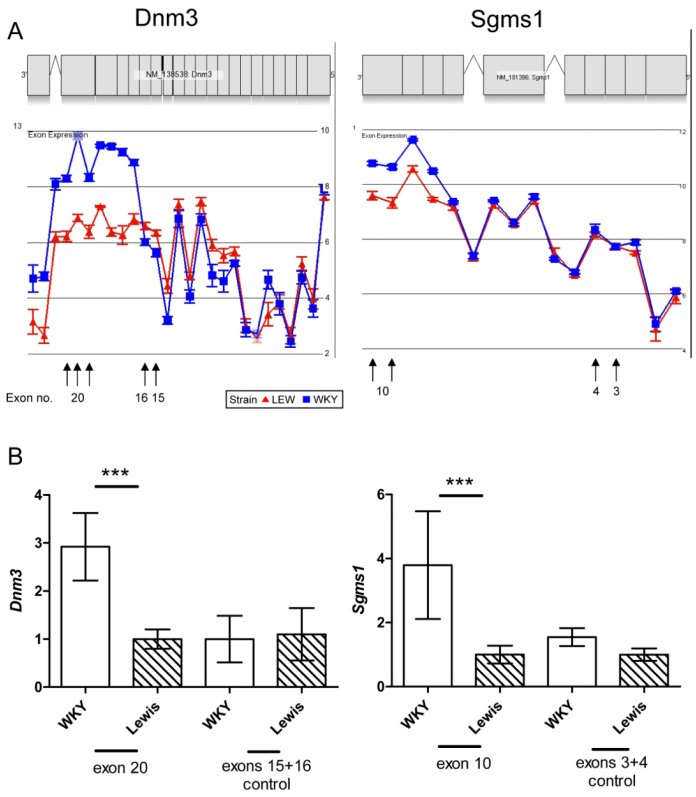

Figure 3. Comparison of alternative splicing microarray data predictions with quantitative PCR of selected transcript isoforms

A) Microarray data showing increased expression of probe-sets that correspond to *Dnm3* exons 17-21, in WKY basal macrophages and increased expression of probe-sets that correspond to *Sgms1* exons 8-10, for WKY LPS-stimulated macrophages. Data is illustrated as a gene view plot showing mean $\log_2$ signal probe-set intensities by strain. Error bars represent standard errors. B) qPCR data confirming the microarray predictions. Relative expression, compared to *Hprt*, for WKY and LEW basal macrophages of *Dnm3* exon 20 and control exons 15-16, together with WKY and LEW LPS-stimulated macrophages of *Sgms1* exon 10 and control exons 3-4. All samples were amplified using an independent set of biological duplicates with three technical replicates per sample. ***P < 0.001, using a one-way ANOVA with Bonferonni's correction. Error bars represent standard deviation.

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

Table 1

Microarray results of transcripts showing greater than 5-fold difference in expression between WKY and LEW, in basal macrophages, with <1% false discovery rate (FDR). Data are sorted according to fold change

Affymetrix transcript identity	Gene name	Gene Symbol	QTL position	p-value (LEW vs. WKY basal)	Fold-Change (LEW vs. WKY basal)
7113955	Fc receptor, IgG, low affinity III, related sequence	*Fcgr3-rs*	*Crgn1*	2.30E-07	52.4
7028278	Leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 3-like	*Lilrb3l*		6.91E-09	36.7
7332712	Matrix metallopeptidase 7	*Mmp7*		4.34E-06	12.1
7216236	Olfactory receptor 1684	*Olr1684*		1.64E-07	12.0
7047485	Paired-Ig-like receptor B	*Pirb*		3.49E-09	11.6
7332774	Transient receptor potential cation channel, subfamily C, member 6	*Trpc6*		4.35E-05	11.2
7317031	Nephroblastoma overexpressed gene	*Nov*		4.46E-09	10.2
7367852	Rattus norvegicus cDNA clone IMAGE:5598800 5-, mRNA sequence	IMAGE:5598800		1.97E-05	7.9
7147913	Ligase IV, DNA, ATP-dependent	*Lig4*		2.31E-07	6.0
7080736	Chemokine (C-C motif) ligand 9	*Ccl9*		1.04E-05	5.4
7294643	Pleckstrin homology domain containing, family H (with MyTH4 domain) member 2	*Plekhh2*		3.56E-06	−5.0
7037483	HtrA serine peptidase 1	*Htra1*		3.28E-06	−5.7
7270146	Immunoreceptor Ly49si2	*Ly49si2*		3.14E-06	−5.8
7334400	Rho GTPase-activating protein	*Grit/Rics*		1.39E-07	−6.8
7025757	Arginase 1, liver	*Arg1*		1.54E-05	−6.9
7080663	Schlafen 8	*Slfn8*		4.64E-06	−8.3
7374731	Carbonic anhydrase VB, mitochondrial	*Ca5b*		1.30E-07	−10.0
7081895	Insulin-like growth factor 2 mRNA binding protein 1	*Igf2bp1*		1.81E-08	−11.3
7270138	Immunoreceptor Ly49si1	*Ly49si1*		5.04E-08	−12.8

p-value - uncorrected ANOVA p-value; Fold-change - positive values show genes down-regulated in WKY, while negative values show genes up-regulated in WKY compared to LEW macrophages

Europe PMC Funders Author Manuscripts

Table 2

Microarray results of transcripts showing greater than 5-fold difference in expression between WKY and LEW, in LPS-stimulated macrophages, with <1% false discovery rate (FDR). Data are sorted according to fold change

Affymetrix transcript identity	Gene name	Gene Symbol	QTL position	p-value (LEW vs. WKY LPS)	Fold-Change (LEW vs. WKY LPS)
7113955	Fc receptor, IgG, low affinity III, related sequence	*Fcgr3-rs*	*Crgn1*	1.35E-07	40.58
7028278	Leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 3-like	*Lilrb3l*		2.44E-09	37.47
7332712	Matrix metallopeptidase 7	*Mmp7*		2.04E-07	29.46
7216236	Olfactory receptor 1684	*Olr1684*		2.92E-07	7.22
7047485	Paired-Ig-like receptor B	*Pirb*		9.15E-09	6.33
7367852	Rattus norvegicus cDNA clone IMAGE:5598800 5-, mRNA sequence	IMAGE:5598800		2.01E-05	5.97
7080742	Chemokine (C-C motif) ligand 6	*Ccl6*		1.88E-07	5.52
7361223	Similar to putative protein (5S487)	RGD1310819		2.34E-07	5.31
7334400	Rho GTPase-activating protein	*Grit/Rics*		1.23E-07	−5.39
7270146	Immunoreceptor Ly49si2	*Ly49si2*		1.60E-06	−5.40
7238766	Rho family GTPase 3	*Rnd3*		8.74E-07	−5.84
7132879	Stathmin-like 4	*Stmn4*	*Crgn7*	1.58E-07	−7.85
7270138	Immunoreceptor Ly49si1	*Ly49si1*		5.03E-08	−9.07
7025757	Arginase 1, liver	*Arg1*		7.09E-07	−14.09
7081895	Insulin-like growth factor 2 mRNA binding protein 1	*Igf2bp1*		5.65E-10	−31.52

p-value - uncorrected ANOVA p-value; LPS - lipopolysaccharide; Fold-change - positive values show genes down-regulated in WKY, while negative values show genes up-regulated in WKY compared to LEW macrophages

Europe PMC Funders Author Manuscripts

Table 3

Candidate genes for crescentic glomerulonephritis (CRGN) quantitative trait loci

QTL	Chr	Markers	Position (Mb)	Phenotypes	Candidate Genes Basal macrophages	Candidate Genes LPS stimulated macrophages
Crgn3	1	*D1Rat246 - D1Rat4*	2.4 - 11.86	Proteinuria	RGD1309759, *Map3k7ip2*, ***Stxbp5***, *Pex3*, ***Aig1***, *Gpr126*	*Map3k7ip2*, *Stxbp5*, RGD1564259, *Pex3*, *Aig1*, *Gpr126*
Crgn4	2	*D2Rat234-D2Rat70*	201.2 - 254.9	Crescents, Proteinuria	*Tmem77*, ***Csf1***, *Gstm1*, *Slc25a24*, *Ptbp2*, *Alpk1*, *Manba*, *Gbp2*, *Clca4*, ***Mcoln3***	*Tmem77*, *Kcna3*, *Csf1*, *Sars*, *Slc25a24*, *Slc44a3*, *Bcar3*, *Camk2d*, LOC691931, *Manba*, *Ppp3ca*, *Eif4e*, *Mcoln3*, *Mcoln2*
Crgn5	5	*D5Rat22 - D5Rat33*	102 - 143.5	Crescents	*Adfp*, *Cdkn2b*, *Cyp2j4*, *Atg4c*, *Jak1*, *Zcchc11*, *Kti12*, *Eps15*, ***Spata6***, *Cmpk1*, *Mmachc*, *Atp6v0b*, *Ipo13*, *Med8*, RGD1309802, *Zmpste24*, *Mycl1*	RGD1305797, *Cdkn2b*, *Cyp2j4*, *Atg4c*, *Jak1*, *Ak3l1*, *Zfyve9*, *Btf3l4*, *Kti12*, *Osbpl9*, *Spata6*, *Toe1*, *Plk3*, *Atp6v0b*, *Ipo13*, RGD1309802, *Cap1*
Crgn6	10	*D10Rat186-D10Rat45*	0 - 20.2	Proteinuria	RGD1563547, *Mgrn1*, *Zfp263*, *Thoc6*, LOC100158225, *Tsc2*, *Clcn7*, RGD1307381, *Tmem8*, *Dusp1*	*Abcc1*, *Rrn3*, RGD1306568, *Emp2*, *Crebbp*, *Tmem204*, RGD1307381, *Tmem8*, RGD1311343, *Dusp1*
Crgn7	15	*D15Rat54 - D15Rat96*	14.1 - 71.5	Proteinuria	*Flnb*, *Lrp10*, *Slc7a8*, *Zfhx2*, *Irf9*, *Kpna3*, *Mtmr9*, *Ephx2*, *Ptk2b*, *Trim35*, *Stmn4*, *Hr*, *Htr2a*, *Cog3*, *Slc25a30*, ***Epsti1***, *Elf1*, *Pcdh8*	*Flnb*, *Wdhd1*, *Slc7a7*, *Acin1*, *Slc7a8*, *Ripk3*, *Spata13*, *Kpna3*, *Mtmr9*, *Ccdc25*, *Ephx2*, *Trim35*, *Stmn4*, *Dpysl2*, *Hr*, *Dok2*, *Lcp1*, *Slc25a30*, *Nufip1*, *Epsti1*, *Diap3*

Genes highlighted in bold were tested by quantitative PCR. Chr - Chromosome

Europe PMC Funders Author Manuscripts

Table 4

Canonical pathways enriched for genes differentially expressed between Lewis and WKY macrophages, with EASE score values less than 0.01

Pathway database	Pathway	Genes (n)	p-value	%	Differentially expressed genes
Basal macrophages					
KEGG	rno04144:Endocytosis	22	6.89E-04	2.9	*Igf1r, Vps37c, Pip5k1b, Tfrc, Ap2m1, Git2, Cxcr4, Kit,Fam125a,Psd3, Grk6, Chmp1b, Pld1, RT1-CE10, Itch, Met, Agap3, Ret, Eps15, Erbb3, Ldlr, Rab31*
KEGG	rno04640:Hematopoietic cell lineage	12	1.25E-03	1.6	*Anpep, Tfrc, Cd55, Kit, Cd14, Itga1, Csf1, H2-Ea, Cd24, Cd44, Cd8b, Cd4*
KEGG	rno04810:Regulation of actin cytoskeleton	20	5.99E-03	2.6	*Itgal, Pip5k1b, Myh10, Baiap2, Ssh1, Arpc1b, Arpc3, Pxn, Enah, Cd14, Pdgfc, Itga1, Pak6, Slc9a1, Pik3cd, Pik3cg, Scin, Itgb8, Rac2, Arhgef12*
KEGG	rno04670:Leukocyte transendothelial migration	13	9.65E-03	1.7	*Itgal, Sipa1, Pxn, Cxcr4, Rassf5, Ptk2b, Ocln, Pik3cd, Pik3cg, Rac2, Icam1, Esam, Cybb*
KEGG	rno04666:Fc gamma R-mediated phagocytosis	11	9.90E-03	1.4	*Pip5k1b, Arpc1b, Arpc3, Pla2g4a, Fcgr2b III Fcgr3 III Fcgr3-rs, Prkcd, Pld1, Pik3cd, Pik3cg, Scin, Rac2*
LPS-stimulated macrophages					
KEGG	rno04666:Fc gamma R-mediated phagocytosis	15	1.98E-04	1.8	*Pak1, Pip5k1b, Arpc1b, Pla2g4a, Fcgr3-rs, Prkcd, Syk, Ppap2a, Myo10, Pld1, Gsn, Pik3cg, Asap1, Pla2g6, Was*
KEGG	rno04144:Endocytosis	22	2.20E-03	2.6	*Igf1r, Vps37c, Adrbk1, Pip5k1b, Arrb2, Cblb, Tfrc, Git2, Grk4, Fam125a, Chmp1b, Dab2, Pld1, RT1-N2, Psd4, Ehd4, Ret, Erbb3, Mdm2, Asap1, Rab31, Sh3kbp1*
PANTHER	P00016:Cytoskeletal regulation by Rho GTPase	15	5.57E-03	1.8	*Pak1, Myh10, Cdgap, Ssh1, Arpc1b, Stmn4, Diaph3, Tubb6, Diaph1, Tubb5, Pak6, Rics, Arhgef12, Was, Diaph2*
KEGG	rno04810:Regulation of actin cytoskeleton	21	7.50E-03	2.5	*Pak1, Actn4, Pip5k1b, Myh10,* LOC685269, *Ssh1, Arpc1b, Pxn, Vcl, Diaph3, Fgd3, Diaph1, Gsn, Pak6, Gng12, Slc9a1, Pik3cg, Golga4, Arhgef12, Was, Diaph2*

p value - modified Fisher's exact test (EASE score); % - percentage (differentially expressed genes/total genes in pathway) involved genes

Europe PMC Funders Author Manuscripts

Table 5

Enrichment of functional annotation terms using DAVID for genes differentially expressed between Lewis and WKY macrophages, with values less than 0.05 after Benjamini multiple testing correction

BP_FAT GO Term	Genes (n)	p-value	Benjamini
Basal macrophages			
GO:0001775~cell activation	29	6.13E-07	1.80E-03
GO:0045321~leukocyte activation	26	1.89E-06	2.78E-03
GO:0002250~adaptive immune response	14	2.07E-06	2.03E-03
GO:0002460~adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains	14	2.07E-06	2.03E-03
GO:0046649~lymphocyte activation	22	4.66E-06	3.43E-03
GO:0010033~response to organic substance	67	5.20E-06	3.06E-03
GO:0006955~immune response	38	6.04E-06	2.96E-03
GO:0043067~regulation of programmed cell death	52	2.13E-05	8.93E-03
GO:0010941~regulation of cell death	52	2.40E-05	8.78E-03
GO:0002684~positive regulation of immune system process	25	2.63E-05	8.56E-03
GO:0002443~leukocyte mediated immunity	13	4.64E-05	1.36E-02
GO:0051338~regulation of transferase activity	28	9.21E-05	2.44E-02
GO:0001666~response to hypoxia	21	1.03E-04	2.50E-02
GO:0043549~regulation of kinase activity	27	1.05E-04	2.35E-02
GO:0030155~regulation of cell adhesion	16	1.06E-04	2.20E-02
GO:0045859~regulation of protein kinase activity	26	1.19E-04	2.31E-02
GO:0042981~regulation of apoptosis	49	1.22E-04	2.21E-02
GO:0001816~cytokine production	10	1.39E-04	2.37E-02
GO:0048871~multicellular organismal homeostasis	13	1.44E-04	2.32E-02
GO:0042127~regulation of cell proliferation	48	1.55E-04	2.38E-02
GO:0002449~lymphocyte mediated immunity	11	1.61E-04	2.34E-02
GO:0042110~T cell activation	15	1.79E-04	2.48E-02
GO:0051174~regulation of phosphorus metabolic process	34	1.85E-04	2.44E-02
GO:0019220~regulation of phosphate metabolic process	34	1.85E-04	2.44E-02
GO:0050778~positive regulation of immune response	17	2.00E-04	2.52E-02
GO:0070482~response to oxygen levels	21	2.37E-04	2.86E-02
GO:0009611~response to wounding	34	2.78E-04	3.22E-02
GO:0051347~positive regulation of transferase activity	21	3.27E-04	3.64E-02
GO:0002252~immune effector process	15	4.03E-04	4.30E-02
GO:0042592~homeostatic process	49	4.25E-04	4.37E-02
GO:0042325~regulation of phosphorylation	32	4.42E-04	4.39E-02
GO:0033674~positive regulation of kinase activity	20	5.00E-04	4.79E-02
GO:0048584~positive regulation of response to stimulus	22	5.27E-04	4.89E-02
LPS stimulated macrophages			
GO:0001775~cell activation	30	2.01E-06	5.87E-03
GO:0046649~lymphocyte activation	23	8.36E-06	1.21E-02

BP_FAT GO Term	Genes (n)	p-value	Benjamini
GO:0045321~leukocyte activation	26	1.46E-05	1.41E-02
GO:0048872~homeostasis of number of cells	17	2.15E-05	1.56E-02
GO:0006955~immune response	38	7.69E-05	4.40E-02

BP_FAT - subset of Biological Process Gene Ontology (GO) terms, generated by the DAVID team. This subset is missing the broadest GO terms for ease of data interpretation; p value - modified Fisher's exact test (EASE score); Benjamini - Benjamini multiple testing correction value.

Europe PMC Funders Author Manuscripts

Table 6

Microarray results of transcripts showing showing isoform differences between WKY and LEW, in basal or LPS-stimulated macrophages

Gene Symbol	Ensembl ID	Transcript ID	Probe-set ID	p-value	Fold-Change (LEW vs. WKY)	Differential exon
Basal macrophages						
Hes1	ENSRNOT00000002346	7094386	6372610	1.52E-03	1.61	1
Dnm3	ENSRNOT00000067653	7113169	6694062	1.69E-04	−7.83	18
Dnm3	ENSRNOT00000067653	7113169	6589493	1.98E-08	−4.57	19
Dnm3	ENSRNOT00000067653	7113169	5968420	1.52E-06	−8.40	19
Dnm3	ENSRNOT00000067653	7113169	5697034	4.25E-04	−3.87	20
Dnm3	ENSRNOT00000067653	7113169	5799664	4.59E-06	−7.75	20
Ndrg4	ENSRNOT00000017413	7182656	6239394	6.97E-04	2.94	11
Ndrg4	ENSRNOT00000017413	7182656	6202670	1.03E-03	4.75	13
Rtel1	ENSRNOT00000055030	7236395	5939980	2.49E-05	2.16	novel 5′utr
Rtel1	ENSRNOT00000055030	7236395	5780635	7.71E-04	2.96	1
Rtel1	ENSRNOT00000055030	7236395	6399147	7.43E-06	4.78	1
Rtel1	ENSRNOT00000055030	7236395	6522856	1.64E-04	2.33	2
LPS macrophages						
Ube3a	ENSRNOT00000021366	7032220	5976529	2.17E-04	2.30	11
Ube3a	ENSRNOT00000021366	7032220	6269903	5.78E-05	12.24	novel extention of 11
Mical2	ENSRNOT00000021858	7035525	6277897	2.99E-03	−1.93	8
Mical2	ENSRNOT00000021858	7035525	5940132	5.82E-04	−3.07	9
Mical2	ENSRNOT00000021858	7035525	6161609	2.91E-04	−3.08	novel 3′ UTR
Sgms1	ENSRNOT00000054761	7061894	6030014	9.26E-06	−2.03	8
Sgms1	ENSRNOT00000054761	7061894	5676967	1.03E-04	−2.10	9
Sgms1	ENSRNOT00000054761	7061894	6269809	4.10E-04	−2.25	10
Sgms1	ENSRNOT00000054761	7061894	6528432	7.29E-04	−2.47	10
Polr1a	ENSRNOT00000013587	7254811	6282280	4.42E-04	−1.62	31
Polr1a	ENSRNOT00000013587	7254811	6206810	3.05E-05	−1.70	32
Polr1a	ENSRNOT00000013587	7254811	6591259	3.45E-05	−1.85	33

ID - identity; p-value - ANOVA p-value; Fold-change - positive values show exons down-regulated in WKY, while negative values show exons up-regulated in WKY compared to LEW macrophages; Differential Exon - Exon differentially expressed between strains. No other exons in each gene showed statistically significant differences between WKY and LEW.