

Transcriptional profiling of dendritic cells in a mouse model of food-antigen induced anaphylaxis reveals the upregulation of multiple immune-related pathways

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Abbreviations

DCs: dendritic cells

DE: differential expression/differentially expressed

FC: fold change

FDR: false discovery rate

GO: gene ontology

KEGG: Kyoto Encyclopedia of Genes and Genomes

LPS: lipopolysaccharide

PBL: peripheral blood leukocyte

PBS: phosphate-buffer solution

RNA-Seq: RNA sequencing

sd: standard deviation

slgE: specific immunoglobulin E

slgG1: specific immunoglobulin G1

ABSTRACT

Scope

Much of our knowledge about gene expression during anaphylaxis comes from candidate gene studies. Despite their potential role, expression changes in dendritic cells (DCs) have not been studied in this context using high throughput methods. We investigated the molecular mechanisms underlying food-antigen induced anaphylaxis using DCs from an animal model.

Methods and results

We used RNA sequencing to study gene expression in lymph node derived DCs from anaphylactic mice sensitized intranasally with the major peach allergen Pru p 3, during the acute reaction phase, induced intraperitoneally. In total, 237 genes changed significantly, 181 showing at least two-fold changes. Almost three quarters of these increased during anaphylaxis. A subset was confirmed using RT-PCR in a second set of samples obtained from a new batch of mice. Enrichment analysis showed an overrepresentation of genes involved in key immune system and inflammatory processes including TGF- β signalling. Comparison with a study using anaphylactic human subjects showed significant overlap.

Conclusions

Our findings provide a comprehensive overview of the transcriptional changes occurring in DCs during anaphylaxis and help elucidate the mechanisms involved. They add further weight to the putative role of these cells in anaphylaxis and highlights genes that may represent potential therapeutic targets.

INTRODUCTION

Anaphylaxis is a potentially life threatening allergic reaction of rapid onset characterized by low blood pressure, respiratory compromise and gastrointestinal problems ^[1]. It is frequently triggered by food, with around 3% of children in the United States experiencing severe reactions ^[2], including anaphylaxis. Anaphylaxis per se has been estimated to have a prevalence of 3% in Europe and 0.5-2% in the USA ^[3]. Food allergy in general has been estimated to affect nearly 5% of adults and 8% of children ^[4]. Common culprits include peanut/tree nuts, fruits, vegetables, shellfish, egg and milk ^[1, 4]. Peach is an important trigger within Mediterranean regions, largely because of sensitization to the panallergen Pru p 3 ^[5, 6]. Pru p 3 is a non-specific lipid transfer protein that can induce dendritic cell (DC) maturation and T cell proliferation in peach allergic individuals ^[7]. As well as cross-linking of IgE receptors on mast cells, systemic activation of the immune system, including mediator production and release of preformed mediators, plays a role in the spread of the reaction. Much of what we know about the underlying mechanisms has been obtained through studies of pre-selected genes and mediators, with roles for multiple cell types ^[8-10]. Nevertheless, little research has been performed using high-throughput analysis. A handful of studies have used transcriptomic techniques to bridge this gap by looking at gene expression in allergic patients, both during a reaction ^[11, 12], and for unprovoked individuals ^[13]. Stone et al., examining peripheral blood leukocytes (PBLs) with gene expression microarrays, found a gene expression signature associated with interferon-producing killer DCs that was upregulated in anaphylactic patients during the acute phase, suggesting a key role for DCs, although direct studies on these cell types exclusively were not performed here ^[11]. DCs have been shown to play multiple roles in allergy sensitization, both through direct activation ^[14, 15], and the regulation of complement and anaphylatoxin-related pathways ^[16]. We have previously found increases expression for multiple DC markers in peach-allergic individuals upon stimulation, with no changes observed in

tolerant controls ^[7, 17], however their relevance in anaphylaxis requires further exploration ^[9]. These efforts are crucial to our understanding of the immune system and other processes in anaphylaxis, and may lead to the discovery of biomarkers for prognosis and treatment.

However, such patient-based studies are limited by lack of access to immunological tissue and organs. DCs are thought to play a key role in food allergy and the acquisition of tolerance through various mechanisms ^[7, 18], but cannot be studied in detail in humans due to lack of access to lymphatic tissue. To overcome this limitation, we have performed studies on monocyte-derived dendritic cells ^[7, 17]. However, although these cells share similarities with myeloid DCs, there are also differences due to *in vitro* transformation, moreover experiments are performed outside of the physiological context of Pru p 3 stimulation.

To circumvent these constraints we have performed *in vivo* studies by developing a mouse model of food-antigen induced anaphylaxis, specifically peach-anaphylaxis ^[19] that involves intranasal sensitization with peach extract and intraperitoneal allergen challenge. This model has also been used to test novel constructs for antigen specific immunotherapy ^[20]. To further our understanding of anaphylaxis and the role of DCs, we performed RNA-Seq transcriptome-profiling using lymph node derived DCs obtained from anaphylactic mice induced using this model, comparing gene expression to control mice that were treated with vehicle only (phosphate-buffered saline, PBS), in both cases following Pru p 3 challenge. DCs were chosen based on the aforementioned increased expression of DC markers upon stimulation in human samples ^[17], and their potential roles in the acquisition of tolerance to food allergens ^[18] and anaphylaxis ^[11]. The changes in genes expression were explored using RT-PCR in samples obtained from new sets of animals that underwent similar procedures but without inducing an allergic response. Within the resulting list of genes changing between anaphylactic mice and controls we found enrichment for genes encoding secreted proteins as well as inflammation, cytokine

response, TGF- β receptor signalling and complement-related processes. We also found genes involved in proliferative processes including angiogenesis and smooth muscle cell regulation. There was significant overlap with previous human studies. These results provide further evidence of a role for DCs in food-antigen induced anaphylaxis.

Materials and Methods

Anaphylaxis Induction

Female Balb/c mice aged 4-5 weeks (Janvier Lab, Saint-Berthevin Cedex, France), were sensitized to peach using intranasally administered Pru p 3 in combination with LPS and challenged intraperitoneally as described previously ^[19]. These will be referred to as anaphylactic mice. Littermates, treated with intranasally administered PBS (instead of Pru p 3 and LPS), and later given an intraperitoneal challenge as per the anaphylactic mice, were used for comparison; these will be referred to as controls. Temperature was recorded 30-40 minutes following intraperitoneal challenge with Pru p 3. Full details in Supporting Information (Methods Section). Physical and behavioural symptoms were measured using a previously described scoring system ^[21]. All experimental animal procedures conform to international standards of animal welfare and were approved by the Animal Experimentation Ethics Committee of BIONAND, Malaga, Spain, approval reference 07/2017.

Immunological Changes

Levels of Pru p 3-specific serum antibodies (sIgE and sIgG1) were measured one hour after challenge using ELISA. Pru p 3 antibody secreting cells were quantified using ELISpot ^[22]. Further details in Supporting Information.

Lymph node cell extraction and dendritic cell enrichment

Following sacrifice, lymph nodes were removed to obtain DCs and total RNA was isolated (more details in Supporting Information). Dendritic cells were purified from axillary, cervical, inguinal and mesenteric lymph nodes. RNA quality was verified using an Agilent Bioanalyzer with a RNA integrity number cut-off of 8, in line with previous studies ^[23].

RNA sequencing

Poly-A enrichment was performed on the total RNA obtained from the mouse DCs. The resultant mRNA was reverse transcribed, fragmented into smaller sequences and amplified using the SMARTer Universal Low RNA Kit (Clontech, Mountain View, CA). The resulting library was then sequenced using the Illumina HiSeq 2500 (Illumina, San Diego, CA) sequencing machine to produce 100-bp paired-end reads.

Read alignment and statistical analysis

Bioinformatics analysis was performed using a modified protocol based on that presented in ^[24]. Full details are available in the Supporting Information. Reads were aligned to the mouse genome (GRCm38/mm10) using the TopHat software ^[25] and RefSeq gene models were used for transcriptome mapping ^[26]. Expression was quantified using HTSeq ^[27]. Differential expression (DE) analysis was performed using DESeq2 and false discovery rate (FDR) was calculated ^[28] by adjusting p-values to control for false positives. An adjusted p-value threshold of 0.05 was chosen to denote significance. Genes with an absolute log fold change (FC) of 1 or more were used for enrichment analysis.

Validation of RNA-Seq results

A subset of the DE genes was chosen for verification using quantitative RT-PCR using samples from a new batch of mice. Six underwent sensitization following the procedure described above; six control littermates that were treated with intranasally administered PBS and challenged intraperitoneally with Pru p 3 were used for comparison to assess DE. An additional group of 6 mice were given Pru p 3 (without LPS) for 6 weeks in the same manner as the anaphylactic mice. Eleven genes were chosen for validation, based on evidence for DE using RNA-Seq and biological relevance. RT-PCR was carried out via the TaqMan gene expression assay, using commercial primers (Thermo Fisher, Waltham, MA). Expression was

quantified using the Cq cycle threshold method ^[29]. DE p-values were calculated using the Mann-Whitney U test, using a single tailed test when comparing anaphylactic and control animals, as the expected direction of change was already established. Data analysis was conducted using ReadqPCR/NormqPCR ^[30]. A further comparison was made between mice treated with LPS only and control mice to assess differences due to LPS treatment only, using an additional set of animals (five controls, ten LPS only). Table 1 describes the different groups of animals, including their naming throughout this manuscript, details of what they were administered for the “sensitization” procedure and how they were challenged.

Homologue Mapping

Mouse genes were mapped to their human homologues to compare results with a previous study of human anaphylactic patients using the biomaRt R package, an interface to the BioMart resource ^[31]. In brief, the getLDS function was used to link the human and mouse Ensembl datasets and to search for human genes based on the Mouse Genome Informatics mouse gene symbol. The lists of DE genes were compared to look for significant overlap by using the hypergeometric distribution to calculate the probability of overlap by chance, using as the total number of genes (background) the genes with orthologues in both species that are measurable by both technologies (RNA-Seq for our data; Affymetrix Human Gene 1.0 ST microarrays for the human data of Stone et al).

Functional analysis

Genes deemed significantly DE between anaphylactic mice and controls following the above criteria were used as input for the Database for Annotation, Visualization and Integrated Discovery (DAVID) tool for functional enrichment. This tool compares the DE genes with groups of functionally related genes, such as members of biological pathways, to find overrepresentation ^[32].

RESULTS

Mice were sensitized intranasally via repeated administration of Pru p 3 and LPS. Anaphylaxis was induced by peritoneal injection of Pru p 3 one week after the sensitization period. Anaphylactic mice underwent a statistically significant drop in body temperature of around 4°C; this did not occur in control mice, whilst Pru p 3-only receiving (sensitized) mice underwent changes of less than 0.4°C; this change was not statistically significant (Fig 1A). Similarly, sensitized mice showed very mild symptoms after challenge, such as scratching and rubbing/puffiness (levels 0-2), whilst anaphylactic mice showed very severe symptoms, i.e convulsion and death (levels 4-5), with control mice showing no symptoms (Fig 1B). Where mice showed heavy symptoms of anaphylaxis, they were immediately anaesthetised in line with ethical guidelines, after which body temperature was measured and they were then sacrificed.

Humoral and cellular immune response

Anaphylactic mice showed higher levels of Pru p 3-sIgE and sIgG1 secreting cells compared to controls (Fig 1C, $p=1.01 \times 10^{-4}$ and $p=5.59 \times 10^{-4}$, respectively). Pru p 3-only receiving animals also showed higher levels than controls, ($p=0.02$ and $p=0.04$, respectively) although the difference was less. Higher numbers of Pru p 3-sIgE secreting were also found for anaphylactic animals compared to Pru p 3 only receiving animals. In terms of sera Pru p 3-specific Ab levels, there was a significant increase for Pru p 3-specific IgG1 only in anaphylactic mice compared to controls (Fig 1D, $p=1.62 \times 10^{-5}$). The lack of increase in Pru p 3 specific IgE levels suggests the increased IgE is attached to cells, although further experiments will be needed to investigate this, as there is a clear tendency for increased levels in the anaphylactic animals. These findings are in line with previous studies using this model^[19, 20, 33] however other allergy models have found IgE secreting cell numbers to be less than

IgG1 secreting cells, for example in a murine model of allergic asthma^[34]. Prup 3-slgG1 levels were also higher for anaphylactic animals vs. Prup 3 only receiving animals; interestingly this is also in contrast with the antibody-secreting cell numbers, which were higher for slgE.

RNA sequencing and read alignment

After RNA extraction the mean purity measured by 260:280 absorption ratio was 1.89 (sd=0.13); mean RNA Integrity Number was 8.94 (sd=0.60). Then, RNA was sequenced, producing an average of 45,107,952 reads per sample (sd=6,368,798). An average of 39,778,272 (sd=5,947,526) of these could then be mapped to the mouse genome (GRCm38/mm10), corresponding to 88.2% percent of the total sequenced reads. Of these mapped reads, an average of 34,211,635 (sd=4,321,366) overlapped with exonic regions, representing 86.0%, the rest mapping to intronic/intergenic regions. Of the 24,421 RefSeq genes (including 3,166 that do not yet have canonical names), 12,815 showed evidence of expression, defined as having >10 reads mapping to exonic regions of the gene in >4 of the anaphylactic or >4 of the control samples. The alignment bam files can be found in Gene Expression Omnibus TO ADD ^[35].

Differential Expression

Differential Expression (DE) was analysed using DESeq2. A total of 237 genes were deemed to change significantly between control and anaphylactic mice using an FDR cut-off of 0.05. Almost three quarters (175 genes) showed higher expression in the anaphylactic animals (upregulated), the remaining 62 showing higher expression in the control animals (downregulated). Of these, 181 showed an absolute log FC of at least 1 (129 upregulated; 52 downregulated). Interestingly, several downregulated genes showed a highly negative log FC (i.e. were downregulated) due to the expression of only two samples Control 2 and Control 3,

suggesting a natural variation in these genes in control animals, but clear loss of expression in the anaphylactic animals (Fig 2A). Importantly, considering all genes that showed non-zero expression, 47.2% showed a positive log FC and 52.8% showed negative log FC, showing there was no bias towards over or under-expression across all genes (Fig 2B). The Volcano Plot (Fig 2C) shows the FC values for all genes in relation to the adjusted p-value for DE. The genes to the far left in Figure 2C were very highly expressed in a number of controls, however they were not deemed significant due to high variation. A full list of DE genes, including FC and FDR is given in Supporting Information Table S1 and details of the differences between samples in Supporting Information Figure S1.

Replication in new samples

We chose 11 genes to verify using RT-PCR in a new set of animals. Significant differences were found for eight of them (Figure 3). The remaining genes showed the same direction of change as the RNA-Seq results, however these were not significant. We also measured the expression of these genes in animals that received Pru p 3 only, without LPS (Supporting Information Figure S2) finding no significant differences for the majority of these genes, except for *Cxcl1*, *Cxcl2*, *Rnf124* and *Fosl2*, which were found to be downregulated. Finally, we measured expression of the genes in animals that received LPS only, and compared this to naïve (control) animals in a further set, finding no significant changes in expression between groups (Supporting Information Figure S3).

Enrichment of functional categories amongst differentially expressed genes

Functional enrichment analysis was performed on the 181 DE genes with at least a two-fold change in expression, by looking for overrepresentation of functional categories. Annotation could be found for 159 genes, using the DAVID functional enrichment tool and the most significant results, along with the genes and the

direction of change are shown in Table 2. The remaining 22 genes with no annotation in the DAVID database are provided in Supporting Information Table S2. The Gene Ontology (GO) functional categories most highly enriched among the DE genes were “extracellular region” and “extracellular space”, corresponding to genes that code for proteins found in the space external to the plasma membrane, including those secreted from a cell, and found in the interstitial fluid or blood. As might be expected, there was a clear overrepresentation of genes related to inflammatory processes and immune system related signalling including TGF- β receptor signalling. The most significant GO biological processes were “Inflammatory response”, due to multiple chemokines (*Cxcl1*, *Cxcl2*, *Ccl8*), interleukins (*Il6*, *Il10*) and NF- κ b related genes (*Nfkbiz*, *Rel*); and “Response to cytokine”, due to cytokine receptors (*Lifr*), enzymes (*Ptgs2*) and secreted proteins (*Serpina1b*, *Serpina1d*). The full list of functionally enriched categories is shown in Supporting Information Table S3. Perhaps surprisingly, there was enrichment for various diseases according to the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, this appears to be due to the complement related genes in the list of DE genes, *C1qa*, *C1qb* and *C1qc* and the immunostimulatory cytokine, *Il6*. This is a common issue in functional enrichment analysis, where genes with multiple functions lead to the appearance of seemingly unrelated process. In terms of KEGG biological pathways, there was enrichment for genes involved in “Complement and coagulation cascades”. There was also enrichment for the GO category “Response to molecule of bacterial origin”, due to *Cxcl1*, *Cxcl2*, *Il10*, and the LPS co-receptor, *Cd14*. In terms of protein domains, a significantly high enrichment for genes encoding proteins with growth factor like domains was found.

Comparison with Human Data

The list of DE genes was compared to those of Stone et al.^[11] who compared gene expression between patients and controls during anaphylaxis and who sent us

their full list of changing genes to facilitate a thorough comparison. They detected 67 genes as DE in PBLs taken from humans suffering anaphylaxis one hour after arrival at the emergency room (44 upregulated, 23 downregulated), of which 60 had mouse orthologues measurable in this current experiment. Eight of these were also found among the 237 genes deemed as DE in our anaphylaxis model (Table 3), representing significant overlap between gene lists ($p=8.73 \times 10^{-8}$, based on the hypergeometric distribution). Moreover, all changes were in the same direction for both experiments. At the functional level, there was overlap in terms of pathways related to the innate immune response. There was also overlap in terms of Interleukin 1 and LPS related pathways, although these were not significant in our dataset following multiple testing correction ($p=0.0036$ and $p=0.0047$, respectively). Despite being found in the human dataset, there was no enrichment for prostaglandin E2 related processes in our dataset, although the *Ptgs2* gene itself was DE.

Stone et al. also detected 2801 genes as DE after 3 hours in the same patients, of which 2490 had mouse orthologues that could be measured in our experiment. A total of 38 of these genes overlapped with the DE genes detected in our model, giving p-value of 8.26×10^{-4} . There was more overlap at the pathway level, with significant enrichment in both datasets for functional groups and pathways related to immune cell responses, cytokine signalling and response to molecules of bacterial origin, moreover the genes encoding CD14 were DE in both human and mouse datasets.

DISCUSSION

DCs play multiple roles in orchestrating the immune response, and a key role in allergic sensitization. Here we examined their involvement in the immunological mechanism of anaphylactic reactions induced by food-antigens, motivated by recent findings looking at gene expression in anaphylactic patients and showing the role of DCs in the process of sensitization ^[11, 17, 18]. We performed high-throughput transcription profiling of lymph node-derived DCs using RNA-Seq technology. In addition to increases in antibody producing cells in these animals, we found differences in expression between anaphylactic mice and controls for 237 genes. The majority of these were upregulated, suggesting that increased DC activity may play a role in anaphylaxis, in keeping with previous findings ^[8].

The strongest expression changes are shown by the labelled genes in Figure 2. These include two members of the Fos gene family, *Fos* and *Fosl2*. These leucine zipper proteins form part of the transcription factor complex, AP-1, along with *Jun* (also overexpressed here), which regulates expression in response to cytokines and growth factors. Interestingly, AP-1 production also occurs as a result of IgE cross-linking in stimulated mast cells ^[36, 37]. Similar results were found for anaphylactic humans, with DE for *FOS* at a comparable time point, and *FOS*, *FOSL2* and *JUNB* later ^[11]. *Vps37b*, another highly overexpressed gene, is known to function as part of the ESCRT-I complex, which regulates vesicular trafficking, important for inflammatory mediator secretion ^[38], a key mechanism in anaphylaxis. Moreover, expression of *Vps37b* decreases in the T cells of patients receiving peanut oral immunotherapy ^[39], but increases here, raising the prospect of a role in the protective effects of immunotherapy. The Histidine Decarboxylase gene *Hdc*, which encodes a protein that catalyzes histamine synthesis, is also highly overexpressed. Whilst the role of histamine in inflammation and IgE mediated allergic reactions is undisputable, reasons for its overexpression in DCs are less obvious, nevertheless, histamine can

be both i) produced by and ii) influence DC function ^[40]. Interestingly, *Plaur* is highly overexpressed here; variants in this gene have been associated with asthma ^[41].

The most significantly overrepresented functional categories are related to proteins secreted into the extracellular space. Although much is known about the release of proteins and other substances from mast cells during anaphylaxis ^[42, 43], DCs have not been studied in as much detail. Given these findings, further studies would appear to be worthwhile.

Somewhat perplexing, we see the upregulation of the cytokine encoding genes *Il6* and *Il10* in anaphylactic mice. These encode cytokines that can have anti- or pro-inflammatory roles, depending on context. Gut-mucosal DCs have been shown to secrete IL-10 and induce its expression in T cells, however this has been associated with tolerance, rather than inflammatory responses ^[44, 45]; moreover IL-10 has been shown to cause DCs to induce tolerance through regulatory T cell differentiation ^[46]. Nevertheless, IL-10 can have pro-inflammatory effects in some contexts ^[47, 48]. This ties in with the overrepresentation of TGF- β related processes in the functional enrichment results – in fact it has been shown that DCs promote tolerance through regulatory T cell differentiation under the influence of TGF- β from intestinal epithelial cells ^[49]. Previous work examining changes in expression in DCs from the peripheral blood of cow's milk allergy patients also found increased levels of IL-6 and IL-10 ^[50]. Another possible explanation is related to the physiology of the anaphylactic response, as muscular contractions have been shown to increase IL-6, IL-10 and IL-1RA ^[51, 52]. IL-1 mediated signalling was overrepresented both here and in humans ^[11]. *Il22* was also found to be overexpressed in anaphylactic mice, almost by four-fold. This gene encodes a cytokine from the same family as *Il10* and is thought to have a role in allergy ^[53]. Other cytokine genes showing DE include *Cxcl1*, *Cxcl2* and *Ccl8*. The transcripts for these cytokines appear to show strong association with the RNA binding protein HuR, in inflammatory lung tissue ^[54]. Moreover, deletion of TLR4 has been shown to abolish CXCL secretion and

therefore neutrophil recruitment in a model of pollen allergy ^[55]. Interestingly, we find the cytokine encoding gene *Ccl8* downregulated in anaphylactic animals: this cytokine has roles in activation of the immune system including mast cells and basophils and is secreted by DCs; as such its downregulation here is somewhat surprising. It is tempting to speculate this is related to attempts to control the reaction through some kind of negative feedback route. In favour of this hypothesis, it has been shown that DCs activated by the transcription factor PPAR γ produce less of this molecule ^[56]. However this result highlights a limitation related to this type of global analysis: although many genes can be found to be changing, it can be difficult to ascertain whether these genes are involved in the mechanisms leading to anaphylaxis, a consequence of them, or even part of attempts to control it. As such, further experiments are essential to further disentangle the underlying processes these genes are involved in.

Several complement genes were downregulated, specifically the C1q subunits, *C1qa*, *C1qb* and *C1qc*. Whilst these are known to be expressed by DCs, the reason for the decreased expression here is unclear, although they have been shown to increase in DC cells during pollen specific immunotherapy ^[18] indicating a role in tolerance responses.

We found DE for several genes associated with LPS-related signalling pathways. There is evidence for the role of these pathways in allergy induction, as has been shown for Der p 2 and Par j 1 ^[57, 58]. It must be stressed that LPS was not used here in the challenge to induce anaphylaxis. Moreover, LPS-related genes were also found to be differentially expressed in humans during anaphylaxis ^[11], indicating that these pathways may be intrinsically involved. This finding is given further weight by the additional comparison made here using mice given intranasal LPS only during the “sensitization” procedure, for which no significant changes in expression were found compared to control mice that did not receive LPS.

Clearly, there are many genes found here that were not found in the human data-set, and vice versa. These discrepancies can be explained by: i) the use of different species, human vs. mice, ii) patients beginning their reactions at different times before arriving at the ER iii) heterogeneous causal agents vs. food allergen-induced anaphylaxis and iv) use of PBLs vs. DCs. Moreover, changes in PBLs may be due to either change in transcriptional regulation or changes in the composition of cell types. Nevertheless, there was still a significant overlap of DE genes, particularly at the first time-point; moreover, the direction of change was the same in both cases for all overlapping genes.

An important caveat here is that most animals reacted very strongly to challenge, and no medicine was given to resolve the symptoms. Moreover, despite intranasal sensitization and challenge using food extract, the challenge was performed intraperitoneally. This route was chosen in order to ensure a response could be obtained; intragastric challenge has been shown as less reliable due to immediate allergen digestion. Despite these limitations, we demonstrate for the first time many changes in DCs that occur in the context of anaphylaxis, including genes encoding proteins involved in signal detection (e.g. *Cd14*), relaying this signal (Interleukins and Cytokines) and effecting changes (*Fos*, *Fosl2*), as well as showing a putative role for mast cell activation, vesicular trafficking and protein secretion, proinflammatory response and cell recruitment, whereas complement genes related with the tolerant responses are downregulated. Taken together the results suggest various roles for DCs in the anaphylactic response. This study adds to the growing compendia of anaphylaxis profiling experiments and suggests multiple potential targets for further study, which we have made publicly available in their entirety. Such approaches could involve time-series studies investigating gene-expression at multiple points during sensitization and before and after challenge; other approaches might investigate the sensitization procedure using knockout mice to investigate the role of the knocked out gene. Given the overlap with humans, this study also shows

the value of the mouse model for anaphylaxis. Future research should address the cross talk between these cells and other important players including T cells and mast cells.

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Raw and processed data along with the lists of differentially expressed genes from the RNA-Seq experiment are available from the Gene Expression Omnibus TBA ^[35].

Author contributions

JRP and CM designed the study. MJR, CM and JR were involved in the animal model and MJR was responsible for obtaining the dendritic node samples. MJR and FP were responsible for RNA extraction and immunological studies. JRP, CM, MJR, FP, GB, JR, MJT and AD were responsible for analysis and critical interpretation of results. FP, MCP and AR performed the RT-PCR analysis. CO and JRP performed the bioinformatics analysis. All authors helped draft and revise the manuscript and approved the final version.

Statement regarding conflicts of interest

None of the authors declare any conflict of interest.

FIGURE LEGENDS

Figure 1. Immunological and physiological data following intraperitoneal challenge. * represents p-value < 0.05; ** represents p-value < 0.01; *** represents p-value < 0.001; A) Changes in temperature 30-40 minutes following challenge for anaphylactic, control and Pru p 3 only receiving animals. Comparisons made using paired t-tests. B) Combined symptom score upon challenge for the three groups. C) Number of allergen-specific antibody secreting cells for IgE and IgG1 for the three groups. Comparisons made using ANOVA followed by Tukey's test. D) Sera allergen-specific antibody levels of IgE and IgG1 for the three groups. Comparisons made using ANOVA followed by Tukey's test.

Figure 2. Differential expression data from the RNA-Seq analysis. A) Expression for the Anaphylactic and control animals. Darker colour = higher expression. B) Relationship between normalised expression level and log2 fold change for all genes measured. Triangles represent genes with a high fold change than can be shown here, the darker (red) points show significantly differentially expressed genes. C) Log2 fold change vs. Adjusted p-value (FDR) (volcano) plot for all genes measured. Again, triangles represent genes with a higher fold change than could be shown here. The genes with the lowest adjusted p-values are shown here.

Figure 3. Verification of RNA-Seq results using RT-PCR. Data shown as ΔCq values for the RT-PCR data (top row) and as normalized expression values (using the DESeq2 normalization function as described in Methods) for the RNA-Seq data (bottom row) for 8 of the 11 genes replicated. P-values represent a one-tailed test, with the expected direction of change corresponding to that detected by the RNA-Seq analysis.

TABLES

Table 1. Groups of animals used to measure gene expression using RNA-Seq (Anaphylactic and Control) and RT-PCR (Anaphylactic, Control, Pru p 3 only, and LPS only).

Group	Treatment (Intranasal)	Challenge (Intraperitoneal)
Control	PBS (12 μ l)	Pru p 3 (100 μ g) in 50 μ l PBS
Anaphylactic	Pru p 3 (20 μ g) + LPS (20ng) in 12 μ l PBS	
Pru p 3 only	Pru p 3 (20 μ g) in 12 μ l PBS	
LPS only	LPS (20ng) in 12 μ l PBS	

Table 2. Top and most relevant functional categories from the DAVID software, using all differentially expressed genes with a log₂ fold change less than -1 or greater than 1 and with an adjusted p-value below 0.05.

Group Type	Group Name	P-value	Genes
Cellular Component (GO)	Extracellular region	7.08E-13	Upregulated: CXCL1, IL1R2, CXCL2, JCHAIN, IL10, WISP1, CEACAM16, THBS1, LOXL2, FLRT3, IL6, LIFR, IL22, PLAUR, HBEGF, ADAMTS1, RELN, CD14, HBEGF Downregulated: ENPP2, ENPP3, LTBP4, COL3A1, CCL8, C1QC, MMRN2, CHIA1, GLYCAM1, SERPINA
	Extracellular space	1.34E-07	Upregulated: CXCL1, CXCL2, JCHAIN, IL10, CEACAM16, CPA3, THBS1, LOXL2, FLRT3, IL6, IL22, THBD, HBEGF, RELN, CD14 Downregulated: ENPP2, LTBP4, COL3A1, CCL8, C1QC, MMRN2, CHIA1, SERPINA1B, CST10, LRG1, SERPINA1D, PIP, BGLAP3, AMY1, LPO, EGFL7, CAR6
Biological Process (GO)	Inflammatory response	3.27E-06	Upregulated: CXCL1, NFKBIZ, IL6, PTGS2, REL, CXCL2, THBS1, IL10, CD14, EPHA2 Downregulated: CHIA1, CCL8, CD5L, CHST4
	Response to cytokine	3.40E-06	Upregulated: FOS, PTGS2, REL, JUN, LIFR Downregulated: SERPINA1B, SERPINA1D, COL3A1
	Transforming growth factor beta receptor signaling pathway	2.72E-05	Upregulated: FOS, GCNT2, SMAD7, JUN, BAMBI, Downregulated: LTBP4, COL3A1
	Positive regulation of smooth muscle cell proliferation	3.93E-05	Upregulated: EGR1, IL6, WISP1, PTGS2, JUN, HBEGF, THBS1 Downregulated:
	Response to cAMP	4.89E-05	Upregulated: FOS, THBD, DUSP1, JUN, CREM, PER1 Downregulated:
	Immune response	5.85E-05	Upregulated: CXCL1, IL6, CXCL2, MCPT4, CTLA4, THBS1, IL22, IL10 Downregulated: ENPP2, ENPP3, CCL8
	Angiogenesis	1.15E-04	Upregulated: PTGS2, JUN, EFNB2, HBEGF, RORA, EPHA2 Downregulated: EMCN, EGFL7, TIE1, MMRN2
	Response to molecule of bacterial origin	1.28E-04	Upregulated: CXCL1, CXCL2, IL10, CD14 Downregulated:

	Cell chemotaxis	3.71E-04	Upregulated: CXCL1, CXCL2, HBEGF, EPHA2 Downregulated: ENPP2, CCL8
Pathway (KEGG)	Pertussis	6.01E-06	Upregulated: FOS, IL6, JUN, IL10, CD14 Downregulated: C1QA, C1QB, C1QC
	Complement and coagulation cascades	8.12E-05	Upregulated: THBD, PLAUR Downregulated: C1QA, C1QB, SERPINA1B, SERPINA1D, C1QC
	Prion diseases	2.28E-04	Upregulated: EGR1, IL6 Downregulated: C1QA, C1QB, C1QC
	Chagas disease (American trypanosomiasis)	4.33E-04	Upregulated: FOS, IL6, JUN, IL10 Downregulated: C1QA, C1QB, C1QC
Protein Domain (InterPro)	Epidermal growth factor-like domain	3.81E-05	Upregulated: ADAM11, THBD, PTGS2, HBEGF, RELN, THBS1, MEGF11 Downregulated: EGFL7, LTBP4, TIE1
	EGF-like, conserved site	6.50E-05	Upregulated: ADAM11, THBD, HBEGF, RELN, THBS1, MEGF11 Downregulated: EGFL7, LTBP4, TIE1
Molecular Function (GO)	Transcription regulatory region DNA binding	4.64E-04	Upregulated: EGR1, FOS, FOSL2, CEBPB, SMAD7, JUN, KLF11 Downregulated: SALL2, KLF15

GO: Gene Ontology, KEGG: Kyoto Encyclopedia of Genes and Genomes

Table 3. Differential expression of gene (mouse) and orthologue (human), with differential expression and p-value data. Human data taken from Stone et al ^[11].

Gene Symbol (mouse)	Human Orthologue	Log2 FC (mouse)	P-value (mouse)	Log2 FC (human)	P-value (human)
<i>Crem</i>	<i>CREM</i>	1.83	4.55E-08	0.322	1.92E-04
<i>Dusp1</i>	<i>DUSP1</i>	1.51	3.48E-04	0.916	1.44E-04
<i>Fos</i>	<i>FOS</i>	3.19	2.05E-14	0.556	1.96E-04
<i>Il1r2</i>	<i>IL1R2</i>	2.02	2.71E-06	1.12	1.49E-05
<i>Pde4b</i>	<i>PDE4B</i>	0.839	2.08E-05	0.298	1.59E-04
<i>Plaur</i>	<i>PLAUR</i>	2.49	1.49E-09	0.585	2.92E-04
<i>Stk17b</i>	<i>STK17B</i>	0.879	4.08E-04	0.233	3.98E-04
<i>Tnfaip3</i>	<i>TNFAIP3</i>	0.83	2.36E-04	0.518	1.25E-04

DE: differential expression

SUPPORTING INFORMATION

Supporting Methods. Full details of anaphylaxis induction, Immunological change measurement, lymph node extraction and dendritic cell enrichment.

Table S1. List of all significant differentially expressed genes.

Table S2. List of differentially expressed genes for which no functional annotation could be found.

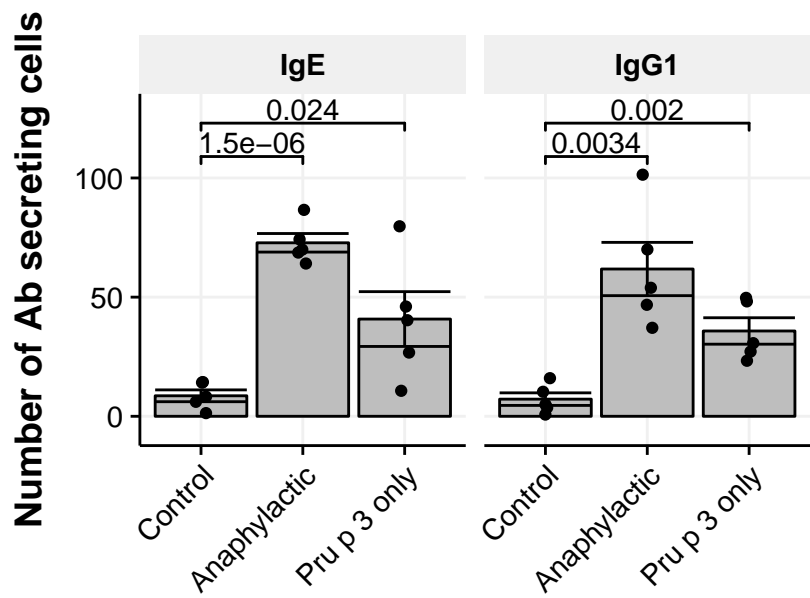
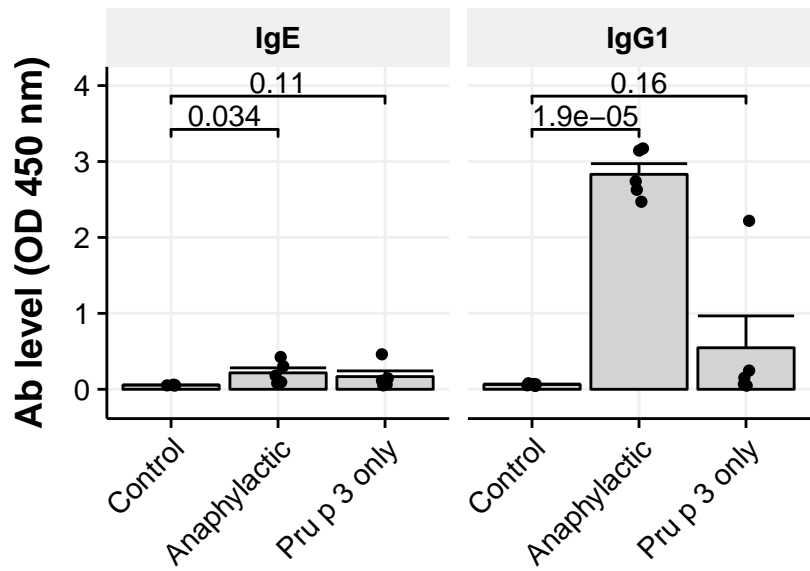
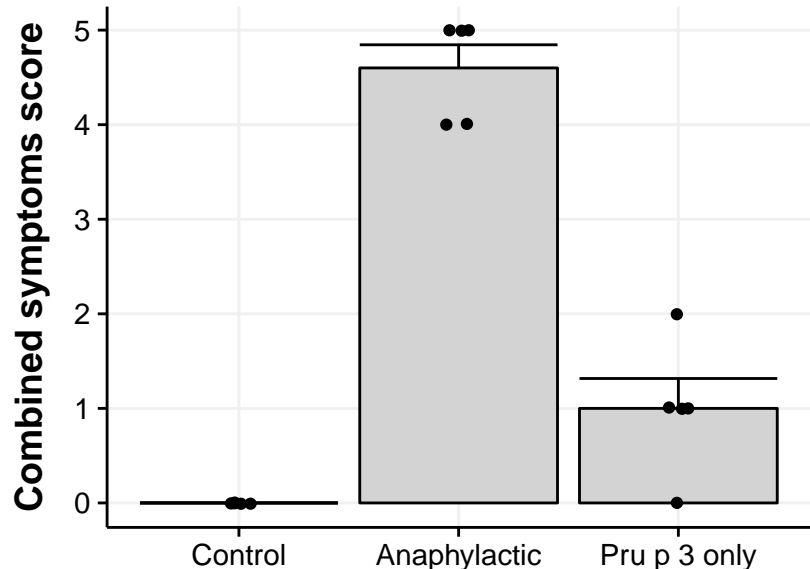
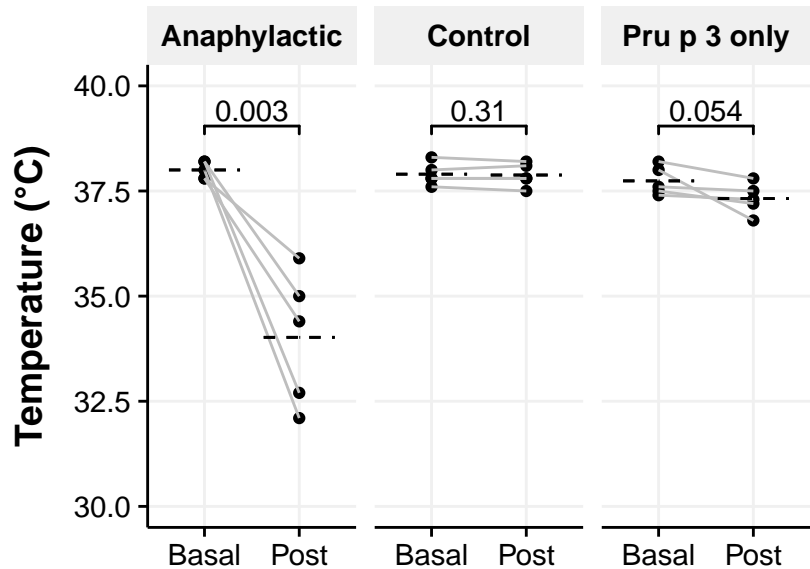
Table S3. List of all significant DAVID categories.

Figure S1. RNA-Seq principal component analysis (PCA) and multidimensional scaling (MDS) plots showing distance between samples in two dimensions. PC1: first principal component; PC2, second principal component.

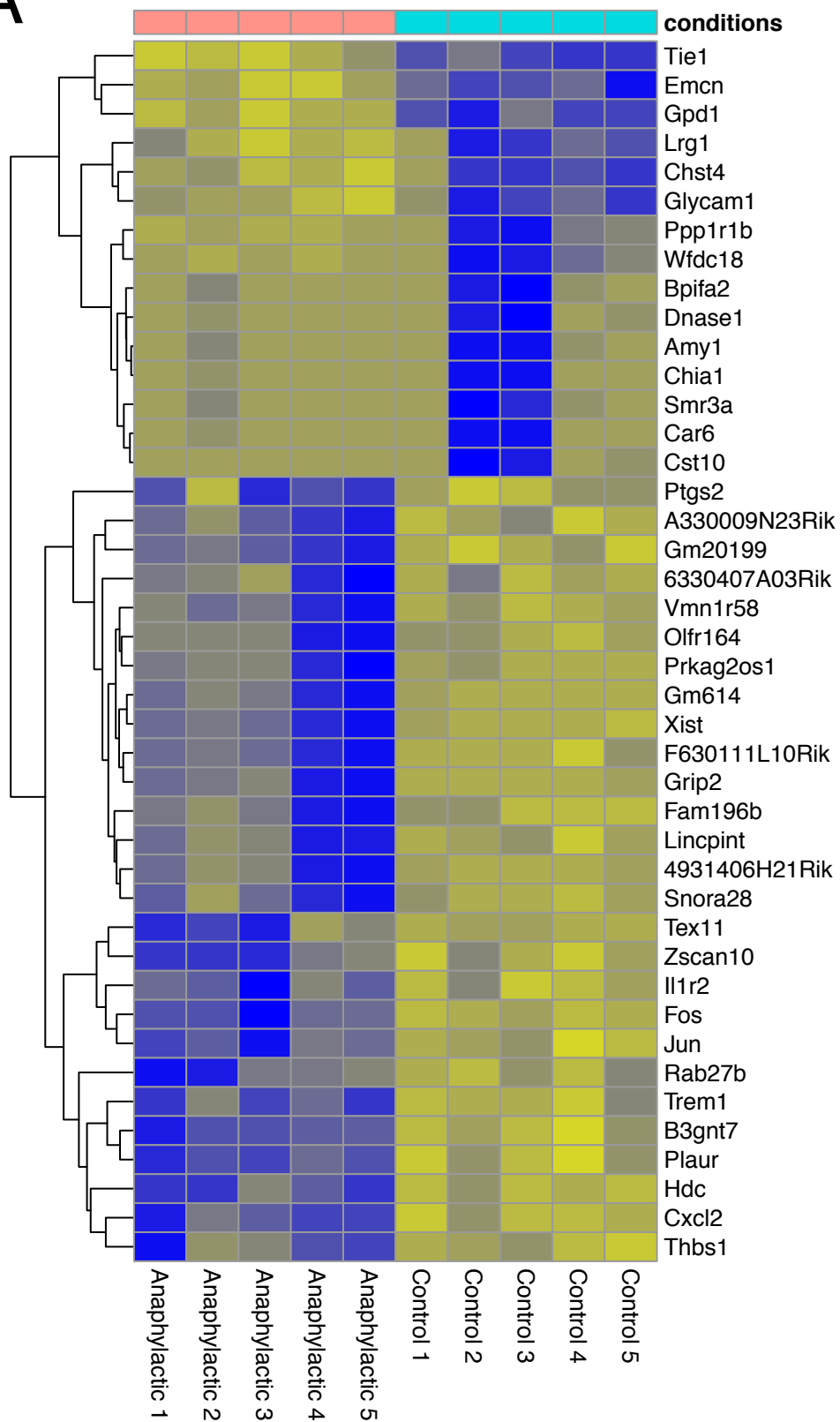
Figure S2. RT-PCR ΔCq levels for the genes tested in anaphylactic, control, and animals receiving Pru p 3 only, compared to naïve control animals. P-values calculated using the Mann-Whitney U test.

Figure S3. RT-PCR ΔCq levels for the genes tested in anaphylactic, control, and animals receiving LPS only, compared to naïve control animals. P-values calculated using the Mann-Whitney U test.

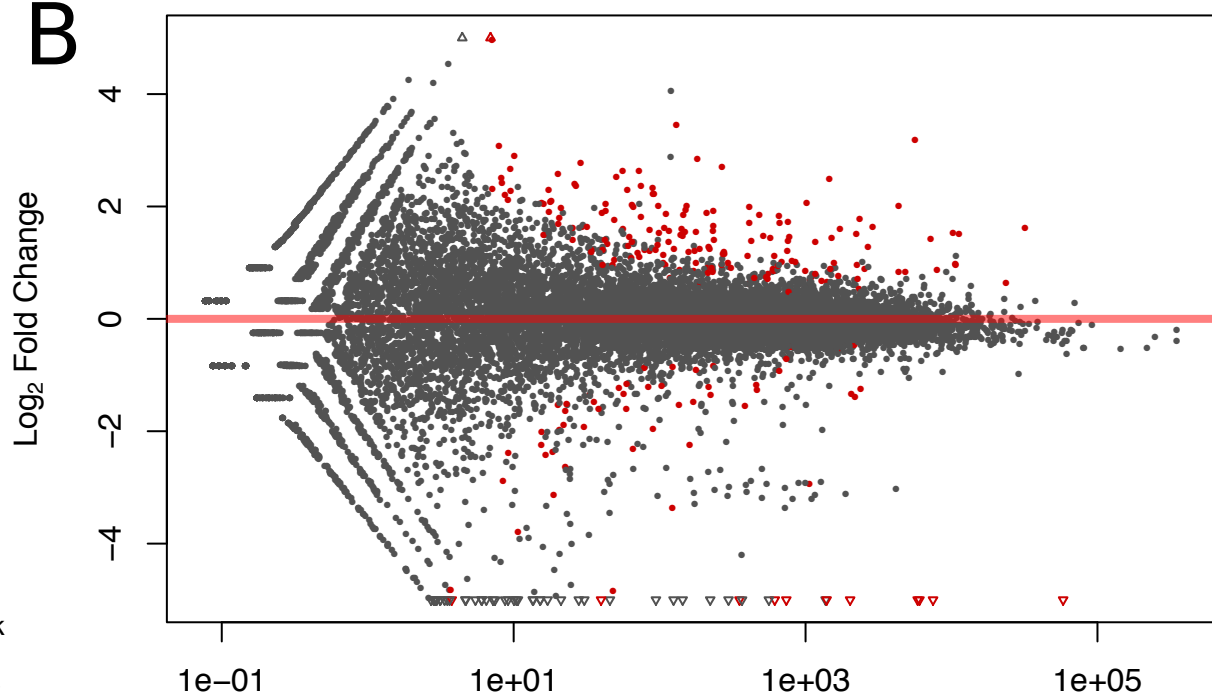
Figures 1 to 3 in order



A



B



C

