RESEARCH ARTICLE

T-Cell Activation and Early Gene Response in Dogs

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Abstract

T-cells play a crucial role in canine immunoregulation and defence against invading pathogens. Proliferation is fundamental to T-cell differentiation, homeostasis and immune response. Initiation of proliferation following receptor mediated stimuli requires a temporally programmed gene response that can be identified as immediate-early, mid- and late phases. The immediate-early response genes in T-cell activation engage the cell cycle machinery and promote subsequent gene activation events. Genes involved in this immediate-early response in dogs are yet to be identified. The present study was undertaken to characterise the early T-cell gene response in dogs to improve understanding of the genetic mechanisms regulating immune function. Gene expression profiles were characterised using canine gene expression microarrays and quantitative reverse transcription PCR (qRT-PCR), and paired samples from eleven dogs. Significant functional annotation clusters were identified following stimulation with phytohemagluttinin (PHA) (5 μg/ml), including the Toll-like receptor signaling pathway and phosphorylation pathways. Using strict statistical criteria, 13 individual genes were found to be differentially expressed, nine of which have ontologies that relate to proliferation and cell cycle control. These included, prostaglandin-endoperoxide synthase 2 (PTGS2/COX2), early growth response 1 (EGR1), growth arrest and DNA damage-inducible gene (GADD45B), phorbol-12-myristate-13-acetate-induced protein 1 (PMAIP1), V-FOS FBJ murine osteosarcoma viral oncogene homolog (FOS), early growth response 2 (EGR2), hemogen (HEMGN), polo-like kinase 2 (PLK2) and polo-like kinase 3 (PLK3). Differential gene expression was re-examined using qRT-PCR, which confirmed that EGR1, EGR2, PMAIP1, PTGS2, FOS and GADD45B were significantly upregulated in stimulated cells and ALAS2 downregulated. PTGS2 and EGR1 showed the highest levels of response in these dogs. Both of these genes are involved in cell cycle regulation. This study provides a comprehensive analysis of the early T-cell gene response to activation in dogs.
Introduction

The immune system is responsible for neutralizing invading pathogens through activation of a complex protective mechanisms relying on the concerted action of inflammatory and immune cells, regulatory mediators (cytokines), antibodies and complement molecules. T-lymphocytes are a key sub-population of immune cells that are vital for immunoregulation and cytotoxic effector responses. Proliferative responses are a fundamental feature of T-cell biology, and in response to receptor activation, they undergo a high rate of proliferation during development and in response to antigen induced activation. Control of the proliferative response is mediated by temporally programmed gene expression that can be identified as immediate-early, mid- and late phases [1–3]. Amongst other things, the early response genes lead to engagement of the cell cycle machinery, and regulate downstream molecular and cellular events [2]. These events are key determinants of an adaptive immune response and will dictate cell-mediated immunoregulatory and homeostatic outcomes.

Activation of T-cells in humans and mice, using either a combination of receptor agonists or mitogens, leads to intracellular phosphorylation events and induction of signalling cascades [4–6]. These events activate and regulate multiple pathways including those that involve mitogen-associated protein kinase (MAPK), extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinases (JNK), nuclear factor-κB (NF-κB) and nuclear factor of activated T-cells (NFAT) [3–5, 7]. A study investigating gene expression profiles of human lymphocytes revealed groups of functionally similar genes showing distinct expression patterns following activation over a 48hr period [2]. The most immediate changes in expression were seen in transcription factors (EGR1, EGR2, NF-κB p50), followed by cytokines, adhesion molecules and genes involved in signalling pathways (RAB13, TNF receptor associated factor) [2]. After a longer period of activation genes involved in DNA replication (DNA Topoisomerase II), cellular proliferation, metabolic enzymes and structural proteins (ribonuclease) are induced [2]. Similar results have been shown in mice, with significant changes in expression of functional groups affecting transcription, signal transduction, cell division and cell surface receptors [7].

Molecular events during T-cell activation are poorly understood in dogs, with no reported studies investigating the early response of genes to lymphocyte activation on a genome-wide scale. A small number of studies have instead focused on changes in expression of individual genes in diseased states [8]. The present study was undertaken to characterise T-cell proliferation in dogs using global expression analysis to identify and explore the variation in expression of key genes, with a specific focus on the early T-cell gene response. T-cell activation was initiated using phytohemagglutinin (PHA) a widely used mitogen which binds primarily to T-cells subsequently stimulating rapid and robust T-cell proliferation [9, 10]. PHA has been successfully used in multiple species to stimulate T-cell proliferation including dogs [6, 11, 12] and is known to closely reflect the gene response of T-cells activated by TCR [13, 14].

Methods

Blood collection and cell stimulation

A total of eleven dogs (Canis lupus familiaris) were selected for inclusion in the gene expression analysis, both sexes were represented with an age range from eight months to seven years (Table 1). Dogs used in the study were all privately owned pets and were volunteered with informed consent; they remained under the care of their owners and were not housed for the purposes of this study, or held for any duration of time. Blood was collected into EDTA coated vacutainers, and the leukocyte fraction was isolated by gradient centrifugation. A registered veterinarian conducted the blood draw procedure in the presence of the owners with minimal
Cells were divided into two aliquots and each was resuspended in 1 ml Dulbecco’s Modified Eagle Medium media. Both aliquots from each dog were incubated for 4 hours, one in the presence of 5 μg/ml of PHA, a mitogen for T-cell activation (referred to as the stimulated sample), the other aliquot served as the non-stimulated control sample. After incubation the cells were collected by centrifugation, and then lysed in RLT buffer (QIAmp; Qia-gen Aust). Once lysed samples were immediately frozen until further use (within 48 hr).

### RNA isolation

Total RNA was isolated from samples using a modified extraction procedure and micro-spin columns (QIAmp; Qiagen, Melbourne, Vic). The lysate was pipetted directly into a QIAshred-der spin column following incubation in a 37°C water bath until completely thawed and salts dissolved. 70% ethanol was added to the homogenised lysate before it was transferred into a QIAamp spin column and washed using a high-salt buffering system allowing RNA to bind to the QIAmp membrane as contaminants are removed. Purified RNA was eluted in 30 μl of RNase-free water followed by a second elution in 50 μl and stored at -80°C.

### Microarray Analysis

Frozen RNA samples from stimulated and non-stimulated cells from eleven dogs were transported to the core facility for processing (Ramaciotti Centre for Gene Function Analysis, Sydney). RNA quality and integrity was confirmed with a bioanalyzer (Agilent Technologies, CA, USA) providing an RNA integrity number for each sample, all of which were >7. RNA was then amplified and labelled before being hybridised to an Affymetrix Canine Gene 1.0 ST Array according to the manufacturer’s instructions. Array chips were read using the GeneChip.

| Dog No. | Dog age | Dog Sex | Sample | Treatment |
|---------|---------|---------|--------|-----------|
| 1       | 4yrs    | M       | SM1    | Unstimulated |
|         |         |         | SM1P   | Stimulated |
| 2       | 4yrs    | F       | SM3    | Unstimulated |
|         |         |         | SM3P   | Stimulated |
| 3       | 15 months | M      | SM4    | Stimulated |
| 4       | 2.5yrs  | F       | SM9    | Unstimulated |
|         |         |         | SM9P   | Stimulated |
| 5       | 4yrs    | F       | SM13   | Unstimulated |
|         |         |         | SM13P  | Stimulated |
| 6       | 2yrs    | F       | SM14   | Unstimulated |
|         |         |         | SM14P  | Stimulated |
| 7       | 7yrs    | F       | SM16   | Unstimulated |
|         |         |         | SM16P  | Stimulated |
| 8       | 7yrs    | M       | SM17   | Unstimulated |
|         |         |         | SM17P  | Stimulated |
| 9       | 8 months | M      | SM5    | Unstimulated |
|         |         |         | SM5P   | Stimulated |
| 10      | 15 months | F      | SM6    | Unstimulated |
|         |         |         | SM6P   | Stimulated |
| 11      | 1yr     | F       | SM10   | Unstimulated |
|         |         |         | SM10P  | Stimulated |

doi:10.1371/journal.pone.0121169.t001
Scanner 3000 (Affymetrix) and probe intensities obtained using GCOS operating software (Affymetrix). The data discussed in this publication have been deposited in NCBI’s Gene Expression Omnibus [15] and are accessible through GEO Series accession number GSE65158.

**Data Analysis**

The Affymetrix Canine Gene 1.0 ST Array data from dogs one to eleven was first analysed for technical faults; one non-stimulated sample (SM4) failed to amplify and was not included. Background correction and quantile normalisation were performed on the data with probe-set intensities calculated accordingly using the Robust Multichip Average (RMA) [16] algorithm available in the Affymetrix Expression Console. Differential expression (DE) of genes between stimulated and unstimulated T-cells was defined using the limma function (linear model approach) [17] incorporated in the R package [18]. A rank fold-change was used to identify DE genes, with a cut-off of ±2-fold, corresponding to a Benjamini & Hochberg adjusted false discovery rate (FDR) of 0.05 [19]. Differentially expressed genes were then analysed for functional annotation clustering using DAVID (The Database for Annotation, Visualization and Integrated Discovery) with a group enrichment score cut-off of 1.3 (EASE = 0.05) [20]. The EASE score is the geometric mean of all the enrichment p-values of members within each annotation cluster. The group enrichment score represents the EASE score (in—log scale), ranking the clusters according to their biological significance [20]. Cluster analysis of expression data from DE genes and the gene set corresponding to the Toll-like-receptor signaling pathway were performed using Heatmap, an integrated function implemented in R, with additional annotations sourced from the program Heatplus [21].

**Quantitative Reverse Transcription PCR**

Additional quantitative analysis for individual genes was performed using qRT-PCR. Oligonucleotide primers were designed for ten differentially expressed genes, chosen for their roles in cell cycle regulation and proliferation, namely EGR1, EGR2, PMAIP1, PLK2, PLK3, PTGS2, RGS1, FOS, GADD45B and ALAS2, and two reference genes SDHA and GAPDH using Primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/), and then synthesised using a commercial source (Invitrogen, Melbourne, VIC, Australia) (Table 2). First strand cDNA synthesis was performed using RNA from both stimulated and unstimulated samples from six of the eleven dogs by the GoScript Reverse Transcriptase System according to manufacturers instructions (Promega, Table 2. Sequence of primers used in qRT-PCR reactions.

| Primer Set | Forward Primer (5'-3') | Reverse Primer (5'-3') |
|------------|------------------------|------------------------|
| PTGS2      | ATGGGTGTGAAAGGCAAGAA   | TGATGGGTAAAGTGCTGGGC   |
| EGR1       | GCTGGAGGAGTGATGCTGCT   | TGTCGGGAAAAGACCTGCGG   |
| FOS        | GGAACAGGAGACAGACCAAC   | TAGGGAAGCCACGGAGACATC  |
| GADD45B    | CTGTCAACAGCCCTACAC     | TCAACAGCTCTGCTGCTGG    |
| EGR2       | GCCGTAGACAAATCCAGTG    | CCAAGGACCTTCTCTCCA     |
| RGS1       | ATTAGTCTTCTGCTGCTTG    | CGTAGGGGTGTGGTTTTTA    |
| ALAS2      | GGAACAGCTGTAGTTATGC    | GATCTTAAAGCCACAGAGCG   |
| PMAIP1     | CGAAGAGCTGAAGTGGAGGT   | CTGAGGGGAGAGTTTGGCAT   |
| PLK2       | TCCAGCCAACCTACACTACA   | TGTCTTCAAGGCATTACCTGA  |
| PLK3       | TCTGGTATGGTCAAGCTGTTG  | GCACGGCTTTTCTGTTTGGC   |
| SDHA       | TGTCAACAGGAGCCCAATC    | ACCAAGTCCAAGGAGCGAGTT  |
| GAPDH      | GCCAAAAGGGTCACTCATC    | GGCGCATCCACAGTCTTCT    |

doi:10.1371/journal.pone.0121169.t002
Australia). RNA from the additional five dogs was unavailable for use in this experiment. Quantitative PCR was performed in duplicate under the following conditions: 20 μl reaction containing 2 μl cDNA transcript, 8 μl QuantiFast SYBR Green PCR Master Mix (Qiagen, Victoria, Australia) and 8 pmol of each primer, using a Rotor Gene 6000 system (Corbett Research, NSW, Australia). The samples were denatured at 95°C for 5 mins followed by a 35 cycle PCR run of 94°C for 15 secs, 60°C for 20 secs and 72°C for 40 secs followed by a melt curve analysis. Calculation of the relative levels of expression, normalised to the reference genes, between stimulated and unstimulated samples and statistical analysis was determined using the software REST (Qiagen, Australia).

Ethics Statement

This study was approved by The University of Sydney Animal Ethics Committee, under protocol number 444.

Results

Microarray analysis of T-cell response to stimulation

Second generation canine gene expression microarrays have proven to be an effective way to investigate transcriptome level responses in T-cells. In this study PHA stimulation was used for a relatively short time-frame to provide for the identification of the most immediate events, and the most responsive genes. When stimulated and unstimulated cells were compared, a total of 2914 probe IDs (representing 1302 annotated genes) were identified as differentially expressed. Of these 1302 annotated genes 665 were upregulated in stimulated cells and 637 downregulated. Applying a 2 fold-change cut-off, a total of 12 annotated genes remain (Table 3). Two genes were downregulated in stimulated T-cells relative to unstimulated cells, hemogen (HEMGN) and delta-aminolevulinate synthase 2 (ALAS2). Ten genes were upregulated in stimulated T-cells including, prostaglandin-endoperoxide synthase 2 (PTGS2/COX2), early growth response 1 (EGR1), LOC100684961, growth arrest and DNA damage-inducible gene (GADD45B), phorbol-12-myristate-13-acetate-induced protein 1 (PMAIP1), V-FOS FBJ murine osteosarcoma viral oncogene homolog (FOS), early growth response 2 (EGR2), regulator of G protein signaling 1 (RGS1), OTU domain-containing protein 1 (OTUD1) and polo-like kinase 2 (PLK2). Although the fold change of polo-like kinase 3 (PLK3) fell outside the 2 fold-

| Gene Symbol | Gene Name                                      | Fold Change | P-value |
|-------------|-----------------------------------------------|-------------|---------|
| PTGS2       | PROSTAGLANDIN-ENDOPEROXIDE SYNTHASE 2         | 5.86        | <0.001  |
| EGR1        | EARLY GROWTH RESPONSE 1                        | 5.48        | <0.001  |
| LOC100684961| LOC100684961                                   | 3.29        | <0.001  |
| HEMGN       | HEMOGEN                                       | -2.94       | 0.01    |
| GADD45B     | GROWTH ARREST AND DNA DAMAGE-INDUCIBLE GENE   | 2.63        | <0.001  |
| PMAIP1      | PHORBOL-12-MYRISTATE-13-ACETATE-INDUCED PROTEIN 1 | 2.57      | <0.001  |
| FOS         | V-FOS FBJ MURINE OSTEOSARCOMA VIRAL ONCOGENE HOMOLOG | 2.50  | <0.001  |
| EGR2        | EARLY GROWTH RESPONSE 2                        | 2.37        | <0.001  |
| RGS1        | REGULATOR OF G PROTEIN SIGNALING 1             | 2.11        | <0.001  |
| ALAS2       | DELTA-AMINOEVULINATE SYNTHASE 2               | -2.10       | <0.001  |
| OTUD1       | OTU DOMAIN-CONTAINING PROTEIN 1               | 2.03        | <0.001  |
| PLK2        | POLO-LIKE KINASE 2                            | 2.01        | <0.001  |
| PLK3        | POLO-LIKE KINASE 3                            | 0.90        | <0.001  |

Table 3. Top differentially expressed genes between stimulated and unstimulated T-cells in dogs from the microarray analysis.

doi:10.1371/journal.pone.0121169.t003
change cut-off, it was included in investigations due to its role in cell division and significant P-value. The identification of these genes provides a good indication of the nature of the immediate response, and the level of response in dogs. The functional annotation clustering analysis revealed that within the significantly higher DE genes in stimulated cells there were three functional annotation clusters that had enrichment scores of more than 1.3 and were considered biologically significant. Of these one is associated with cellular structures such as organelle lumen, another receptor signaling pathways and the third phosphorylation (enrichment scores 1.5, 1.43 and 1.33 respectively) (Table 4). In contrast no functional annotation clusters with a score above 1.3 were identified in significantly lower DE genes in stimulated cells.

Hierarchical cluster analysis

Hierarchical cluster analysis is a useful method for investigating consistent and robust expression profiles between treatment groups. Variation in the gene expression patterns is highlighted by hierarchical cluster analysis of DE genes. There was tight clustering of expression profiles from DE genes between stimulated and non-stimulated cells (Fig. 1). The expression profile of nine genes, representative of 76 genes in the TLR signaling pathway, resulted in a significant enrichment score in the functional annotation cluster analysis. Hierarchical cluster analysis of these upregulated genes shows distinct clusters between stimulated and non-stimulated cells with the exception of SM1 and SM9 which cluster with stimulated cells (Fig. 2).

Table 4. Genes belonging to significantly upregulated functional annotation clusters in stimulated T-cells.

| Functional Annotation Cluster                        | Gene Name                                                                 |
|-----------------------------------------------------|---------------------------------------------------------------------------|
| Organelle Lumen                                     | CYTOCHROME C, SOMATIC                                                     |
|                                                     | SURVIVAL MOTOR NEURON                                                     |
|                                                     | ACIDIC (LEUCINE-RICH) NUCLEAR PHOSPHOPROTEIN 32 FAMILY, MEMBER A          |
|                                                     | DIHYDROLIPAMIDE DEHYDROGENASE                                              |
|                                                     | MITOGEN-ACTIVATED PROTEIN KINASE 8                                        |
|                                                     | TRANSMEMBRANE EMP24 PROTEIN TRANSPORT DOMAIN CONTAINING 7                 |
|                                                     | INTERLEUKIN 1, BETA                                                       |
|                                                     | FBJ MURINE OSTEOSARCOMA VIRAL ONCOGENE HOMOLOG                            |
|                                                     | JUN PROTO-ONCOGENE                                                        |
|                                                     | INTERLEUKIN 8                                                             |
|                                                     | TUMOR NECROSIS FACTOR                                                     |
|                                                     | LIPOPOLYSACCHARIDE BINDING PROTEIN                                        |
|                                                     | INTERFERON ALPHA-1/2-LIKE                                                  |
| Toll-like Receptor Signaling Pathway                | MITOGEN-ACTIVATED PROTEIN KINASE 8                                        |
|                                                     | TRANSMEMBRANE EMP24 PROTEIN TRANSPORT DOMAIN CONTAINING 7                 |
|                                                     | INTERLEUKIN 1, BETA                                                       |
|                                                     | FBJ MURINE OSTEOSARCOMA VIRAL ONCOGENE HOMOLOG                            |
|                                                     | JUN PROTO-ONCOGENE                                                        |
|                                                     | INTERLEUKIN 8                                                             |
|                                                     | TUMOR NECROSIS FACTOR                                                     |
|                                                     | LIPOPOLYSACCHARIDE BINDING PROTEIN                                        |
|                                                     | INTERFERON ALPHA-1/2-LIKE                                                  |
| NOD-like Receptor Signaling Pathway                 | INTERLEUKIN 1, BETA                                                       |
|                                                     | INTERLEUKIN 8                                                             |
|                                                     | MITOGEN-ACTIVATED PROTEIN KINASE 8                                        |
|                                                     | TUMOR NECROSIS FACTOR                                                     |
|                                                     | TUMOR NECROSIS FACTOR, ALPHA-INDUCED PROTEIN 3                            |
| RIG-I-like Receptor Signaling Pathway               | MITOGEN-ACTIVATED PROTEIN KINASE 8                                        |
|                                                     | TUMOR NECROSIS FACTOR                                                     |
|                                                     | INTERLEUKIN 8                                                             |
|                                                     | INTERFERON ALPHA-1/2-LIKE                                                  |
|                                                     | INTERLEUKIN 5                                                             |
|                                                     | BCL2-ASSOCIATED X PROTEIN                                                  |
|                                                     | SUPPRESSOR OF CYTOKINE SIGNALING 3                                        |
|                                                     | INTERLEUKIN 1, BETA                                                       |

doi:10.1371/journal.pone.0121169.t004
Six of the ten genes were significantly upregulated in stimulated T-cells when compared to unstimulated T-cells; these include \textit{EGR1}, \textit{EGR2}, \textit{PMAIP1}, \textit{PTGS2}, \textit{FOS} and \textit{GADD45B} (Table 5). ALAS2 was significantly downregulated in stimulated T-cells. \textit{EGR1} and \textit{PTGS2} had the greatest change in expression with \textit{EGR1} upregulated in stimulated cells by 7.5-fold and \textit{PTGS2} by 4.4-fold. While greater differences were observed across nine of the ten genes in the qRT-PCR when compared to those from the microarray, the patterns seen across the genes were consistent with that of the microarray (Fig. 3).

**Discussion**

The present study characterises the immediate-early molecular T-cell stimulatory responses in a cohort of healthy dogs. Microarray and qRT-PCR analysis of gene expression following stimulation identified key genes that control activation and cellular proliferation. Prominent amongst these were \textit{EGR1} and \textit{PTGS2}, with an additional seven of the top DE genes associated with pathways that relate to cell proliferation and differentiation. These genes represent the most sensitive T-cell early response genes to stimulation in dogs and may be useful sensitive quantitative markers of activation.

Functional annotation clusters, identified within the most responsive genes, highlight cellular processes that may play a key role in T-cell activation and proliferation. These clusters
included the NOD-like receptor signaling pathway, the RIG-I-like signaling pathway and the Toll-like receptor (TLR) signaling pathway. Similar intracellular signaling pathways have been highlighted in both humans and mice, and play a significant functional role in T-cell activation [2, 3, 7]. NOD-like receptors (NLRs) are pattern recognition receptors found in lymphocytes, dendritic cells, macrophages and other non-immune cells. They recognise pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) and play a

![Hierarchical clustering and heat map of expression levels of genes involved in TLR signaling pathways. Expression levels are colour coded with shades of red, green and black corresponding to an increase, decrease and no change in gene expression, respectively.](image)

doi:10.1371/journal.pone.0121169.g002

Table 5. Analysis of differentially expressed genes between stimulated and unstimulated T-cells using qRT-PCR.

| Gene Symbol | Gene Name                                                                 | Fold Change (Log2) | P-Value |
|-------------|---------------------------------------------------------------------------|--------------------|---------|
| EGR1        | EARLY GROWTH RESPONSE 1                                                  | 7.54               | 0.001   |
| PTGS2       | PROSTAGLANDIN-ENDOPEROXIDE SYNTHASE 2                                    | 4.40               | 0.001   |
| FOS         | V-FOS FBX MURINE OSTEOSARCOMA VIRAL ONCOGENE HOMOLOG                     | 3.39               | 0.001   |
| GADD45B     | GROWTH ARREST AND DNA DAMAGE-INDUCIBLE GENE                              | 2.91               | 0.001   |
| EGR2        | EARLY GROWTH RESPONSE 2                                                  | 1.62               | 0.012   |
| PMAIP1      | PHORBOL-12-MYRISTATE-13-ACETATE-INDUCED PROTEIN 1                        | 1.62               | 0.029   |
| ALAS2       | DELTA-AMINOLEVULINATE SYNTHASE 2                                         | -2.67              | 0.047   |
| PLK2        | POLO-LIKE KINASE 2                                                       | 1.57               | 0.081   |
| RGS1        | REGULATOR OF G PROTEIN SIGNALING 1                                        | 1.33               | 0.146   |
| PLK3        | POLO-LIKE KINASE 2                                                       | 0.77               | 0.165   |

doi:10.1371/journal.pone.0121169.t005
role in the regulation of the innate immune response. Activation contributes to regulation of the apoptotic response, inflammasome assembly and the production of proinflammatory cytokines [22]. RIG-I-like receptors also induce inflammatory cytokines, particularly in response to viral infection [23], and although a role in regulation of intrinsic mRNA is intimated, the importance of this in T-cell activation is not clear.

The nine upregulated DE genes representing the TLR signaling pathway had distinct expression patterns between stimulated and non-stimulated cells. These genes play important roles in the TLR signaling pathway. *FOS*, cytokines interleukin 1-beta, interleukin 8 and tumor necrosis factor, phosphorylation mitogen-activated protein kinase 8 and transmembrane emp24 protein transport domain containing 7 are responsible for transcription, cellular signalling, phosphorylation and selection of secretory cargo respectively [24–26]. This pathway is of particular interest for its role in T-cell proliferation and regulation of the cell cycle, which is detected within hours after T-cell activation. TLR proteins recognise PAMPs in structurally conserved molecules from microbes and DAMPs released from stressed cells, and as a result activate an innate immune response. Upon activation they induce production of proinflammatory cytokines and elevate expression of costimulatory molecules. They are linked to cell proliferation, cell survival, apoptosis and tissue remodelling and repair [27–30]. The expression of TLRs on CD8 T-lymphocytes and interactions with TLR adaptor molecule myeloid differentiation factor (MyD88) has been shown to facilitate rapid T-cell activation in response to invading pathogens [31]. TLR signaling pathways influence the regulation of proteins, such as D-type cyclins and
cyclin dependent kinases, which play key roles in cell cycle kinetics [32]. The TLR signaling pathway has been shown to enhance cell survival and proliferation. As seen in a study looking at TLRs in CD4 T-cells in which the engagement of TLR1/2, TLR5, TLR7/8 and TLR9 alters interleukin 2 (IL-2) production and increases proliferation [32].

Individual genes of interest included EGR1 and PTGS2, both relate to a role in cell cycle regulation and cell proliferation. One of the genes with the greatest response to stimulation in the present study was EGR1, with expression increasing 5.48 and 7.54-fold in stimulated cells based on results from the microarray and qRT-PCR respectively. EGR1 is an inducible transcription factor and plays an important role in gene transcription and T-cell development [33, 34]. Previous studies have identified EGR transcription factors as genes induced by T-cell activation in both humans and mice [2, 3, 35]. EGR1 interacts with the key transcriptional regulator, NFAT, and acts as a switch in the progression of the cell cycle from the resting G0 to primed G1-phase in T-lymphocytes whilst also playing an important role in the regulation of cytokine expression, including the gene for T-cell growth factor IL2, promoting proliferation [33, 36, 37]. Further, it has previously been linked to the development of cancer, and is involved in the proliferation and survival of cancer cells through its direct involvement in the regulation of cyclin D2 (CCND2), p19 and Fas [38]. EGR1 is linked to over-expression of CCND2, a positive regulator of the cell cycle, which may result in deregulated cell cycle progression.

PTGS2, also highly expressed in stimulated cells regulates prostaglandin synthesis in association with biological processes such as inflammation and proliferation. PTGS2 has been identified in other species as an immediate-early response gene following T-cell activation and is associated with T-cell signaling pathways, regulating proliferation of T-cells, activation of transcription factors NF-AT and NF-kB and production of IL-2, TNF-α, and IFN-γ [39]. PTGS2 plays an active role in the immune response, regulating production of prostaglandin E2 (PGE2). PGE2 promotes local vasodilatation and the attraction, as well as activation of neutrophils, macrophages and mast cells during active inflammation [40]. PGE2 also promotes immune suppression by suppressing proinflammatory cytokines [40]. High doses of PGE2 can also suppress the production of IL2 and in lower doses can regulate the shift from aggressive Th1 cells towards TH2 and TH17 responses [40–44]. The immunosuppressive action of PGE2 may also contribute to the over-expression of PTGS2 by some tumors [40, 45, 46].

Additionally, amongst the most responsive genes in this study were: FOS, PMAIP1, PLK2/3, EGR2, GADD45B and HEMGN. FOS is a well known transcriptional regulator that codes a leucine zipper protein that dimerises with proteins of the JUN family to form the activator protein-1 (AP-1) transcription factor complex [47–49]. Genes from the Jun-Fos family have previously been implicated as rapidly induced transcription factors in response to T-cell activation [3], some studies in both humans and mice however report down regulation of JunB and C-Fos after 4 hours of stimulation, which is probably a result of a delayed timeframe for collection of data [2, 7]. Over-expression of this transcription factor is associated with increased proliferation of cells and oncogenic potential. PMAIP1 is a negative regulator of cell cycle and a mediator of p53-dependent apoptosis, expression of this gene during T-cell activation helps to ensure that cells do not multiply uncontrollably [50, 51]. Disruption of PMAIP1 has been shown to reduce DNA damage-induced apoptosis [51]. Genes involved in the negative regulation of cell cycle progression, signaling pathways, and transcription have been shown previously to be induced early following mitogenic stimulation and play a vital role in immune modulation [2, 3]. PLK2 and PLK3 belong to the ‘polo’ family of serine/threonine protein kinases that play a role in cell division. PLK2 is a transcriptional target for p53 after genotoxic stress [52, 53]. PLK3, also known as CNK, has been implicated in the phosphorylation of CDC25C, a phosphoprotein activated in the G2 phase of cell division and essential for cell cycle progression [54]. EGR2 is another member of the EGR transcription factor family that has
been shown to become upregulated in response to T-cell activation [2, 3]. EGR2 is a negative regulator of cell growth [55] and is involved in regulating cell survival during the positive selection of T-cells [56]. It attenuates the immune response through negative regulation of T-cell activation, [57, 58], plays a regulatory role at different stages of T-cell development [56], and is required for initiation of T-cell anergy [57]. GADD45B genes are highly expressed in stimulated cells, and are activated in response to DNA damage and environmental stresses. Studies in mice have shown that activation of GADD45B by NF-κB downregulates the pro-apoptotic JNK signaling cascade, thereby temporarily protecting cells from apoptosis [59]. HEMGN is down-regulated in stimulated cells. It plays a role in the proliferation, differentiation and survival of hematopoietic cells [60] and is associated with the negative regulation of lymphocyte development [61]. Over-expression of HEMGN has been shown to promote proliferation and enhance survival through the activation of NF-κB in myeloid cells and impede lymphoid development and differentiation [60, 61].

In conclusion, this study provides a comprehensive analysis of the early T-cell gene response to activation in dogs and improves our knowledge on the genetic mechanisms behind T-cell activation. Key differentially expressed genes and biological pathways identified here may provide quantitative markers of T-cell response.

Acknowledgments
We thank Katherine Howard and David Simmons for their generosity and support. We would also like to thank Mrs Kim Rogers for sharing her knowledge and providing support, and the owners who volunteered their dogs.

Author Contributions
Conceived and designed the experiments: SM PW. Performed the experiments: SM PW. Analyzed the data: SM JW PW. Contributed reagents/materials/analysis tools: SM JW PW. Wrote the paper: SM JW PW.

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