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Genetic control of the innate immune response

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Abstract

Background: Susceptibility to infectious diseases is directed, in part, by the interaction between the invading pathogen and host macrophages. This study examines the influence of genetic background on host-pathogen interactions, by assessing the transcriptional responses of macrophages from five inbred mouse strains to lipopolysaccharide (LPS), a major determinant of responses to gram-negative microorganisms.

Results: The mouse strains examined varied greatly in the number, amplitude and rate of induction of genes expressed in response to LPS. The response was attenuated in the C3H/HeJ*lps*^{d} strain, which has a mutation in the LPS receptor Toll-like receptor 4 (TLR4). Variation between mouse strains allowed clustering into early (C57Bl/6J and DBA/2J) and delayed (BALB/c and C3H/ARC) transcriptional phenotypes. There was no clear correlation between gene induction patterns and variation at the *Bcg* locus (Slc11A1) or propensity to bias Th1 versus Th2 T cell activation responses.

Conclusion: Macrophages from each strain responded to LPS with unique gene expression profiles. The variation apparent between genetic backgrounds provides insights into the breadth of possible inflammatory responses, and paradoxically, this divergence was used to identify a common transcriptional program that responds to TLR4 signalling, irrespective of genetic background. Our data indicates that many additional genetic loci control the nature and the extent of transcriptional responses promoted by a single pathogen-associated molecular pattern (PAMP), such as LPS.

Background

Susceptibility to infection is determined by the nature of the pathogen, and by the fitness of an individual to respond appropriately. The nature of the host response is controlled in part by the appropriate recognition of PAMPs by cells of the innate immune system [1,2]. Ineffective PAMP recognition, or an inappropriate response underlies clinical complications such as circulating bacterial load or septic shock. Lipopolysaccharide (LPS), a component of bacterial cell walls, is the predominant trigger of adverse clinical consequences of infection with

gram-negative bacteria, including host procoagulant response and septic shock [3].

Susceptibility to gram-negative bacteria in human populations has been associated with allelic variation at *TLR4* [4,5], with consequences for infectious, inflammatory and cardiovascular disease [6]. Yet the complexity of the innate immune response to PAMPs constrains large-scale experimental analysis in outbred human populations. Microarray experiments comparing PAMPs from different organisms on human peripheral blood monocytes have shown a remarkably stereotyped response, even between different TLR ligands [7–9]. By contrast, LPS responses studied by high-density microarray in the mouse transformed monocyte line RAW264 establish that in a controlled cell culture system there is transcriptional diversity between different TLR agonists [10], and that even this controlled cell culture system demonstrates heterogeneity of response to a single PAMP [11].

As a model for human variation, inbred mouse strains have been central to our understanding of the function of innate and acquired immune systems. Individual strains have differing susceptibilities to disease onset and pathology in a wide number of experimental disease models. In this study we have chosen to use five inbred mouse strains, with a spectrum of LPS susceptibility as genetic tools to determine the nature and diversity of innate immune responses to a single pathogenic stimulus. The strains chosen (C57Bl/6J, DBA2, BALB/c, C3H/ARC*lps*^{n} and C3H/He*Jlps*^{d}) differ on at least two genetic loci known to influence innate immune responses. For example, polymorphism in Slc11A1 at the *Bcg* (also known as *Ity* or *Lsh*) locus controls susceptibility to intracellular pathogens. C57Bl/6J and BALB/c carry the susceptible allele (*Bcg*^{s}), a G169D substitution in the predicted TM4 of the Slc11A1 protein [12], whereas DBA2 and C3H substrains carry the resistant allele (*Bcg*^{r}). Slc11A1 variants exert pleiotropic effects on LPS-inducible cytokine gene expression [13]. The LPS-hypo responsive strain C3H/He*Jlps*^{d} has a P712H substitution in the cytoplasmic Toll-IL1 receptor (TIR) domain of TLR4, which prevents activation of the Myd88 signalling cascade shared with many other Toll-like receptors [14,15]. The comparison of C3H/He*Jlps*^{d} with the near-isogenic line C3H/ARC*lps*^{n}, which has functional TLR4, provides an important control for specificity of TLR4 signalling, since LPS contaminants may signal via TLR2 [16].

In this study we show that macrophages from each strain display an idiosyncratic gene expression profile upon LPS activation, indicating that loci other than *Tlr4* profoundly affect LPS responsiveness. We have also identified a core set of genes that respond to LPS in a TLR4-dependent fashion, regardless of genetic background. This set describes a conserved transcriptional program underlying inflammatory responses to LPS.

Results and discussion

To determine the effect of genetic background on macrophage responses to LPS, we exposed primary populations of bone marrow-derived macrophages (BMM) from each mouse strain to LPS over a 21 h time course. For the purposes of cDNA microarray analysis, this system had the additional advantage that pure populations of cells undergo large, relatively synchronous, and reproducible changes in gene expression. We have previously shown that macrophages derived from the bone marrow of three of these strains cluster as a single tissue subtype when compared to 49 other mouse and embryonic tissues. In this system, C3H/He*Jlps*^{d} mice do not express inflammatory transcripts in response to our LPS preparation, and cluster on a separate branch of the macrophage lineage tree to activated LPS-responsive strains [17].

BMM were stimulated in the presence of macrophage growth factor, CSF-1, which has been shown to amplify induction of key pro-inflammatory cytokines by LPS [18]. The LPS concentration (10ng/ml) is saturating, and the times chosen (30 min, 2, 7, 21 hours) distinguish temporal classes of inducible genes in a sequential cascade of gene activation. We interrogated the 19,000 element arrays arising from the RIKEN mouse gene encyclopaedia project [19], using 17.5dpc C57Bl/6J embryo RNA as a reference for each point of the time series.

LPS stimulation of macrophages elicits global transcriptional changes

Analysis was restricted to elements that were detected in every hybridisation on the reference channel. This filter provided confidence that variation in expression between time points or mouse strains was not due to spot artefacts, and although this eliminated some genes that are expressed in macrophages but undetectable in the reference embryo RNA, it greatly increased the reproducibility of the data. 3612 array elements passed filters for reproducibility, signal detection 2 fold above background and reliability of reference signal across all of the hybridisations. The normalized distribution of cDNA signal intensities is displayed for each time point and each mouse strain in Fig 1. Each element was coloured with respect to the unstimulated C57Bl/6J time point to illustrate the rapid changes in gene expression upon LPS activation. Across the C57Bl6J time course alone, 918 (25%) of genes expressed at the median level (yellow) or below the median (blue) at time zero were substantially induced by LPS, and 696 (19%) of genes expressed at the median level (yellow) or above (red) were repressed at some time point, using a 2 fold cut-off from the median. Table 1 lists the numbers of elements that were induced or repressed

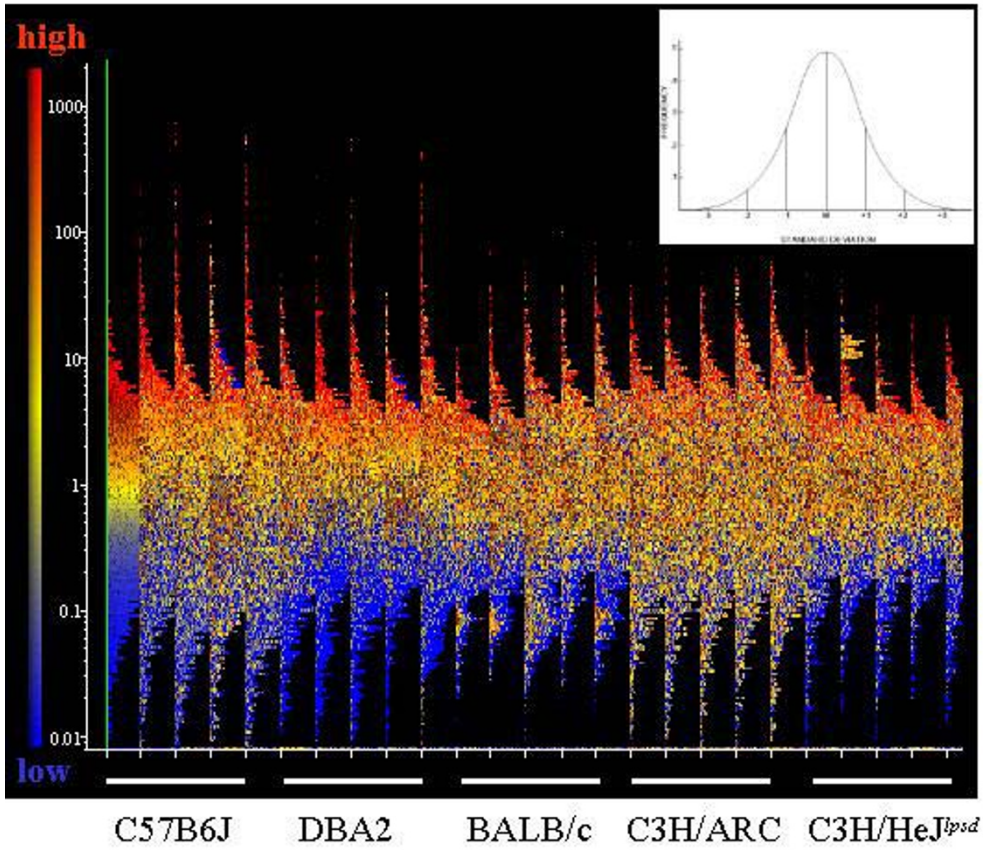

Figure 1

Temporal distribution of the entire 19,000 RIKEN cDNA dataset. Figure 1 describes the distribution of expression of 19,000 RIKEN full-length cDNAs across a 21-hour time course for each of 5 mouse strains, C57Bl/6J, DBA2, BALB/c, C3H/Arc and C3H/HeJ*lps*^{d}. A normal distribution plot allows standard deviations (SD) from the mean of the population to be calculated (inset). The graph describes a series of overlaid normal distribution plots; each time point is plotted as a normal distribution of signal intensities on the y-axis, frequency on the x-axis, with a log median intensity of 1 across the dataset. Each element is coloured with respect to its expression in the unstimulated C57Bl/6J time point, where blue is expressed at least 2SD below the median, yellow is considered within 2SD of the median and red is at least 2SD above the median.

Table 1: The number of probes on the 19 K RIKEN array that were 2 fold induced or repressed compared to the unstimulated time point, after the addition of LPS. The data is also expressed as a percentage (%) of the 3612 genes that were reliably expressed across the entire experimental series.

STRAIN	Induced	Repressed
C57Bl/6J	918 (25%)	696 (19%)
DBA/2J	438 (12%)	1275 (35%)
C3H/ARC	619 (17%)	409 (11%)
BALB/c	563 (15%)	791 (21%)
C3H/HeJ	779 (21%)	200 (5%)

in any given activated macrophage population, and shows that this global pattern was recapitulated for each of the LPS responsive mouse strains. Each strain presented an idiosyncratic gene expression profile in response to LPS. Only 11% of elements (415/3612) were co-regulated in all 4 responder mouse strains. This variation in global gene induction between the mouse strains is obvious in Fig. 1, particularly in the C57Bl/6J and C3H/ARC comparison.

Identification of a transcriptional program describing the core Tlr-4 dependent pathway

415 elements were identified (Table 1 Additional file: 1) as inducible in a temporally regulated manner in all four of the LPS responsive strains, but were not regulated by LPS in C3H/HeJ*Lps*^{d} mice. This set represents candidate targets specifically of the TLR4/MyD88/NFk B regulated signalling pathway, as the TLR4 mutation carried by these mice is known to prevent NF-Kappa B activation in response to LPS [14,16,20]. Hierarchical clustering of the core inflammatory set (Fig. 2) illustrates temporal conservation of gene induction between the responder mouse strains for most, but not all clusters of genes. Notably, DBA2 and C57Bl6J shared the most similar temporal profiles, whereas BALB/c showed a universal delay in induction of this set. Due to redundancy in the RIKEN set these 415 elements compress to 383 unique transcripts, and approximately 40% of these (146 transcripts) are uncharacterised. 237 genes were annotated as part of the following cellular processes (Fig. 3): cytoskeleton (43 genes); growth and differentiation (35 genes); phagocytosis (33 genes); cell signalling pathways (32 genes); transcription (26 genes); cytokine or chemokine (19 genes); oxidative stress (14 genes); apoptosis/anti-apoptosis (13 genes); antigen presentation (12 genes) and lipid metabolism (9 genes). All describe inducible processes known to accompany macrophage activation, but this is the first and most comprehensive assembly of the individual parts of each process in macrophage activation by LPS. The largest proportion (32%) of known genes that were induced upon LPS activation were cytoskeletal components or components of the phagosome, which correlates with the dramatic morphological changes that occur in BMM upon activation with LPS [21]. This dataset provides new candidates for the still poorly understood processes involved in changes to secretory machinery induced by activation of macrophages and dendritic cells [22].

Impact of LPS on proliferation and differentiation transcriptional programs

Macrophages were differentiated from bone marrow progenitors *in vitro* in the presence of CSF-1. Many *in vitro* studies that characterise immunological responses of macrophages are performed in the absence of CSF-1. CSF-1 is, however, constitutively present *in vivo*, and is further induced upon activation with LPS. CSF-1 has itself been shown to enhance the activation of some genes by LPS [18]. *In vitro* studies aimed at assessing macrophage activation should therefore be performed in the presence of CSF-1.

LPS has been shown to down-modulate the CSF-1 receptor, leading to growth arrest, but at the same time providing a signal favouring cell survival [18]. We looked for transcriptional evidence of this LPS modulated survival pathway in the set of transcripts conserved between the LPS-responsive mouse strains. 30 elements corresponding to 22 genes with roles in regulating growth, cell cycle and differentiation were identified as part of the conserved TLR4 dependent transcriptional pathway, and these clustered together in the earliest temporal group, induced within 30 mins (immediate – early) of LPS exposure. Amongst this set were known targets of NF-κB in human and mouse monocytes, including the anti-apoptotic genes BCL2-related protein A1 (BFL-1) [23], immediate-early gene 3 [24], Gadd45β [25,26] and myeloid cell leukaemia [27] which have been reported as mediators of cell survival upon an inflammatory stimulus. Haematopoietic growth and differentiation factors, including CSF-1, granule cell differentiation protein (gdf3), the myeloid oncogene PIM1, glia maturation factor (gmfg) and the interferon-inducible stem cell marker Ly6e were also actively induced by LPS as part of the core TLR4-mediated transcriptional program. The inclusion of fibroblast growth factor 1 (fgf1) and its intracellular binding protein (fgfbp) in this early cluster suggests a novel role for basic fibroblast growth factor in LPS mediated macrophage growth and survival. We observed little regulation of cell cycle genes early in the time course, however these were clustered together and were induced in the late temporal group. Potentially pro-apoptotic transcripts were also present in this set – 5 elements corresponding to 4 genes, although these did not cluster together. These were the Mitochondrial ribosomal protein S30 (induced late in the time course), Caspase 7, a downstream target of LPS-inducible capsase11 (induced 7 hours post-LPS), and Fas antigen (induced 2 hours post LPS-induction). Alix, (ALG-2-interacting protein X), was initially repressed at 2 hrs post-LPS, but induced by the 21 hr time point. The later induction of these elements suggests that they are induced by secondary events in the inflammatory cascade. These data are consistent with LPS activating a survival pathway in macrophages, even in the presence of CSF-1, through TLR4 signalling that is fundamentally conserved between different genetic backgrounds.

Temporal modulation of the inflammatory cascade

The examination of the gene expression profile with time provided information about clusters of genes with similar regulatory patterns and inferred functions. Figure 4 shows

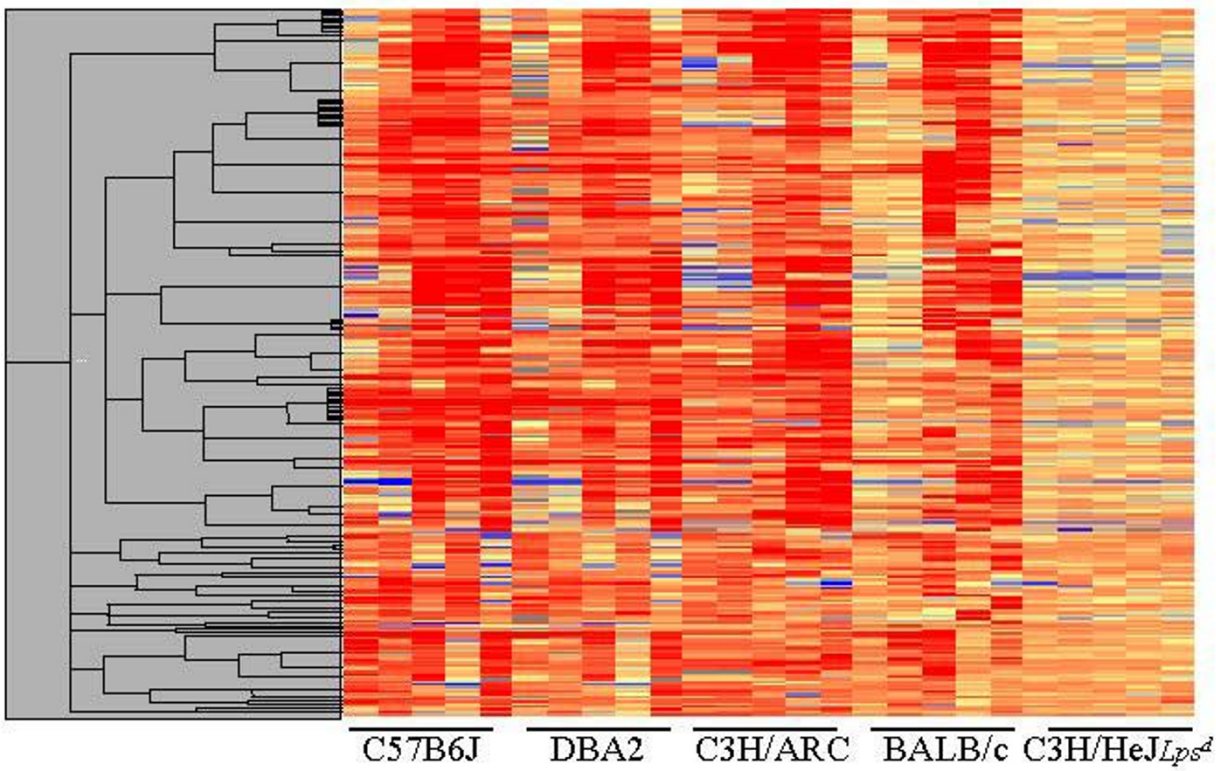

Figure 2
Hierarchical clustering of the core transcriptional program. Unsupervised hierarchical clustering of 415 elements that were determined to be co-induced across the time course in all responder mouse strains tested, but not in the Tlr4-null mice C3H/HeJ*lps*^{d}. The temporal samples (from left to right 0, 0.5, 2, 7, 21 h) for each strain are shown as columns across the X-axis, and each row on the Y-axis represents a single cDNA. The colour indicates expression levels of the cDNAs, where blue is low expression, red is high expression, and yellow is the same expression relative to the embryo control.

the principal component analysis for the core data set and summarizes the transcriptional program underlying the core inflammatory processes shared by the LPS responsive strains, further illustrated in fig. 5. The PCA analysis segregated genes induced within 30 mins (immediate-early), induced within 2 hours (early), induction peaking by 7 hrs (mid), and induction continuing across the time-course (late). The earliest sets of genes, induced by 30 mins, activated protective mechanisms against oxidative challenge, survival/anti-apoptosis signals and cellular adhesion molecules involved in macrophage mobilization. Inflammatory mediators such as the cytokines IL-1α, IL-18, components of the complement system such as H2-Bf, and chemoattractants such as galectin-9 [28] were induced in the early and mid clusters (peaking between 2 and 7 hours post-LPS). A large cluster of elements, including arginase 1 and 2, the transcription factor Hif-1α and lysosomal enzymes cathepsin C, D, L and Z were induced rapidly, and their transcription remained elevated even at 21 hours. This temporal cluster provided the most information on pathways that feed-back into the inflammatory cascade. Previous studies have indicated that LPS induces a complex transcriptional regulatory cascade leading to an adapted transcriptional steady state that depends upon continuous LPS exposure [29]. The cluster of elements expressed highly at 21 hours

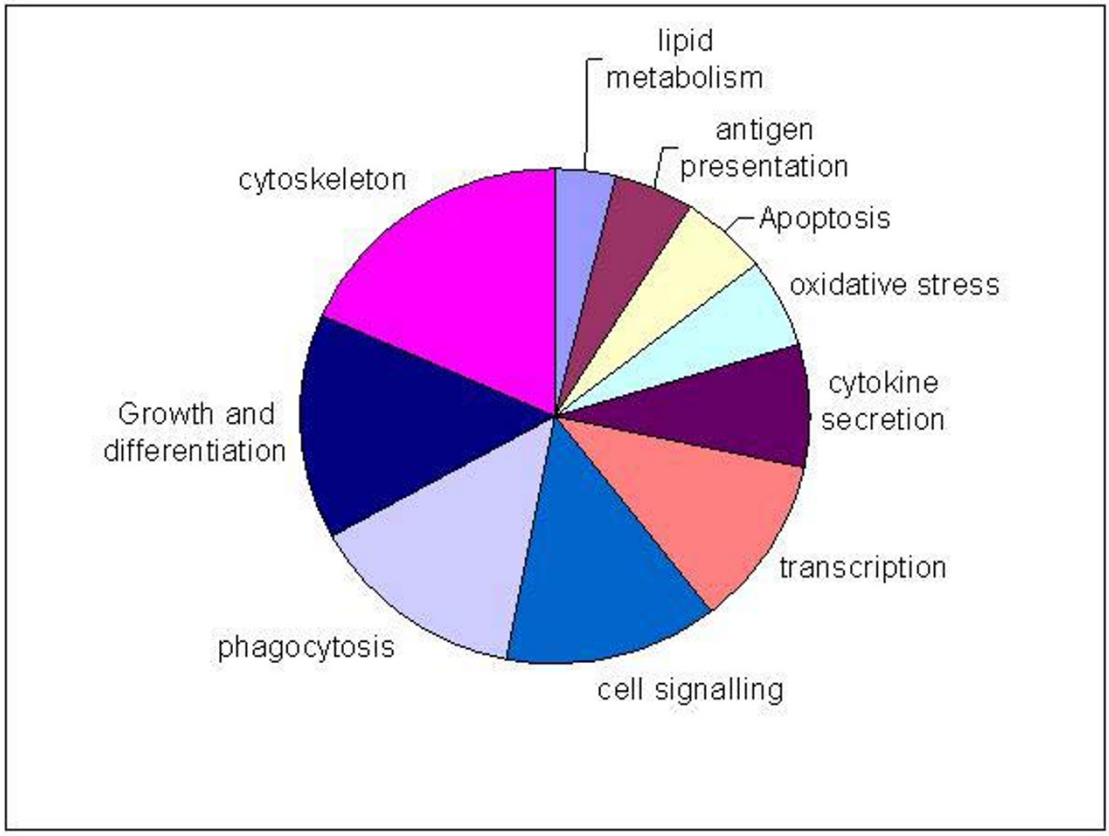

Figure 3
Functional classification of the core transcriptional program The core inflammatory program consisted of 383 unique transcripts including 146 with no known function. 237 known genes were annotated using GO terms, and the results displayed as a pie graph. Genes were classified into the following groups: cytoskeleton (pink, 43 genes); growth and differentiation (navy, 35 genes); phagocytosis (lavender, 33 genes); cell signalling pathways (blue, 32 genes); transcription (orange, 26 genes); cytokine or chemokine (aubergine, 19 genes); oxidative stress (aqua, 14 genes); apoptosis/ anti-apoptosis (yellow, 13 genes); antigen presentation (maroon, 12 genes) and lipid metabolism (pale blue, 9 genes).

demonstrated the continuing amplification of the inflammatory cascade through continual exposure to LPS, and through induction of secondary autocrine factors such as TNF-alpha and nitric oxide (NO). Arginase is induced by nitric oxide [30], and acts as a negative regulator of NO synthesis by competing with inducible nitric oxide synthetase for substrate. The induction of both positive and negative regulators of inflammation demonstrated the dynamic nature of the transcriptional cascade.

Mapping inducible transcripts to genomic clusters

LPS stimulation of the macrophages in all strains produced a very marked induction of genes with in 30 minutes of exposure. The induction of these transcripts in the LPS^d strain and the absence of obvious NF-k B transcription factor sites in a substantial proportion of the promoters examined (Matt Takovic, personal communication) makes it unlikely that this early gene induction phenomenon is specifically directed, but rather may be due to transitory changes in chromatin structure [31], RNA transport [32] or stability of inducible messages [33].

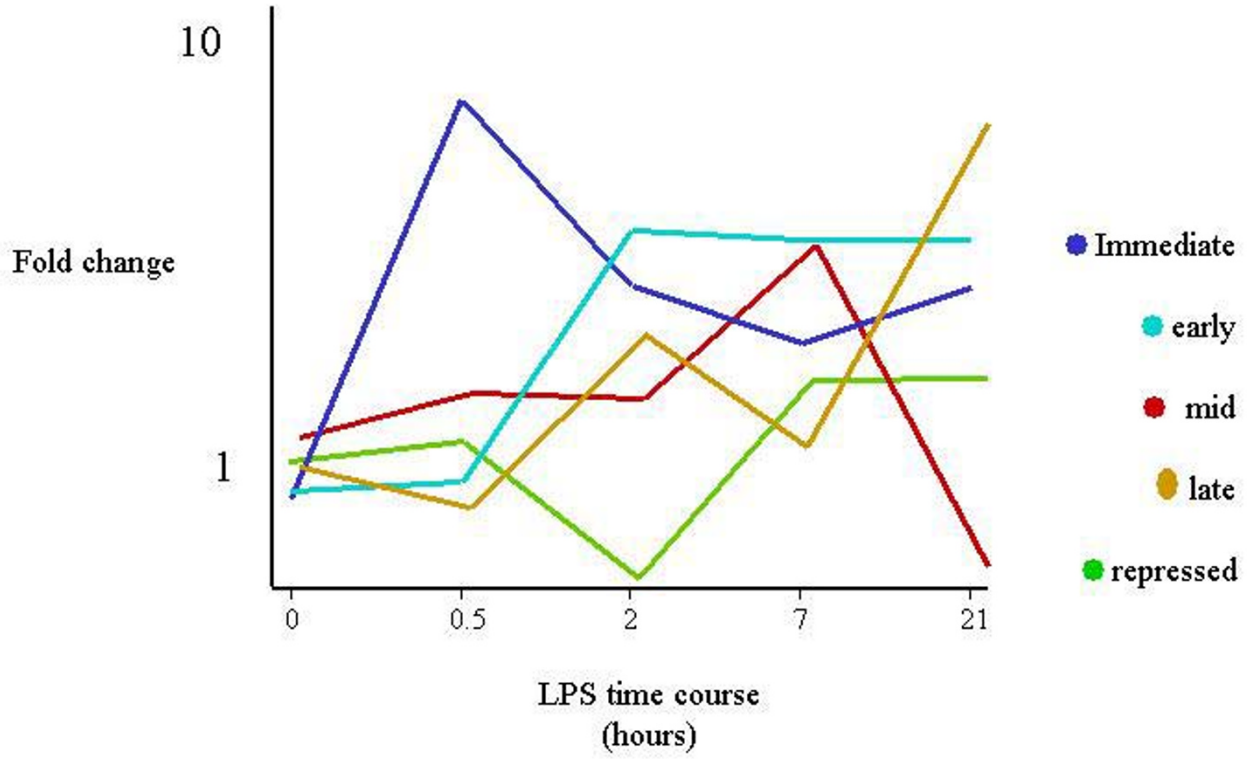

Figure 4
Temporal profiling of the core transcriptional program. Temporal clustering of the inflammatory template by Principal Component Analysis (PCA) revealed a highly regulated set with early (30 min–2 hours), mid (2–7 hours) and late (21 hours) onset of gene induction. Average relative intensity of expression is displayed on the Y-axis, time in hours on the x-axis.

LPS has been reported to rapidly alter chromatin structure at the MHC locus [34], and chromatin remodelling has been shown to induce transcription of closely mapped genes [35,36]. There are many examples of functional grouping of immune related genes in the same genomic region, including the cytokine-receptor cluster on mouse chromosome 16 [37] and a group of cytokine-related genes associated with IL-4 on mouse chromosome 11 [38]. We mapped 379 of the 415 conserved LPS responsive transcripts to the mouse genome. The transcripts mapped across all 19 autosomal chromosomes, as well as the X chromosome. Transcripts grouped into clusters across most of the chromosomes, particularly Chromosomes 2 and 17 (Fig. 6 and table 2 Additional file: 2), and these genomic clusters did not segregate with the temporal expression patterns.

Most of the genes associated together in genomic groupings in this study were novel, and did not necessarily share the same temporal expression patterns. This suggests that while chromatin remodelling may contribute to rapid transcription in inducible systems, temporal regulation of at least the conserved LPS-responsive set provides evidence for specific amplification of these transcripts within the inflammatory cascade.

Strain specific sensitisation of the LPS response

LPS transcriptionally modified 800 array elements in two or more LPS-responsive strain (Fig. 7). 385 transcripts

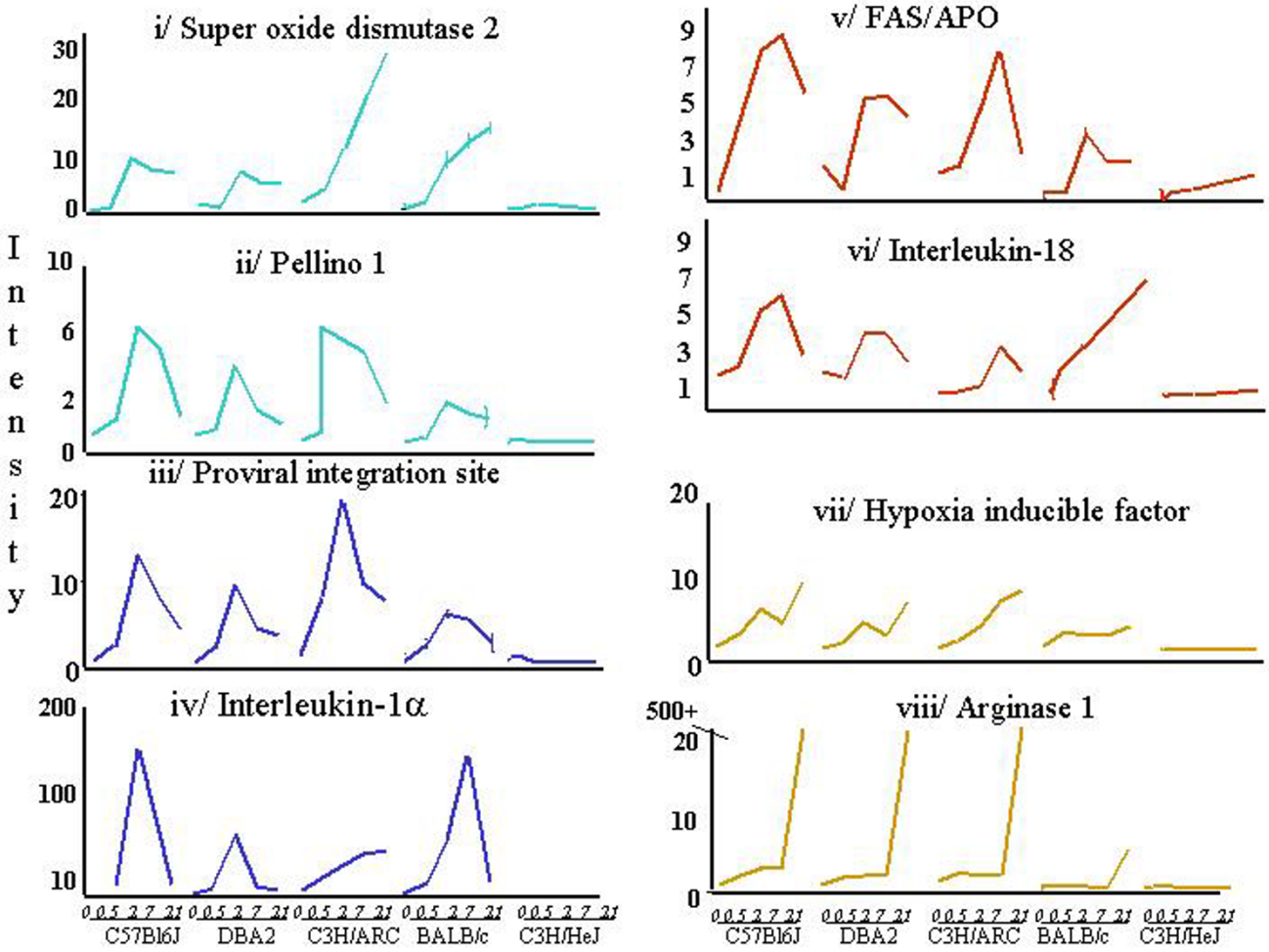

Figure 5
Temporal profiling of the core transcriptional program. The profiles of 8 key genes representing each temporal cluster. Immediate (30 min peak) IL1α, SOD2, Pellino and EVI Early (2 hr peak) IL18, FAS; Late (24 hr peak) Hif1α, Arginase I.

were co-regulated in two or more mouse strains, and of these 305 elements were co-responsive in DBA2 and C57Bl6/J alone. 415 transcripts were regulated in all LPS responsive strains, described above as the candidate targets of TLR4-dependant signalling. Even within this highly conserved set C57Bl6/J and DBA2 BMM were delineated particularly with a subset of 18 genes induced so rapidly that they are presumed to be direct targets of the LPS signalling cascade, evident in Fig. 2 and 5. The delayed induction of this set in C3H/ARC and BALB/c macrophages suggested that the transcripts were induced as a secondary response in these strains, implying the initial lack of a necessary signalling factor which itself was LPS inducible. Given the continuous LPS exposure in our system, it is likely that expression of this group of genes is co-dependent on both this unknown autocrine factor and LPS, and implies that C57Bl6J and DBA2 BMM were sensitised to LPS by the endogenous production of this factor. Many LPS inducible genes are known to be dependent upon priming of the cells with either type 1 or type II interferon [39]. 55 elements corresponding to 28 proteins associated with interferon signalling are present on the RIKEN 19 K array. The average profile of these interferon regulated genes in C57Bl6J and BALB/c mice is shown in figure 8, where a delay in induction from 30 mins to 7 hours is evident in the BALB/c profile. Strain specific differences in endogenous type 1 interferon production, or a downstream target of type 1 interferons could underlie the two patterns of gene induction. A knockout of the IFN-α-receptor 1 (IFNAR1) desensitises BMM to LPS-mediated

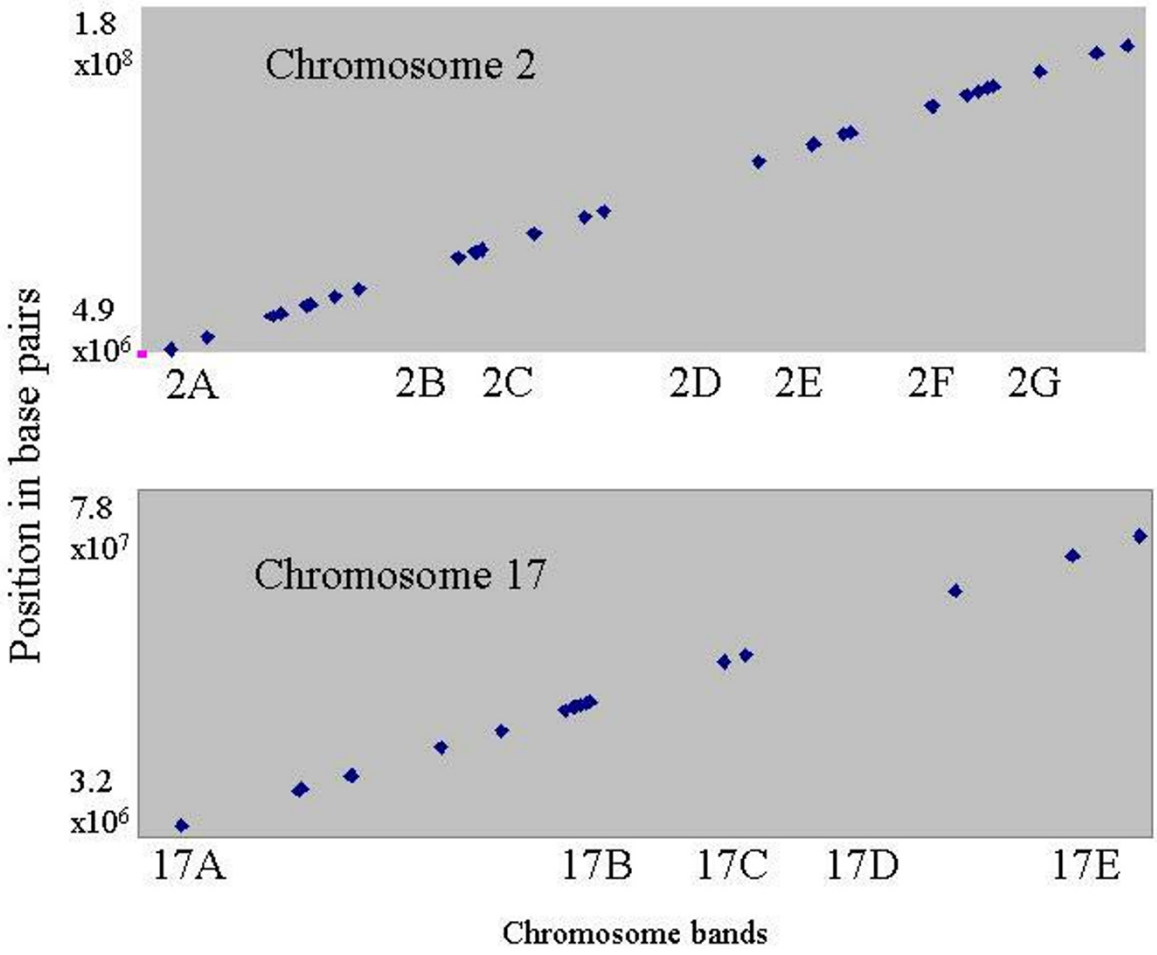

Figure 6
Genomic clustering of conserved LPS inducible transcripts. Full length cDNA sequences corresponding to the conserved LPS responsive set were mapped to the mouse genome using the BLAST function of the FANTOM2 server at http://fantom.gsc.riken.go.jp/viewer/ Map positions (in base pairs) were plotted against the position of chromosome band boundaries to identify sequences mapping within the same chromosomal region. Chromosomes 2 and 17 are given as examples, the remaining data may be found in the supplementary information accompanying this paper.

growth inhibition [40] and C57Bl/6J and BALB/c differ in their ability to produce type 1 interferon in response to pathogen challenge [41–43]. Consistent with this hypothesis, 7 of the 13 interferon-responsive genes found in the conserved LPS responsive set distinguish C57Bl6/J and DBA/2 from BALB/c and C3H/ARC and include Isg20 (interferon stimulated gene (20 kD)), interferon α induced gene Ifi56 and guanylate-binding protein-2 (Gbp2) (Figure 8); Interferon-γ induced GTPase (GTPI); torsin-3, an ATP-dependent interferon responsive gene [44]; IFIT-1 and IFIT-2, a family of interferon-induced genes with tetratricopeptide repeats. Also delayed in C3H/ARC and BALB/c were Tpl2/Cot, and the NF-κB-regulated myeloid differentiation primary response gene MyD118/GADD45β, which are necessary for LPS induced proinflammatory signalling in macrophages [26,45] as well as the chemokine Ccl-6, and six ESTs with no annotated function. The interferon receptor IFNAR2 was also elevated in C57Bl6/J macrophages compared to BALB/c (Supplementary Table 2).

Human microarray studies have shown remarkable consistencies of response between individuals to a range of PAMPs, including LPS. The massive variation that we have observed between mouse strains appears diluted in the heterogeneity of an outbred human population. One

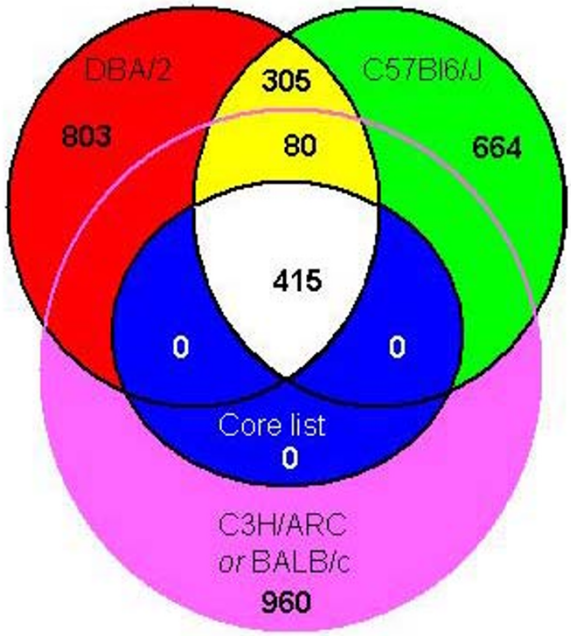

Figure 7
The distribution of LPS-responsive transcripts between mouse strains. Venn Diagram showing the number of LPS-responsive elements shared between the 4 *LPS*^{n} mouse strains. 960 were inducible in C3H/ARC and/or BALB/c alone, 803 were expressed in DBA/2 alone and 664 were expressed in C57Bl/6J alone. 415 elements were expressed in all 4 LPS-responsive strains. 80 transcripts were expressed in 3 of the 4 LPS responsive strains, including C57Bl6J and DBA/2. 305 elements were shared only between DBA and C57Bl/6J.

study did find a "donor variant response" among healthy individuals in a set of interferon-responsive transcripts [7]. Our data suggests that genetic variation in an autocrine factor modulating the type1 interferon signalling pathway may be critical for appropriate induction of an innate response to LPS.

Evidence for novel genetic loci underlying strain specific LPS responses

The cytokine expression profiles of stimulated T lymphocytes differ between mouse strains. Such variation led to the discovery of T helper cell phenotypes associated with cell-mediated immunity (Th1) or humoral immunity (Th2) [46]. C57Bl/6J and BALB/c are archetypal Th1 and Th2 strains respectively [47,48]. We directly compared these strains to look for transcriptional differences in the macrophage populations that may prime the nature of any subsequent Th1/Th2 polarization. This smaller, focused analysis allowed 5039 elements to pass restrictions between BALB/c and C57Bl6J across the time course and in all replicates. Of these, 436 differed significantly ($p < 0.05$) between the two strains (Table 3 Additional file: 3). We saw no evidence for endogenous differences in expression of known Th1/Th2-regulating cytokines IL-12 or 18, although IL-18 was induced earlier in C57Bl6J than in BALB/c (Fig. 5). In their definition of M1-M2 macrophage phenotypes, Mills et al. [47] identified the regulation of arginase as a key distinguishing factor in the propensity to bias Th1 or Th2 T-cell responses. It is the alternative metabolism of arginine to nitric oxide or ornithine that predicts M1 or M2 development. Accordingly, BALB/c and DBA/2 mice fall into the M2 class whilst C57Bl6J is an M1 strain. Arginase I and II also distinguished C57Bl/6 from BALB/c in our study. Surprisingly, arginase mRNA was induced 10–50 fold less in BALB/c macrophages than in C57Bl/6J BMM or either of the other LPS-responsive strains (Fig 5). The model proposed by Mills et al was generated on enzyme levels, where our gene expression data contradicts the M1/M2 hypothesis. Our observation that C57Bl/6J and DBA/2

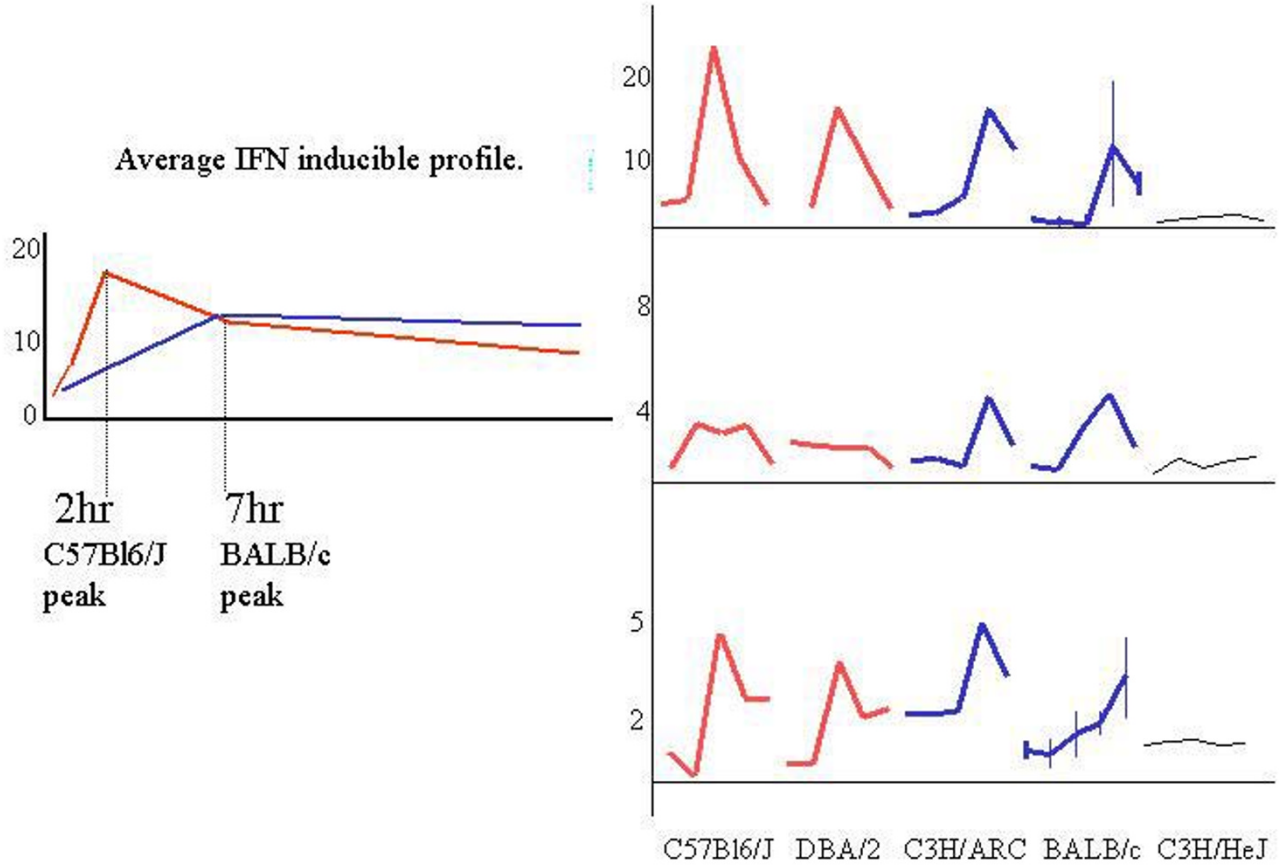

Figure 8
The sensitisation of interferon-responsive transcripts in C57Bl6J mice. The averaged temporal profile of 13 interferon-responsive genes compared between C57Bl/6J and BALB/c mice, where the Y axis represents the fold induction from the unstimulated timepoint and the X-Axis categorises the time points for both mouse strains. The C57Bl6/J profile (red) is induced by 2 hours, whereas the BALB/c profile is delayed at 7hours. This segregation of temporal induction was observed in 18 genes within the conserved TLR4-dependent set, and 3 examples are given: A/ Isg20; B/ Ifi56 and C/ Gbp2 for all 5 mouse strains.

have similar expression profiles, even in an unactivated state may actually reflect the fact that these two strains are genetically related [49]. These data do not support classification of DBA as an M2 strain, nor indeed the predilection for a strain based M1 or M2 bias, at least at a transcriptional level.

Several effectors of the Rac 1 activation pathway were found to be significantly up regulated in C57Bl6J compared to BALB/c, including Rac 1, the activating enzyme farnesyltransferase, and interactor Profilin. Rac1 has a role in the generation of reactive oxygen species (ROS) in phagocytic and nonphagocytic cells [50], and has been shown to activate STAT3 in response to ROS [51]. Similarly, a number of genes required for ROS induction, including cytochrome b-245 and neutrophil cytosolic factor 4 were also differentially expressed between C57Bl/6J and BALB/c macrophages. These data suggest that C57Bl6/J macrophages are more readily activated to produce cytocidal oxygen free radicals. Interestingly, the redox status of BMM has been shown by others to be critical in supporting polarization of antigen presenting cells [48]. Slc11A1 is predicted to affect redox potential

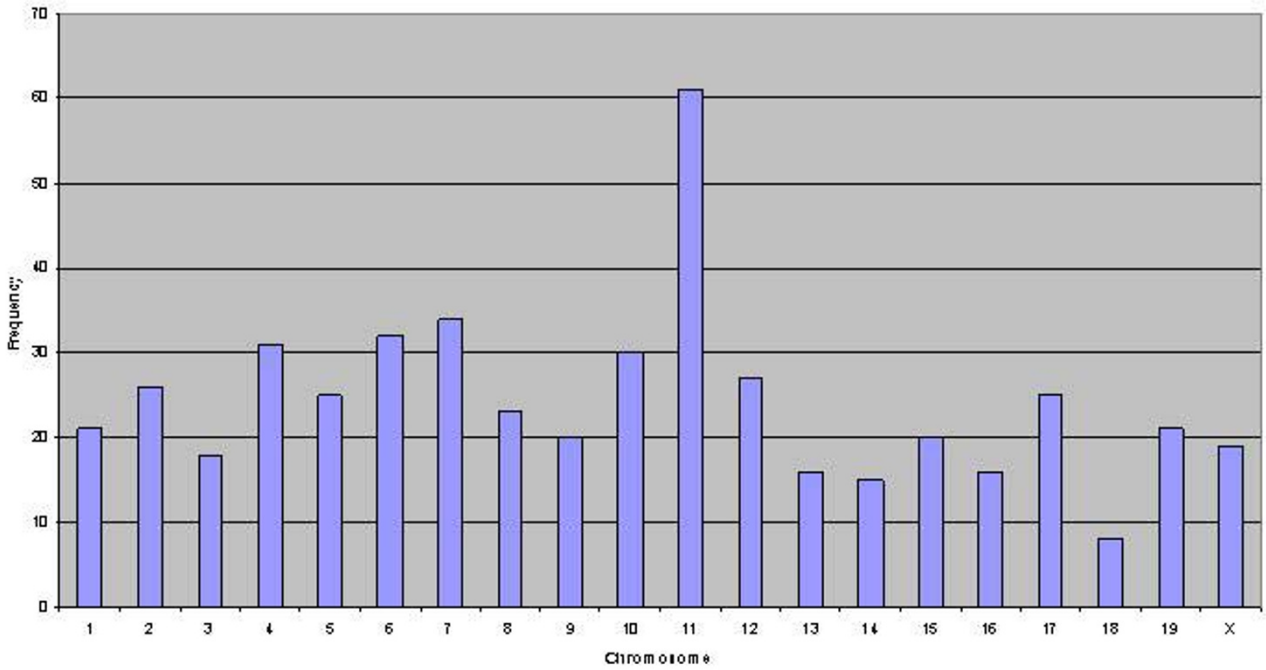

Figure 9
Genomic clustering of transcripts differentially expressed between C57Bl/6J and BALB/c mice. Full-length cDNA sequences corresponding to the 436 transcripts identified as significantly (p = 0.05) differentially expressed were mapped to the mouse genome using the BLAST function of the FANTOM2 server at http://fantom.gsc.riken.go.jp/viewer/. The chromosome locations are graphed as frequency (number of transcripts) vs Chromosome number.

through iron homeostasis in the phagosome (reviewed in [52,53]), but BALB/c and C57Bl6J share the susceptible *Slc11A1* allele at the *Bcg* locus.

61 (12.5%) of the 436 genes differentially expressed between BALB/c and C57Bl/6J mapped to mouse chromosome 11 (Figure 9, table 4 Additional file: 4). 45% of these mapped to a single cytogenetic band, 11b. This region of mouse chromosome 11 was identified as conferring a salmonella-resistant phenotype in a study between resistant C57Bl/6J and the susceptible wild-mouse strain MOLF/Ei [54], although the mapping was of low resolution. Our data further refines the 11b locus to two regions where differentially expressed genes cluster within 10 Mb of each other (Figure 10).

12 candidates cluster at 70 Mb, and these include 7 novel proteins and one putative noncoding RNA. 11 of the 12 transcripts in this cluster had significantly lower expression in the Salmonella-resistant strain C57Bl/6J than in BALB/c. Profilin1 (Pfn1) was the only transcript mapping into this cluster with significantly higher expression in unstimulated C57Bl6J than BALB/c BMM, and was induced by LPS across the C57Bl6J but not BALB/c time course. Pfn1 is a cytoskeletal protein that is essential for cell survival and division and acts in a dose-dependent manner [55]. The three other known genes were Slc25a11,

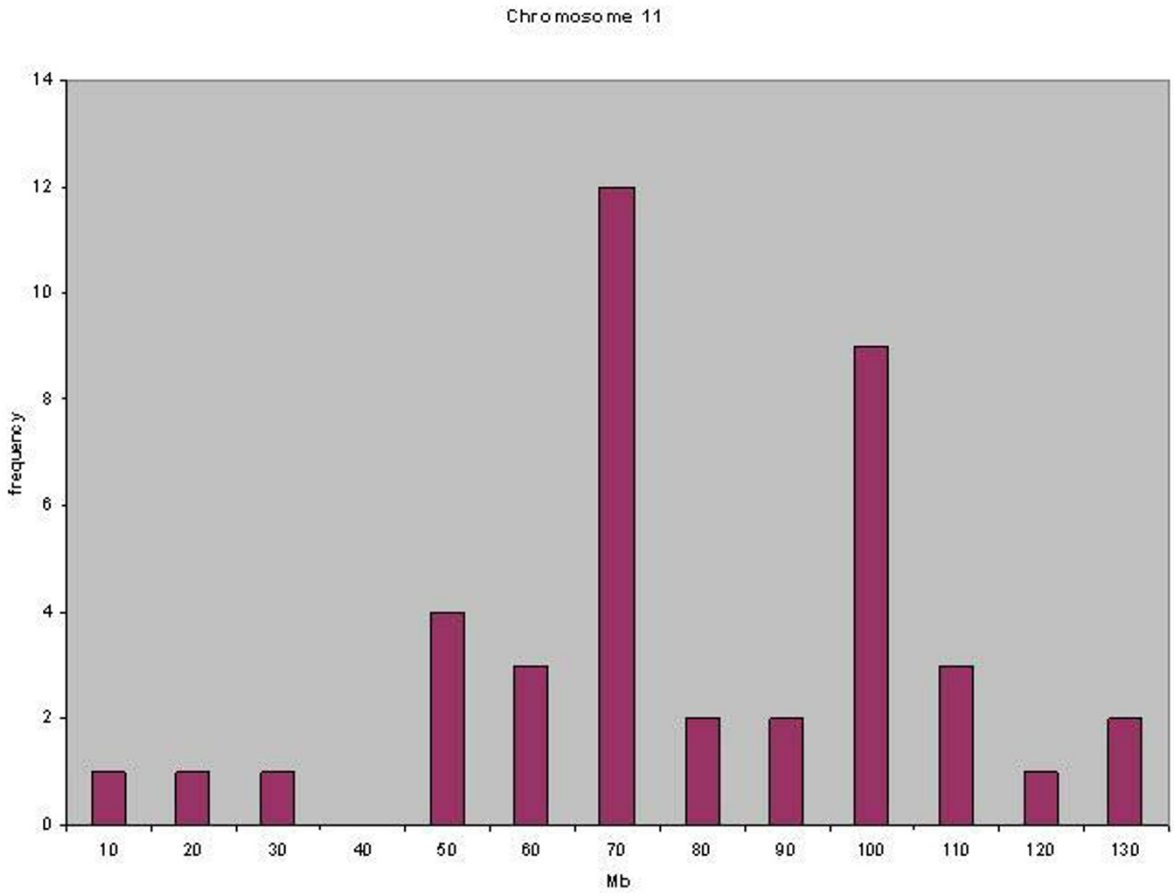

Figure 10
Genomic clustering of transcripts differentially expressed between C57Bl/6J and BALB/c mice. Sixty-one transcripts mapped to chromosome 11. The graph plots frequency (number of transcripts) vs Mb (x-axis).

a mitochondrial transporter; the cell adhesion molecule vitronectin and Supt6h, a chromatin structural protein thought to regulate transcription [56]. Vitronectin is the only protein in this set with a known biology in macrophage adhesion and activation, binding plasminogen activator inhibitor 1 (PAI-1) and urokinase plasminogen activator (u-PA) [57,58], and recruiting ERK1 signalling through alpha(v)beta 3 integrin [59]. Interestingly iNOS also maps into this region, but is not present on the RIKEN 19 K arrays, and its expression may not be regulated in the absence of IFN-γ priming of the BMM [60].

9 genes clustered together around 100 Mb. This region encompasses the keratin complex 1 – a region of 9 acidic keratins, 4 of which are identified as significantly different between BALB/c and C57Bl6/J in this analysis; keratin 1, 13, 17 and 19. The remaining keratins in this complex were present on the array, but did not pass confidence criteria, particularly that of low signal threshold. Also identified in this cluster were the calcitonin receptor modifying protein 2 (Ramp2); granulin; a macropain (Psmc5); and the lymphocyte antigen CMRF35. These data suggest that the dominant genetic regions controlling LPS-induced changes in redox status lie outside the *Bcg* locus, and highlight new candidates for LPS/Salmonella susceptibility in a region of chromosome 11 plentiful in innate-immune mediators.

Conclusions

We provide transcriptional evidence for the enormous potential for variation in individual response to pathogen challenge, which is unsurprising given the infinitely variable and changing face of pathogens. Our data provides a description of the genetic networks activated by LPS induction in primary murine macrophages from different genetic backgrounds. The data shows the many possible transcriptional consequences of macrophage activation by LPS in a strain dependent manner. Functional conclusions drawn solely from gene expression data can be at odds with protein levels – indeed increases in gene expression can be a direct consequence of falls in corresponding protein level. We describe a conserved TLR4/MyD88/NfkB signalling cascade that is amplified at a transcriptional level over the 24 hour time course. These co-ordinately regulated transcripts provide insights into pathways that are likely to function in an innate immune context, and provide candidates for further functional analysis on individual mouse backgrounds. The differences that we have demonstrated between inbred mouse strains contrast to the stereotyped response observed in monocytes of healthy human individuals to a suite of pathogenic materials including LPS [7,8]. Nevertheless, variation in TLR4 and Slc11A1 occurs in humans and underlies susceptibility to infectious disease and arteriosclerosis [6]. The P712H Tlr4 mutation has been shown to act as a dominant negative on some mouse backgrounds (C3H/b^{11r30m}) [61], and the degree of LPS hypo responsiveness in Tlr4 null mice may be modulated by mutations in RAN/Gtpase [62,63] on the C3H background, or in IL-12R on the C57Bl10Cr background [64]. The *Tlr4* locus is clearly important in determining LPS sensitivity, but many additional genetic loci control the extent and the nature of transcriptional response promoted by LPS.

Methods

Mouse Strains

BALB/c, C3H/ARC, C57Bl/6J and DBA2 mice were sourced from the Animal Resources Center, Western Australia and housed at the SPF facility, Biological and Chemical Faculty, University of QLD, Australia. C3H/He*Jlps*^{d} mice were sourced from the Walter and Eliza Hall Institute, Victoria. The *Tlr4* genotype of each strain was confirmed by genomic sequencing.

All procedures were carried out under the NH&MRC guidelines for animal research; animal ethics license number IMB132/00.

Derivation and culture of BMM

Bone marrow derived macrophages were taken from the femurs of a pool of 6–8 week old male mice. Macrophages were differentiated from bone marrow progenitors in RPMI1640 (BRL), 10% FCS and 10^4 U/ml (100 ng/ml) recombinant human CSF-1 (a gift from Chiron, Emeryville, CA) for 6 days [65]. Specific, homogeneous differentiation of macrophages under these conditions has been well characterised by our and other laboratories [18,66–68]. Gross phenotypic changes associated with macrophage differentiation were monitored and by day 5 BMMs from all strains had become semi-adherent on bactoplastic, were of uniform size and displayed characteristic cytoplasmic and nuclear morphology. No gross differences were observed between the strains in cell yield or morphology under these controlled culture conditions. At day 6 macrophages were seeded at 1×10^6 cells/ml and stimulated on the following day with 10 ng/ml LPS *Salmonella minnesota* (Sigma-Aldrich, St. Louis, MO). 3 × 10 cm dishes were harvested for each time point – unstimulated (time 0), 30 min, 2, 7 and 21 h. RNA was extracted using RNeasy midi kit (Qiagen) according to the manufacturers instructions. The optimal LPS dose of 10 ng/ml was determined by assaying iNOS and TNF-α production in the presence and absence of IFN-γ, where 10 ng/ml gave maximal activation in all strains, but no response in C3H/HeJLpsd mice (data not shown). Replica data was derived from independent RNA extractions of a different pool of 6–8 week old mouse femurs.

Microarray labeling, hybridizing, scanning and analysis

The experimental design, using 17.5dpc C57Bl/6J embryo as a common reference, has been described previously [19]. Briefly, cDNA was indirectly labeled with aminoallyl-conjugated Cy3 (time course) or Cy5 (embryo), and hybridized overnight to RIKEN 19,000 full-length cDNA microarrays. Slides were washed and scanned on a ScanArray 5000 confocal laser scanner. Molecularware (Digital Genome) was use to process the images, and data was corrected for local background, and confidence status flagged for empty spots, signal/noise ratio, spot CV ratio and spot morphology. Data was imported into GeneSpring4.2 (Silicon Genetics) for further filtering, clustering and comparative analysis. Hybridisations were performed in duplicate to replicate each data point, shown as an average in the figures. Each element was spotted onto the arrays once, but the redundancy in the RIKEN 19 K set provides multiple hybridisation events for many of the genes represented on the array. These are treated as separate elements in the data analysis, but do present a high level of confidence in the consistency of the hybridisation across each array. Most of the elements (80%) expressed by BMM did not alter across each time course, and many of these (>60%) did not change in expression level between mouse strains. The Global Error Model in GeneSpring4.2 (Silicon Genetics) tested each element for reproducibility against its replica hybridisation, and against the population of invariantly expressed elements on the array using a single sample t-test to gen-

erate a confidence (p) value. Microarray hybridisations are inherently noisy at signal strengths outside the linear range of detection. Elements expressed below 700 units and above 65,000 units were therefore excluded from subsequent analysis. Data was flagged for empty spots, high signal/noise ratio, aberrant spot CV ratio and abnormal spot morphology, and replica confidence (p) value of less than 0.1 (or greater than 90% confident).

Data handling and annotation of clusters

The ratio of the experimental signal/the control signal for each spot was calculated; intensity-dependent normalization was also applied, where the ratio was reduced to the residual of the Lowess fit of the intensity versus ratio curve [69]. The dataset was restricted to those spots passing confidence status on the control channel of every hybridization, across each temporal series and on 5 separate mouse strains. 3612 elements passed these criteria. Figure 1 describes the normalised distribution of elements at each time point, and for each mouse strain. A 2-fold cutoff was employed to identify elements expressed 2 standard deviations (S.D.) from the normalised population median of 1. An additional normalisation was performed to identify those elements that were temporally regulated, where at each post-lps time point the intensity of each element was divided by its average intensity at time 0. A set of genes was identified induced or repressed across the time course if they were found more than 2 S.D. from the sample-normalised population median of 1 and a list was compiled for each mouse strain. A small subset (415) of genes was identified in all 4 responder strains, but not C3H/HeJ*lps*^{d}. This set was clustered using the unsupervised hierarchical clustering tool in GeneSpring, where similarity was measured by Pearson correlation; the separation ratio was 0.5 and the minimum distance was 0.001. Temporally conserved profiles were identified by principle component analysis of the co-expressed subset.

441 genes were identified by Genespring4.2 as statistically different between C57Bl/6J and BALB/c (Table 3 Additional file: 3), using parametric test, variances not assumed equal (Welch t-test), p-value cutoff 0.05, where the multiple testing correction used was the Benjamini and Hochberg False Discovery Rate option. This restriction tested 5,039 genes; 153 genes had insufficient data for a comparison, and 5.0% of the identified genes could be expected to pass the restriction by chance.

Clusters of co-expressed genes were annotated using SOURCE http://genome-www5.stanford.edu/cgi-bin/SMD/source//sourceBatchSearch assisted extraction of Gene Ontologies (GO) from UniGene http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=unigene.

Genomic analysis of LPS-inducible transcripts

Full length cDNA sequences were mapped to the mouse genome using the BLAST function of the FANTOM2 server at http://fantom.gsc.riken.go.jp/viewer/[70]. Array identifiers were first associated with their representative transcript using a local BLAST of the sequence tags from the RIKEN 19 K array and the representative transcript and protein set RTPS version 6.2. 415 RIKEN clones from the conserved TLR4 dependent set were associated with 379 representative transcripts, 35 RIKEN clones were not found in the RTPS v6.2 set. Similarly, of the 436 RIKEN clones identified as statistically different between C57Bl6/J and BALB/c mice, 347 were associated with a corresponding representative transcript, and 89 were not found in the RTPSv 6.2 set. These clones are most likely to have been excluded from the RTPS set because of sequence artefacts associated with the clones – poor available sequence, or no similarity with sequences in the EST and genomic databases, and were annotated as "unclassifiable transcripts" in the FANTOM annotation process.

The FANTOM annotation pipeline included automated mapping of each RTPS with SIM4 and blEST, which are cDNA-genome alignment programs integrated in TIGR Gene Index genomic mapping pipeline. The output used in this analysis was obtained as chromosome number, and base pair position numbered from proximal to distal. Transcript frequency was plotted against chromosome number in excel to determine distribution of transcripts across the genome (figure 8). A number of conventional mouse mapping studies have identified genetic loci associated with susceptibility to pathogens, but these have been based on cytogenetic or genetic maps rather than physical maps. The location of each transcript on its corresponding chromosome was displayed as a function of both physical position (basepairs on the y axis) and cytogenetic position (chromosome band) on the x-axis (figure 6). The number of diamonds plotted on the graph indicates frequency of transcripts at any one position.

List of Abbreviations

CSF-1 colony stimulating factor-1

GO Gene Ontology

dpc Days post coitum

LPS lipopolysaccharide

lps^{d} Defective LPS allele

lps^{n} Normal LPS allele

NO Nitric Oxide

PAMP pathogen associated molecular pattern

PAI-1 plasminogen activator inhibitor 1

PCA Principle Component Analysis

ROS reactive oxygen species

TLR Toll-like receptor

u-PA urokinase plasminogen activator

Authors' contributions

Christine Wells performed experimental design, collection of cell lines, manufacture of RNA and analysis & interpretation of the data, under the supervision of Professor David Hume and Professor Brandon Wainwright. Dr Timothy Ravasi performed hybridizations in the laboratory of Yasushi Okasaki, and contributed to data analysis and interpretation. Geoffrey Faulkner wrote the scripts to extract mapping information for transcripts differentially expressed between BALB/c and C57Bl/6J. He provided locus link information for mouse chromosome 11. Matthew Sweet provided RNA for the DBA/2 analysis. The microarray slides were manufactured in the laboratory of Dr Okasaki under the RIKEN Mouse Genome Encyclopedia project, which is directed by Professor Hayashazaki. The microarray clones were selected from libraries made by Dr Piero Carninci.

Additional material

Additional File 1

A conserved set of 415 cDNAs, identified as candidate targets of the core TLR4/MyD88 signalling pathway, downloadable tab-delimitated text file.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2172-4-5-s1.xls]

Additional File 2

Mapping of the conserved LPS responsive set to the mouse genome. Tab-delimitated text file.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2172-4-5-s2.txt]

Additional File 3

Identification of 436 genes that differed significantly ($p < 0.05$) between C57Bl6/J and BALB/c inbred mouse strains. Tab-delimitated text file.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2172-4-5-s3.xls]

Additional File 4

Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2172-4-5-s4.xls]

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