

TLR expression profiles are a function of disease status in Rheumatoid Arthritis and experimental arthritis

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### **Conflict of interest**

None.

## **ABSTRACT**

The role of the innate immune system has been established in the initiation and perpetuation of inflammatory disease, but less attention has been paid to its role in the resolution of inflammation and return to homeostasis. Toll-like receptor (TLR) expression profiles were analysed in tissues with differing disease status in rheumatoid arthritis (RA), ankylosing spondylitis (AS), and in experimental arthritis. TLR gene expression was measured in whole blood and monocytes, before and after TNF blockade. In RA and osteoarthritis synovia, the expression of TLRs was quantified by standard curve qPCR. In addition, four distinct stages of disease were defined and validated in collagen-induced arthritis (CIA), the gold standard animal model for RA – pre-onset, early disease, late disease and immunised mice that were resistant to the development of disease. TLR expression was measured in spleens, lymph nodes, blood cells, liver and the paws (inflamed and unaffected). In RA whole blood, the expression of TLR1, 4 and 6 was significantly reduced by TNF blockade but the differences in TLR expression profiles between responders and non-responders were less pronounced than the differences between RA and AS patients. In RA non-responders, monocytes had greater TLR2 expression prior to therapy compared to responders. The expression of TLR1, 2, 4 and 8 was higher in RA synovium compared to control OA synovium. Circulating cytokine levels in CIA resistant mice were similar to naïve mice, but anti-collagen antibodies were similar to arthritic mice. Distinct profiles of inflammatory gene expression were mapped in paws and organs with differing disease status. TLR expression in arthritic paws tended to be similar in early and late disease, with TLR1 and 2 moderately higher in late disease. TLR expression in unaffected paws varied according to gene and disease status but was generally lower in resistant paws. Disease status-specific profiles of TLR expression were observed in spleens, lymph nodes, blood cells and the liver. Notably, TLR2 expression rose then fell in the transition from naïve to pre-onset to early arthritis. TLR gene expression profiles are strongly associated with disease status. In particular, increased expression in the blood precedes clinical manifestation.

**KEYWORDS.** Toll-like receptors, monocytes, rheumatoid arthritis, ankylosing spondylitis, osteoarthritis, inflammation

## 1. INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory disease, characterised by cellular infiltration and pro-inflammatory cytokine production within the synovial joints. Pattern recognition receptors are considered to have an important role in the development and perpetuation of RA. In particular, toll-like receptors (TLRs) have been recognised to contribute to disease pathogenesis in both clinical studies and animal models. The expression of TLR3 and 4 in the human synovia in early and

late disease was established by Ospelt C et al. [1], and later observations by Chamberlain ND et al. implicated a role for TLR5 [2]. Synovial fibroblasts express and respond to stimulation of TLR2 [3,4] and the expression of TLR2 is upregulated on monocyte subsets in RA patients [5]. The involvement of leukocytes in the increase in fibroblast TLR expression is evidenced by the upregulation of TLR2 and 4 in the synovium by pro-inflammatory cytokines such as IL12 and IFN $\gamma$  [6]. Leukocytes infiltrating the synovium also express and upregulate TLR expression in response to the pro-inflammatory milieu, and ligation of endogenous TLR ligands potentiates further activation [7]. Indeed, monocyte TLR5 and TLR7 mRNA expression is associated with disease activity in RA patients [2,8]. Studies have examined the kinetics of gene expression in animal models of arthritis but none have included immunised mice that have failed to develop arthritis, as well as paws unaffected

by clinical manifestations [9–11]. The inclusion of these groups permits the comparison of gene expression profiles from inflamed tissues with non-inflamed tissues which nevertheless may be affected by mediators from the arthritic joints. In the absence of inflamed joints, immune-related organs in immunised mice (e.g. lymph nodes) may produce mediators that systemically modulate TLR gene expression. Transgenic mouse models have demonstrated that TLR stimulation may be permissive [12–14] or preventative [15–17] for the initiation or maintenance of the inflammatory process. The role of TLRs in the perpetuation of chronic disease or resolution of inflammation has been less well studied. Although TLR signalling is thought of as pre-dominantly pro-inflammatory, TLR expression has been measured in cells with a regulatory function suggesting TLR-mediated responses encompass a greater role than initiating and amplifying inflammation [18,19]. To contextualise TLR expression profiles, we compared the expression of inflammatory genes, including TLRs, at different stages of experimental arthritis in mice and in human RA patients, and examined the expression of TLRs at different stages of anti-TNF treatment. By assessing a range of disease states, changes in expression that preceded disease were discernible, as were expression profiles associated with reduced disease and differences in responders and non-responders treated with TNF inhibitors.

## **2. Methods and materials**

### **2.1. Collagen-induced arthritis (CIA)**

All procedures were approved by the Animal Welfare Ethical Review Board and were undertaken in accordance with project and personal licences issued by the UK Home Office under the UK Animals (Scientific Procedures) Act, 1986. Mice were housed in pathogen-free conditions with food and water available *ad libitum*. In brief, bovine type II collagen (200 µg) in complete Freund's adjuvant (CFA) was injected subcutaneously at the base of the tail of DBA/1 mice, as previously described [20]. The disease activity for each paw was scored as follows: 0, normal/unaffected, 1 moderate swelling/erythema, 2 severe swelling; paws with a score <1 were defined as “affected”. At each time-point investigated the mice were euthanized and the paws, lymph nodes, spleens and livers were removed and blood was obtained via cardiac puncture. Blood was collected into heparin-coated tubes and erythrocytes were lysed to obtain leukocytes. For analysis of humoral responses and circulating cytokines, serum was used instead. Anti-type II collagen IgG1 and IgG2a were measured as previously described [21]; all samples were expressed relative to an internal titrated standard composed of pooled serum from vehicle-treated mice. Blood cytokines were measured using the Th1/Th2 9-plex Ultra-Sensitive Kit (Mesoscale Discovery).

### **2.2. RNA extraction**

Paws were snap-frozen in liquid nitrogen and pulverized with the BioPulverizer™ (BioSpec). Paw powder was then homogenised in 500 µL of TRIzol reagent (Invitrogen) using the Sample Grinding Kit (GE Healthcare). The aqueous phase of the phenol/chloroform extraction was mixed with isopropanol then added to an RNA extraction column (RNeasy Mini Kit, Qiagen), and RNA extraction completed according to manufacturer's instructions. Spleens, lymph nodes and livers were pressed through a cell strainer, washed, and then RNA was extracted using Trizol in a similar fashion to the paws.

### **2.3. RT-PCR**

Reverse transcription of 500 ng of RNA was performed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) in a 40 µL reaction which was then diluted to a total of 120 µL. Expression of target genes was determined using TaqMan gene expression assays (ThermoFischer Scientific) and expressed relative to housekeeper gene expression using the  $\delta\delta CT$  approximation method; see Supplementary Methods for Taqman qPCR assay details.

### **2.4. Human clinical samples**

Blood and tissues were collected in accordance with approval from the Riverside Research Ethics Committee, the City and East London Ethics Committee, the Ethics Committee of the Ministry of Health of Hungary and Ethics Committee of the University of Szeged; poor response to therapy was defined as a reduction in DAS28 of less than 2.2. Tissues and blood samples were obtained from the Nuffield Orthopaedic Centre (Oxford) and The Royal Free London NHS Foundation Trust (London), Northwick Park Hospital (London), Imperial College Healthcare Trust hospitals (London) and the Department of Rheumatology and Immunology, University of Szeged after receiving informed consent.

## 2.5. Whole blood

Blood was obtained from patients prescribed anti-TNF, before and 3 months after therapy. Blood from RA patients, AS patients and healthy normal controls was collected in Paxgene Blood RNA tubes (PreAnalytiX) and stored at  $-80^{\circ}\text{C}$  until processed for RNA extraction using the Paxgene Blood RNA kit (Qiagen) according to the manufacturer's instructions.

## 2.6. Human RA blood monocytes

Blood for monocyte isolation was obtained from patients prescribed anti-TNF, including adalimumab and etanercept, before and 3 months after therapy. PBMC were isolated after density gradient separation then washed extensively to remove platelets. Monocytes were then isolated immunomagnetically by CD14 positive selection (Miltenyi). Purity was confirmed by flow cytometric staining with antibodies for CD14 (HCD14) and CD16 (3G8) which were obtained from BioLegend Ltd. mRNA was extracted using an Isolate II RNA/DNA/Protein kit (Bioline). Reverse transcription was performed as mentioned above and gene expression was measured with Taqman qPCR assays as detailed in the Supplementary methods.

## 2.7. Human RA tissue

RA and OA synovial membrane cells were isolated from patients undergoing joint replacement surgery as previously described [22]. Where disclosed, the treatments for RA patients included methotrexate (47%), biologics (20%), Prednisone (33%), analgesics (13%), Leflunomide (20%), Sulfasalazine (7%) and Azathioprin (7%). The mRNA was isolated using Qiagen miRNA extraction columns and reverse transcribed as detailed above. Gene expression was measured using a linearised plasmid containing genes of interest as a quantitative standard for qPCR using Taqman assays as detailed in the Supplementary Methods.

## 2.8. Statistical analysis

Data was analysed by ANOVA or Student's t-test as detailed in the figure legends, using Prism (GraphPad). Heat maps were generated using Multi-Experiment Viewer software (TM4 software) [23].

## 3. Results

### 3.1. Characterisation of disease status-associated gene expression

Although no model completely reproduces human RA, collagen-induced arthritis (CIA) is regarded as the gold standard animal model for RA as the development of disease requires humoral and cellular components of the immune system and the clinical symptoms reproduce the human disease at the gross and microscopic level in the affected joints; many treatments are effective for both CIA and RA. To investigate inflammatory mediator expression in paws of mice with CIA we identified key states of disease – pre-onset, the 1st day of disease onset and the 10th day post-onset. In addition, we compared these states of disease with immunised mice that had failed to develop arthritis (“resistant”) several weeks after the last arthritic mice had reached day 10 post onset; it was reasoned that the mechanisms preventing the development of disease are at least as important as those initiating it. Despite showing no clinical signs of arthritis, the resistant phenotype has anti-collagen antibody titres that are comparable to arthritic mice but a serum cytokine profile not dissimilar to naïve mice (IFN $\gamma$ , IL-10, IL-12, IL1 $\beta$ , IL-5 and TNF, Supplementary Figure 1). When analysing inflammatory gene expression in paws from arthritic mice, we compared arthritic paws (“affected”) with paws from the same mouse which displayed no swelling or redness (“unaffected”). It was reasoned that any genes modulated by systemic inflammation would be similarly changed in the “bystander” unaffected paws as the affected paws, and that changes in gene expression caused by cellular infiltrate could be better accounted for by comparing affected paws to non-arthritic paws in different stages of disease.

#### 3.1.1. Arthritic paws at d1 and d10 of arthritis

Within the paw, the first day of arthritic disease is markedly different from day 10. As with many inflammatory processes, there is an initial influx of neutrophils, which are later replaced with macrophage-lineage cells and lymphocytes [24]. Changes in non-haematopoietic cellularity include synovial fibroblast hyperplasia and angiogenesis. Erosions to the cartilage and bone contribute to a loss of joint architecture concurrently with fibrosis and loss of joint space. When comparing affected arthritic paws from the 1st and 10th day of arthritis there was an increase in the expression of Agtr2, Gusb, Il1b and Il6 and a decrease in Stat1 at day 10; there was also a trend for increased expression of Vcam1 at day 10 (Fig. 1, Supplementary Figure 2).

### 3.1.2. Unaffected paws in pre-onset, arthritic and resistant mice

During the pre-onset period (i.e. after immunisation but prior to apparent disease in the joints) much of the immunological activity is confined to lymphoid organs, which increase in size and accumulate cells with pathogenic potential. When comparing paws from immunised mice prior to disease onset we found only a weak trend for lower Bcl2 and higher Nos2 compared to day 1 unaffected paws, but significantly increased expression for Bax and Bcl2l1 in day 10 unaffected paws in arthritic mice compared to pre-onset paws. When comparing pre-onset paws versus resistant paws we found no significant differences in gene expression for the genes measured. Comparisons between unaffected

paws at all stages of disease (pre-onset, day 1, day 10 and resistant) had few differences for the genes measured. Only B2m, Ccl5 and Vegfa were significantly different between the unaffected paws from day 10 of disease and paws from resistant mice. Although resistant mice have no overt clinical manifestations, the average gene expression of the pro-inflammatory chemokine Ccl5 in paws was higher compared to unaffected paws from arthritic mice at day 10 of disease. Conversely, there

was lower expression of B2m and Vegfa in resistant paws compared to arthritic mice at day 10 of disease. For the genes measured, there were no differences between day 1 and 10 in unaffected paws.

### 3.1.3. Arthritic paws in early and late disease

On the first day of disease, several genes (Cd8a, Cd40, Fas, Gusb, Il-1 $\beta$ , Il2ra, Nos2, Prfl, Ptprc, Selp, Stat1) were increased in the affected paws of arthritic mice compared to unaffected paws (Fig. 1, Supplementary Figure 2). At day 10 post onset, affected paws had relatively increased expression of Ccl2, Ccl3, Cd4, Ctla4, Gusb, Il-1 $\beta$ , Il6, Nos2, Ptgs2, Ptprc, Sele, Selp, Stat4, Tgfb1, Tnf, Vcam1 and Vegfa compared to unaffected paws. There was also relatively lower expression of several genes (Bax, Bcl2l1, Cd34, Col4a5, Csf2, Edn1, Gapdh, Ikbkb, Il1a, Il7, Il15, Il18, Stat6, Tfrf and Tnfrsf18) in affected paws. A common set of genes (Ccl2, Cd4, Gusb, Il1b, Il6, Ptgs2, Sele, Selp and Vcam1) were upregulated in day 10 affected paws compared to day 10 unaffected paws, resistant paws and pre-onset paws, with the latter three time-points having further individually specific significant differences (Fig. 1B).

### 3.1.4. Differential expression in early and late disease

To compare differences between affected and unaffected paws in each mouse we calculated the affected:unaffected paw ratio of gene expression at day 1 and day 10 ( $\delta D1$  and  $\delta D10$ ); these values indicate the different processes occurring in inflamed paws compared to unaffected paws in individual mice. By comparing  $\delta D1$  and  $\delta D10$  we sought to reveal differences in gene expression at each time-point that were specific to disease processes. Using this metric, there was decreased Bcl2l1, Ccl19,

Csf2, Il1a, Il15, Il18, Stat1, Stat6 and Tnf for the  $\delta D_{10}$  value compared to  $\delta D_1$ ; of note was that Ccl19 not significantly different in unaffected vs affected paws at d1 or d10 but, due to minor trends in opposite directions these time points, were significantly different using this comparison.

### 3.2. TLR gene expression is associated with disease status

Although inflammatory mediators, such as TNF, IL-6 and IL1 $\beta$ , are potent influencers of inflammatory disease, the intracellular signalling which precedes and perpetuates cytokine production is augmented by TLR signalling. During inflammatory disease, the processes of leukocyte subset infiltration, activation and cell death, synovial membrane proliferation and matrix remodelling release into the inflammatory milieu molecules containing damage-associated molecular patterns (DAMPs); many endogenous DAMPs have been identified and characterised as ligands for TLRs [25,26]. To determine the potential for TLR-mediated inflammatory sequelae we mapped TLR expression in different organs during CIA.

#### 3.2.1. Joints

The expression of Tlr1-9 was measured in paws at all stages of disease and, in general, naïve and unaffected paws at day 1 tend to be similar. (Fig. 2, Sup. Fig. 3). When comparing paws from naïve and resistant mice the former had increased expression of Tlr3, 5, 6, 8 and 9. There were no statistically significant differences in expression between unaffected paws from day 10 arthritic mice and resistant mice. In the pre-onset period (day 5 and/or day 10 post-immunisation) there were significant differences for all TLRs compared to the resistant mice. The greatest differences between unaffected and affected paws at day 1 was nominally decreased expression of Tlr6 in affected paws, whereas at day 10 there was significantly increased expression of Tlr1, 2, 3, 4 and 7 in affected paws compared to unaffected paws. When comparing affected paws from day 1 versus day 10 there was significantly higher expression of Tlr1 and Tlr2 at the latter time-point.

#### 3.2.2. Liver

The liver has a less appreciated role in systemic inflammation as the source of acute phase proteins and although there is a high proportion of mononuclear phagocytes resident (Kupffer cells) in the organ, their phenotype is polarised towards endotoxin tolerant macrophages due to the conditioning effect of the contents of the portal vein [27]. The expression of Tlr4 was lower in resistant mice compared to pre-onset mice (Sup. Fig. 4) and there was a significant trend of decreased expression during the course of disease for Tlr2, 4 and 9 suggesting a systemic response to the ongoing peripheral inflammation in tissue not normally regarded as part of the adaptive immune system.

#### 3.2.3. Spleen



The spleen is a secondary haematopoietic organ and is the reservoir for the majority of murine monocytes in the steady state, although these cells are refractory to TLR stimulation [28]. There were no significant differences during the course of disease for Tlr3, 4, 6, 8 or 9 (Fig. 2B, Sup. Fig. 5). Tlr1, 2 and 7 had reduced expression in immunised mice compared to naïve mice but conversely Tlr5 expression was increased in arthritic and resistant mice compared to naïve mice. Across groups arranged according to time and disease progression there was a significant trend towards reduced expression of Tlr1, 2, 4 and 7 and increased expression of Tlr5.

#### 3.2.4. Lymph nodes

As the site of antigen presentation and lymphocyte proliferation, the lymph nodes, particularly those draining from the affected joint, experience changes in the composition of inflammatory cells during the course of disease. Relative few differences in TLR gene expression were found in the lymph nodes (Fig. 2C, Sup. Fig. 5). Tlr2 gene expression decreased over the course of the disease. Tlr3, 5 and 6 tended to have lower expression in the pre-onset period (day 10 post immunisation) and first day of disease onset (d1) compared to other time-points, although these differences did not reach statistical significance.

#### 3.2.5. Blood

Many clinical studies of autoimmune disease rely on analyses of blood as an indicator or predictor of disease status or response to treatment due the ease of accessibility and repeated sampling [29]. TLR gene expression was particularly variable in circulating leukocytes in erythrocyte-lysed blood (Fig. 2D, Sup. Fig. 7). Expression of Tlr2 was significantly higher in the pre-onset stage compared to naïve mice; this trend was repeated with Tlr4 and Tlr8 albeit with statistically weaker significance. Tlr7 expression was relatively lower in day 1 arthritic mouse blood compared to pre-onset mice.

### 3.3. TNF blockade reduces TLR expression in whole blood

Although combination of methotrexate and TNF blockade is the current treatment recommended for moderate to severe RA, a significant minority of patients do not respond, experience a loss of efficacy over time or rapidly relapse upon cessation of treatment [29]. Much effort is now being directed to replicate for RA the successful elucidation of endotypes as has been achieved for asthma and airway-associated inflammatory disease [30]. A common strategy for phenotyping Responder/Non-Responder (R/NR) status in RA patients is the collection of samples prior to treatment [29,31], in order to identify pathogenic pre-existing mechanisms present in NR which can be potentially targeted.

Whole blood was collected from RA patients prior to and after TNF blockade to measure TLR gene expression. For comparison, samples were collected from normal healthy controls and anti-TNF

treated ankylosing spondylitis (AS) patients (Fig. 3, Sup. Fig. 8), as representative of the steady state and a comparable but distinct autoimmune disease, respectively. The expression of TLR1 was reduced after treatment in R and NR in both AS and RA patients and furthermore TLR2 was reduced in R in RA patients. TLR4 was reduced by treatment in R in AS and RA. TLR5 was reduced in AS R after treatment, but the same trend in AS NR did not reach significance. TLR6 reduced in NR and R in AS, and R in RA. To compare the effect of TNF blockade between R and NR the difference in expression before and after treatment was calculated ( $\delta R$  and  $\delta NR$ , Fig. 8). There was a minor non-significant reduction in  $\delta NR$  vs  $\delta R$  for TLR3 in AS which was in contrast to an increase in the same parameters for RA patients; essentially, AS patients who responded to treatment had a reduction in TLR3 compared to NR whereas RA patients who responded to therapy had a relative greater increase in TLR3 compared to NR. Compared to normal donors, TLR4 expression was reduced after therapy in AS and RA patients. TLR1 was also reduced after therapy in RA patients compared to normal healthy donors.

#### 3.4. Monocyte TLR2 is increased in RA non-responders prior to TNF blockade

Although lymphocytes are more prevalent within PBMC, their greater heterogeneity compared to monocytes makes them a less informative cell lineage in which to measure TLR gene expression. Beyond classifiers of maturity (e.g. CD45RA and CD45RO) there are limited markers to distinguish functionally-defined subsets, particularly the Th17 subset which is increased in inflammatory disease. By comparison, monocytes can be functionally separated into 3–5 subsets based upon surface markers [32,33]. The expression of TLR1-10 was measured in freshly isolated circulating monocytes of RA patients before and after anti-TNF therapy (Fig. 4). Interestingly, prior to therapy the expression of TLR2 was increased in non-responders compared to responders ( $p = 0.02$ ). For all RA patients, anti-TNF therapy caused an average increase in TLR9 expression ( $p = 0.039$ ) and this trend was driven mainly by responders ( $p = 0.052$ ). In non-responding patients there was a trend towards reduced TLR7 expression after treatment which did not reach significance ( $p = 0.057$ ).

#### 3.5. TLR2 and TLR4 expression is higher in human RA synovium compared to OA

The expression of TLR1-10 was measured in tissue isolated from the synovium of RA and OA patients undergoing joint replacement and calibrated against a linearised recombinant standard to measure absolute copies of transcript (Fig. 5A). While there was virtually no difference in the expression of TLR5, 6, 7 and 10 (Fig. 5B), TLR2 and 4 were overexpressed in the human RA synovium compared to OA synovium ( $p < 0.05$ ) and there was also a strong trend for increased expression of TLR1 ( $p = 0.062$ ) and TLR8 ( $p = 0.052$ ).

## 4. Discussion

Autoimmune diseases are often associated with particular genetic susceptibilities [34] and several studies have examined the potential contribution of TLR variants to disease [35–37]. TLRs prime the innate immune system to engage with infection but also detect the tissue damage which may or may not be associated with infection. Tissue damage may indicate that a barrier breach has occurred, and so infiltration of leukocytes is a necessary precautionary measure. The consequent TLR signalling is a putative mechanism that contributes to the perpetuation of inflammation in the apparent absence of infection. TLRs are intimately involved in the inflammatory process and targeting their activity at the level of their ligands, receptors and associated signalling pathways offers a strategy to reduce the severity of chronic inflammatory disease. The characterisation of TLR expression at different stages of disease or in different organs, or whether they are specifically altered in responders or non-responders to current therapies could inform us as to potential mechanisms associated with non-responsiveness. This may also provide biomarkers that would not only allow us to identify those patients most likely to benefit from treatment with TNF inhibitors, but also indicate which, if any, TLRs are associated with disease activity and at which stage and location of disease.

Although more recently developed biologics (rituximab, tocilizumab, anakinra, abatacept) have broadened the options for the treatment of RA, TNF inhibitors in combination with MTX is still the first line treatment for moderate to severe RA that does not respond to non-biologic DMARDs. The discovery was a turning point for the treatment of RA [38] but many patients do not respond, lose their response over time or relapse rapidly upon cessation of treatment [29,39]. After disease severity, the response to TNF blockade treatment is emerging as a defining feature for the stratification for RA (and AS) patients. Although differential responses to TNF blockade presents challenges for clinicians and patients, the heterogeneity of patients' responses provides valuable insight regarding the underlying mechanisms of disease. In addition to endotypes (phenotypes defined by mechanism), the processes present at each stage of disease represent new avenues for therapeutic targeting and provide evidence of the initiators of disease or a succession of regulatory mechanisms that fail to take effect in susceptible individuals.

### 4.1. TLR expression profiles are associated with disease status in experimental arthritis

To deconvolute disease processes, several disease states were identified based upon chronological progression and inflammatory disease status. For systemic effects naïve mice, immunised mice prior to disease onset, mice on the first and tenth day of disease and mice that failed to develop disease were examined. Although arthritic disease onset is defined by oedematous swelling of the paws, preceding immunological activity was evident in the swollen lymph nodes and with the development of collagen-specific antibodies.

CIA is widely regarded to be the gold standard model for RA as the development of disease requires antibody and cell mediated auto-reactivity, and it has clinical features in common with human pathophysiology. As the course of disease tends to be uniform and the use of anti-TNF is highly efficacious, the model could be considered analogous to the anti-TNF-responsive human RA endotype(s). The induction of CIA is not 100% efficient however, and 10–30% of mice do not develop overt clinical signs of disease in a timely fashion. The mice that are resistant to disease induction have been suggested to have a different microbiota, compared to susceptible cage-mates [40]. The influence of microbiota has also been demonstrated by the presence or absence of segmented filamentous bacteria in mice from different suppliers, which determines the susceptibility of mice, and more broadly in the reduced severity and delayed kinetics of disease in mice housed in individually ventilated cages compared to open, “dirty” cages [41].

Resistant mice had a comparable anti-collagen humoral response and anti-collagen IgG2a antibodies were prevalent; this indicated that the cytokine milieu of the B cells may not be dissimilar to between susceptible and resistant mice in the pre-onset period and that iso-type switching had occurred. The determination of anti-collagen auto-antibody levels in the pre-onset period may therefore not be particularly informative of the eventual development of disease. In paws from pre-onset and resistant mice, no differences were observed in the expression of genes examined. When comparing unaffected paws from early (day 1) and late (day 10) arthritis no significant differences in the expression of inflammatory mediator gene expression were observed however Tlr5, 6 and 8 were lower on day 10. When comparing pre-onset paws to unaffected paws from day 10 arthritic mice, the relative upregulation in the latter of two genes that encode proteins that influence apoptosis when associated with the outer mitochondrial membrane (Bcl2l1, Bax) [42] was observed. These genes were also upregulated in the unaffected paws of day 10 arthritic mice compared to day 10 affected paws. Further investigation will be necessary to determine whether the upregulation was due to a systemic effect in late disease that was not present in the pre-onset state.

When comparing unaffected paws from day 10 arthritic mice with paws from resistant mice, there was higher expression of Ccl5 in the latter and lower expression of B2m and Vegfa. As CCL5 is a chemokine, it was not unexpected that it was slightly higher in affected paws compared to unaffected paws at day 10 of arthritis. However, it was not expected to be highest on average in the resistant mice paws compared to all other groups; further investigation will be required to determine whether Tregs responding to CCL5 have played a role in preventing disease, as has been noted previously [43]. The expression of Vegfa was higher in unaffected paws compared to affected paws on day 10 of arthritis and in paws from resistant mice. Although the average differences between unaffected paws and paws from immunised, non-arthritic mice is not substantial, the differences are unlikely to be due to cellular

composition but instead a function of prolonged exposure to inflammatory cytokines borne via the circulation. When comparing differences between affected and unaffected paws in arthritic mice at the early (day 1) and late (day 10) timepoints, differences in many of the genes measured were observed. The genes up regulated at both timepoints in affected paws vs unaffected paws were *Il1b*, *Gusb*, *Nos2*, *Ptpnc* and *Selp* which are all expressed in leukocytes and are thus indicative of leukocyte infiltration. These results are not dissimilar to previous attempts to map inflammatory mediator expression in different states of disease in experimental arthritis [9–11] and re-iterate the importance of IL1 $\beta$  in this model [44,45]. The influence of TLRs in the chronicity of arthritis is supported by several studies which have evaluated the beneficial effect of TLR inhibition [46]. Thus, the effect of pro-inflammatory cytokines such as IL-17 on the expression of TLR2, 4 and 9 [47] and, more recently, the effect of endogenous TLR ligands which may be released from damage matrix during inflammation [48,49] have been shown to be associated with chronicity. TLR signalling is often a precursor to cytokine production and endogenous TLR ligands are increased in the joints and plasma of RA patients [25]. It is not unexpected that TLR gene expression varied greatly between paws with differing disease status, and that the variation was generally greater than that seen in other organs. When examining TLR expression during the course of CIA in arthritic mice, the greatest changes between over time were observed in the expression of Tlr1, 2, 5, 6 and 8 in paws, Tlr4 in the liver, Tlr1, 2, 4, 5 and 7 in the spleen, Tlr2 in the lymph nodes and Tlr2 and 7 in the blood.

#### 4.2. TLR expression profiles are associated with disease status in RA

Compared to normal healthy donors, the expression of TLR1 and 4 was reduced in RA whole blood samples after TNF blockade; the effect of treatment was a reduction in the expression of TLR1, 4 and 6. In monocytes, the effect of treatment was an increase in the expression of TLR9, which was not observed in the whole blood samples. This observation is consistent with the increase in expression of TLR9 (and TLR2) observed on monocyte subsets of RA patients with active disease [50] or total monocytes that express higher levels of TLR4 and TLR7 in RA patients [8,51]. Patients with psoriatic arthritis have also been observed to have upregulated TLR2 on blood monocytes [52]. Comparisons of TLR expression in circulating monocytes and synovial fluid monocytes in RA [53] and juvenile idiopathic arthritis [54] identified differences in the expression of TLR 2, 4 and 8. When comparing R v NR, it was observed that each endotype tended to change TLR expression in the same way although the only statistically significant difference between R and NR prior to treatment was increased expression of TLR2 in NR monocytes. A deeper phenotyping of these patients may reveal whether the increased TLR2 expression is a cause or effect of the mechanisms that reduce the response to anti-TNF, which could be therapeutically targeted. Synovial RA explants tended to have higher mean expression of TLRs compared to OA explants although this was most evident for TLR1, 2, 4, and 8; this pattern of expression mirrors that of the affected and unaffected paws in CIA suggesting

infiltrating cells express these molecules. The effects of TLR ligands are dependent on the cells expressing TLRs within the synovium and the inflammatory milieu, both of which may fluctuate according to the dynamic processes of cell recruitment, activation, cell death and resolution. Infiltrating and activated resident cells in the synovium of RA patients have been phenotyped extensively based upon cytokine profiles [55], single cell transcriptomics [56,57] and cell surface marker expression [57]. It is likely that where there is a failure to resolve inflammation to a degree where surgery is necessary, that genes with relatively increased expression would represent significant components in the mechanism of patho-physiology.

## **5. Conclusions**

To summarise, this study confirms the association of TLRs with inflammatory processes, which we have mapped out in fine detail for collagen-induced arthritis. The findings reiterate the primacy of TLRs in inflammatory disease, and the likelihood of endogenous TLR ligands participating in sterile inflammation. Similarities were observed in the effect of anti-TNF on the expression of TLR expression in the blood of RA and AS patients. In RA monocytes, increased expression of TLR2 was associated with a poor response to anti-TNF. In human RA synovium, robust expression of TLRs was found when compared to OA. These data indicate that therapeutic targeting of TLRs and their ligands may be beneficial in inflammatory disease.

## FIGURE LEGENDS

Figure 1. Gene expression analysis of disease status in collagen-induced arthritis

(A) Gene expression in paws from differing stages of disease – pre-onset ten days after immunisation (PRE), arthritic affected (AA1) and unaffected (AU1) paws on day 1 of clinically apparent disease and arthritic affected and unaffected paws on day 10 of clinically apparent disease (AA10 and AU10 respectively), immunised mice that did not develop disease (“resistant”, RES). Heat map is row normalised. (B) Statistically significant differences in gene expression between time-points. Legend – paws with disease status “A” have relatively greater expression of “gene” compared to “B”. See supplementary Figure 2 for detailed data and statistical analyses.

Figure 2. TLR expression is modulated during collagen-induced arthritis

(A) TLR expression in paws at different stages of disease; naïve (NVE), pre-onset five days after immunisation (P5), pre-onset ten days after immunisation (P10), affected and unaffected paws on the first day of clinically apparent arthritis (AA1 and AU1, respectively), arthritic affected and unaffected paws on day 10 of clinically apparent disease (AA10 and AU10, respectively) and immunised mice that failed to develop disease (RES). TLR gene expression in (B) spleen, (C) lymph nodes, (D) erythrocyte-lysed blood; stages of disease – NVE, P5, P10, RES are labelled the same as (A); A1 first day of disease, (A10) ten days after disease onset. Heat maps are row normalised. See supplementary Figures 3-7 for detailed data and statistical analyses.

Figure 3. TLR expression in whole blood is decreased after TNF blockade in AS and RA patients.

Gene expression in whole blood samples from healthy donors, AS patients and RA patients. (A) Samples grouped as Normal healthy donors (N), RA patients ante-therapy (RA-A) and post therapy (RA-P), AS patients grouped as ante-therapy (AS-A) and post therapy (AS-P). (B) Samples from (A) further subdivided into anti-TNF responders (R) or non-responders (NR). Heat maps are row normalised. Statistically significant differences between RA or AS patients are indicated connecting lines, and significant differences to normal healthy donors are indicated by white diamonds. See supplementary Figure 8 for detailed data and statistical analyses.

Figure 4. TNF blockade non-responders have greater TLR2 expression in monocytes prior to therapy.

Gene expression in purified monocytes from RA patients. Data is plotted from L to R as follows – combined patients ante-treatment (0m (A)), combined patients 1 month after beginning treatment (1m), combined patients 3 months post-treatment (3m (P)), ante-treatment non-responders (A-NR), post-treatment non-responders (P-NR), ante-treatment responders (A-R), post-treatment responders

(P-R), change in expression for non-responders ( $\delta$ -NR) and change in expression for responders ( $\delta$ -R); \*  $p < 0.05$  t-test.

Figure 5. RA synovial cells have higher TLR expression, compared to OA.

(A) A linearized plasmid incorporating sequences recognised by Taqman™ assays was used to measure expression of TLR1-10 by standard curve qPCR. (B) The expression of TLR2 and TLR4 was significantly higher in RA explants and there was a strong trend for TLR1 and TLR8; \*  $p < 0.05$  Student's t-test,  $n=13$  (OA) and  $n=19$  (RA).

Supplementary Figure 1. Analysis of the serum of the resistant phenotype.

(A) Anti collagen responses in arthritic mice (CIA) and immunised mice that fail to develop disease (RES); (i) serum levels of anti-type II collagen IgG1 and IgG2a antibodies at day 10 post-onset and (ii) ratio of IgG2a/IgG1; no significant differences, Student's t-test. (B) Cytokines in serum from naïve mice, arthritic mice (day 10 post onset) and mice that failed to develop disease (resistant); \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$ , ANOVA with Tukey post-hoc test.

Supplementary Figure 2. Inflammatory gene expression in paws

Gene expression in paws from differing stages of disease; stages of disease – pre-onset ten days after immunisation (Pre), arthritic affected and unaffected paws on day 1 of clinically apparent disease (D1Un and D1Aff, respectively) and arthritic affected and unaffected paws on day 10 of clinically apparent disease (D10Un and D10Aff, respectively), immunised mice that failed to develop disease (RES) and the difference between the affected and unaffected paws in the same mouse on day 1 and day 10 post-onset ( $\delta$ D1 and  $\delta$ D10, respectively). Statistically significant differences determined by paired or unpaired t-test are indicated above data (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ); matrices below graphs indicate statistically significant differences between groups; \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$ , ANOVA with Tukey post-hoc test.

Supplementary Figure 3. TLR expression in paws

Gene expression in paws at different stages of disease - naïve (NVE), pre-onset five days after immunisation (P5), pre-onset ten days after immunisation (P10), arthritic affected and unaffected paws on day 1 of clinically apparent disease (AA1 and AU1, respectively) and arthritic affected and unaffected paws on day 10 of clinically apparent disease (AA10 and AU10, respectively) and



immunised mice that failed to develop disease (RES). Matrices below graphs indicate statistically significant differences; \*  $p<0.05$ , \*\*  $p<0.01$ , \*\*\*  $p<0.001$ , \*\*\*\*  $p<0.0001$ , ANOVA with Tukey post-hoc test.

#### Supplementary Figure 4. TLR expression in liver

TLR gene expression was measured in the liver from mice at different stages of disease – day 5 post immunisation pre-onset (P5), day 10 post immunisation pre-onset (P10), day 1 of clinically evident arthritis (1), day 10 post disease onset (10) and mice that fail to develop disease (R); \*  $p<0.05$  – ANOVA with Tukey post-hoc test. Test for linear trend across groups †  $p<0.05$ , ††  $p<0.01$ .

#### Supplementary Figure 5. TLR expression in spleens

TLR gene expression was measured in spleens from mice at different stages of disease – naïve (N), day 10 post immunisation pre-onset (P), day 1 of clinically evident arthritis (1), day 10 post disease onset (10) and mice that fail to develop disease (R); matrices below graphs indicate statistical differences, \*  $p<0.05$ , \*\*  $p<0.01$ , \*\*\*  $p<0.001$ , \*\*\*\*  $p<0.0001$ , ANOVA with Tukey post-hoc test. Test for linear trend across groups †  $p<0.05$ , ††  $p<0.01$ , †††  $p<0.001$ , ††††  $p<0.0001$ .

#### Supplementary Figure 6. TLR expression in lymph nodes

TLR gene expression was measured in lymph nodes from mice at different stages of disease – naïve (N), day 10 post immunisation pre-onset (P), day 1 of clinically evident arthritis (1), day 10 post disease onset (10) and mice that fail to develop disease (R); there were no statistical differences using ANOVA with Tukey post-hoc test. Test for linear trend across groups †  $p<0.05$ .

#### Supplementary Figure 7. TLR expression in blood

TLR gene expression was measured in erythrocyte lysed blood from mice at different stages of disease – naïve (N), day 10 post immunisation pre-onset (P), day 1 of clinically evident arthritis (1) and day 10 post disease onset (10) and resistant mice (R); \*  $p<0.05$ , \*\*  $p<0.01$ , \*\*\*  $p<0.001$ , ANOVA with Tukey post-hoc test. Test for linear trend across groups †  $p<0.05$ , ††  $p<0.01$ .

#### Supplementary Figure 8. TLR expression in whole blood is decreased after TNF blockade in AS and RA patients

Gene expression in whole blood samples from normal donors, AS patients and RA patients. Data is plotted from L to R as follows – normal healthy donors (N), all AS patients ante-treatment (A), all AS patients post-treatment (P), AS non-responders ante-treatment (A-NR), AS non-responders post-treatment (P-NR), AS responders ante-treatment (A-R), AS responders post-treatment (P-R), change in expression for AS non-responders ( $\delta$ -NR) and change in expression for AS responders ( $\delta$ -R). RA

patients – same order as AS patients; \*  $p < 0.05$  t-test, †  $p < 0.05$  compared to normal healthy donors, ANOVA.

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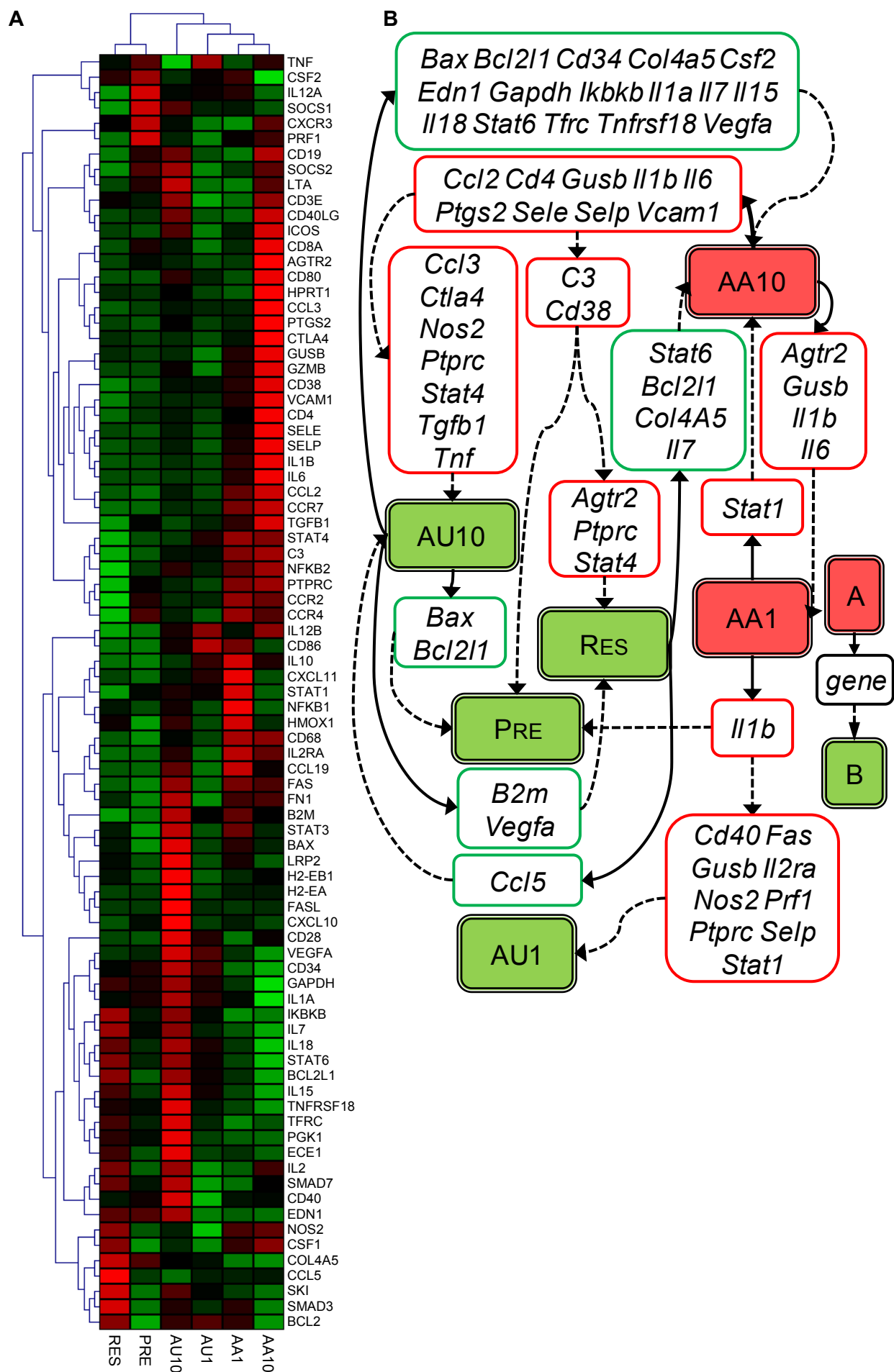


Figure 1

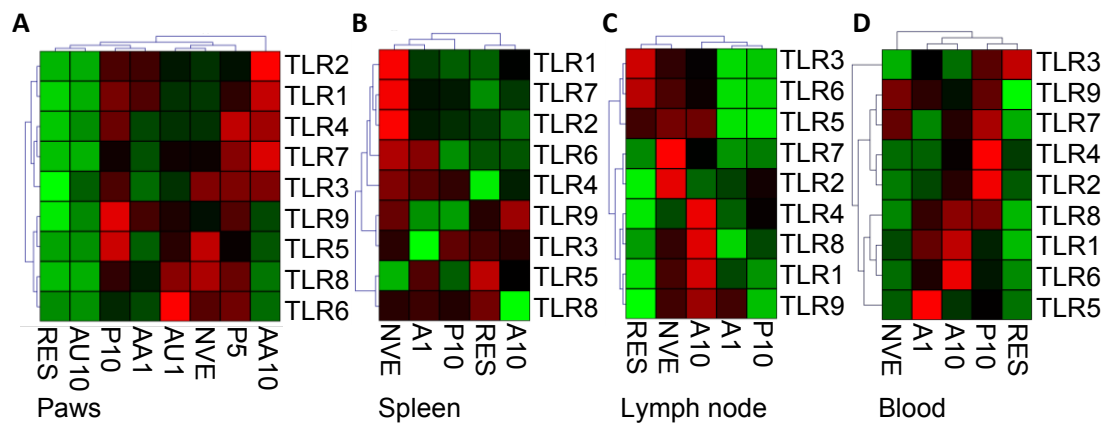


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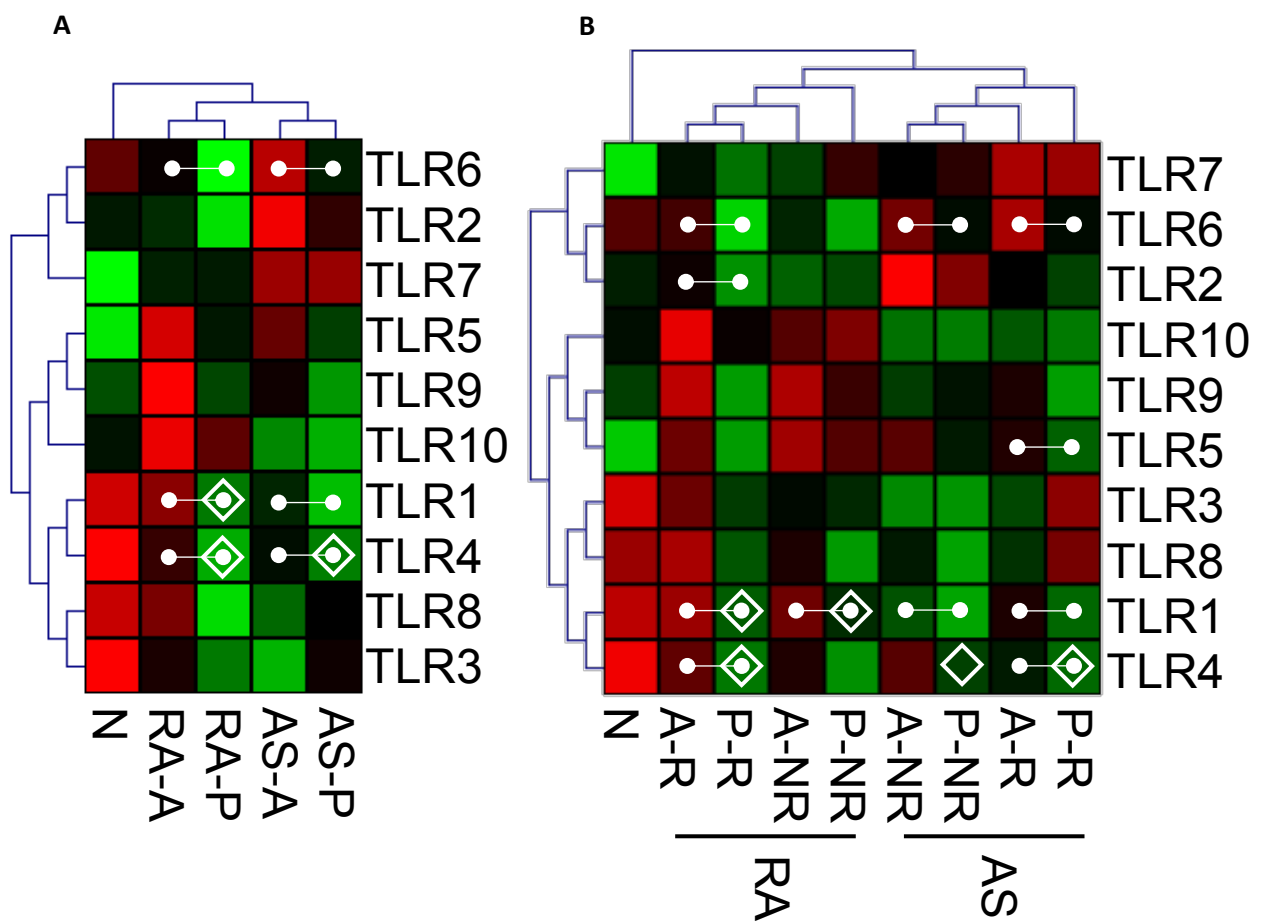


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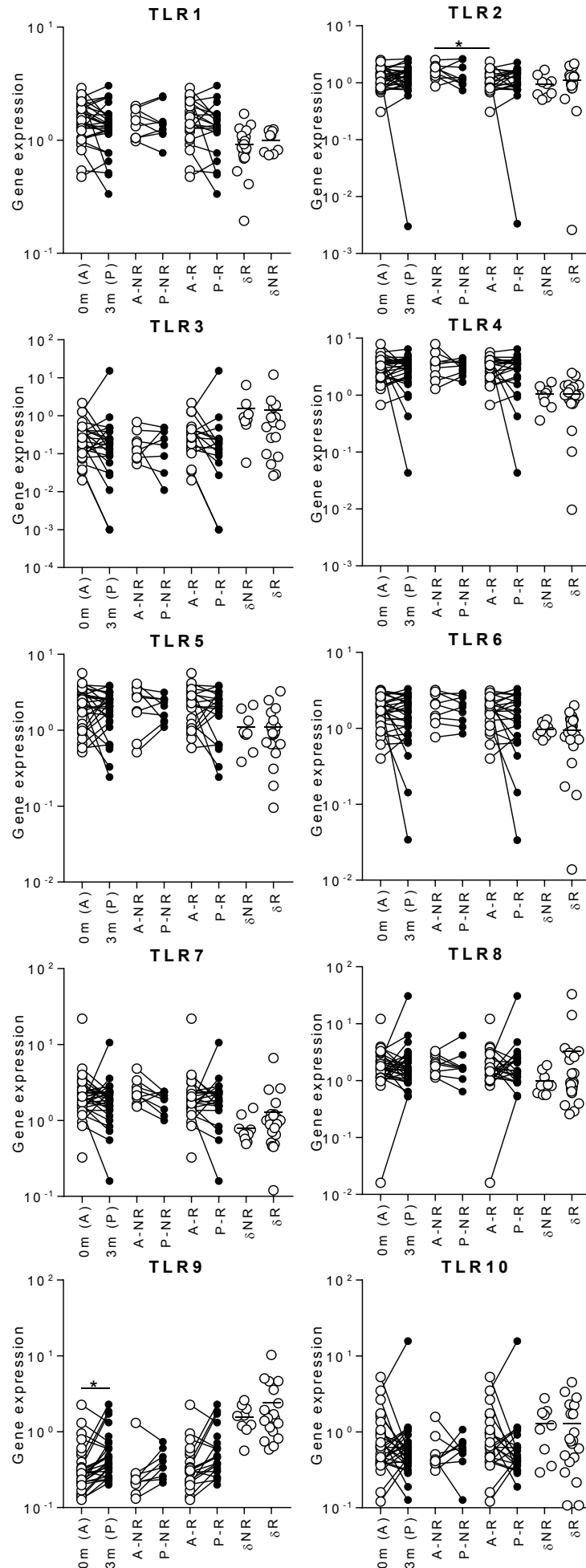


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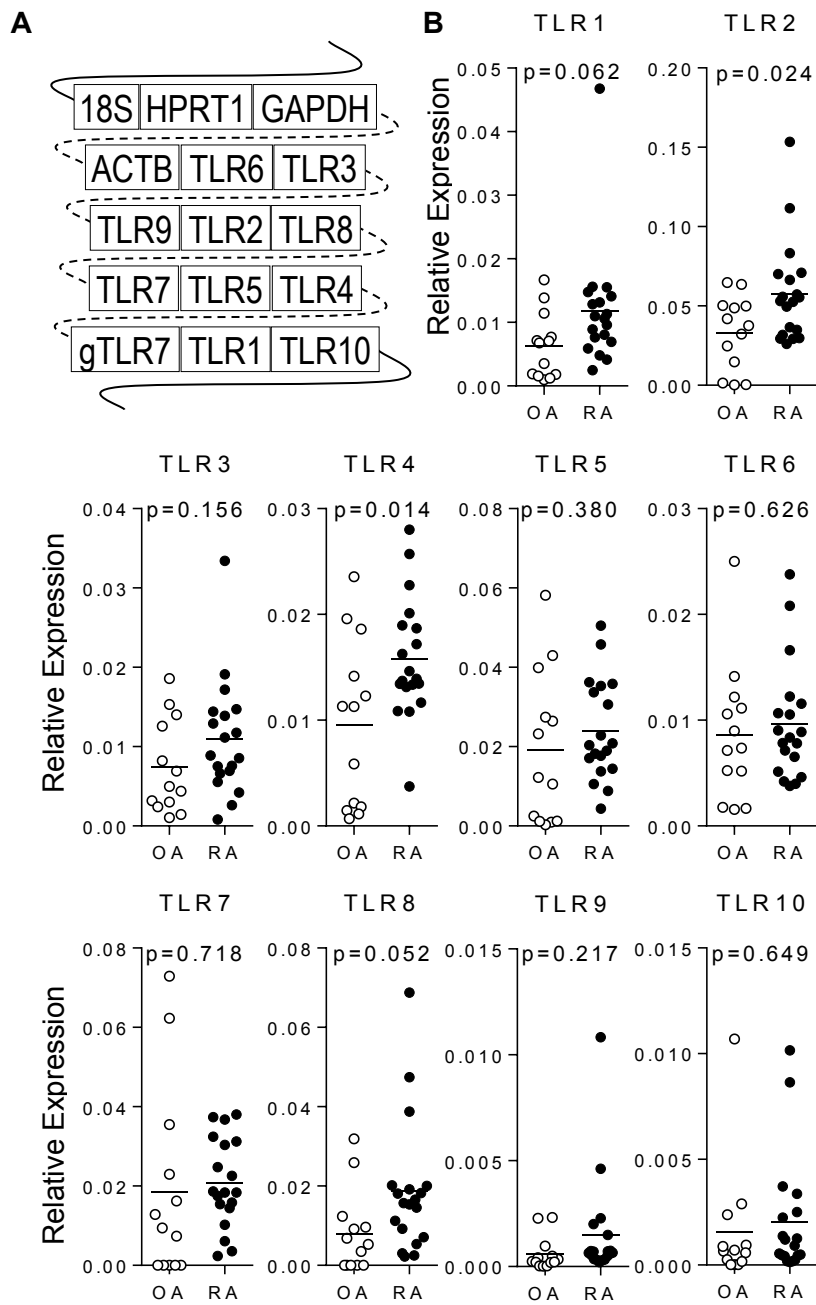
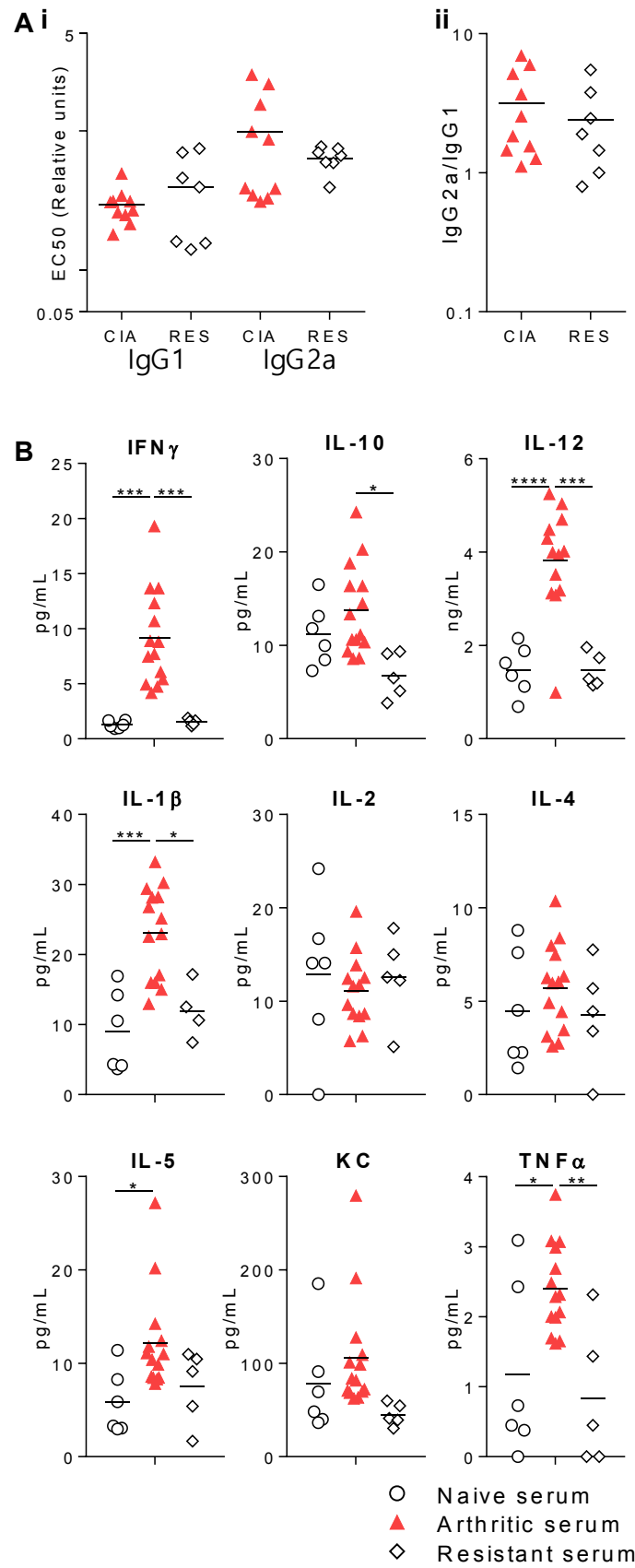


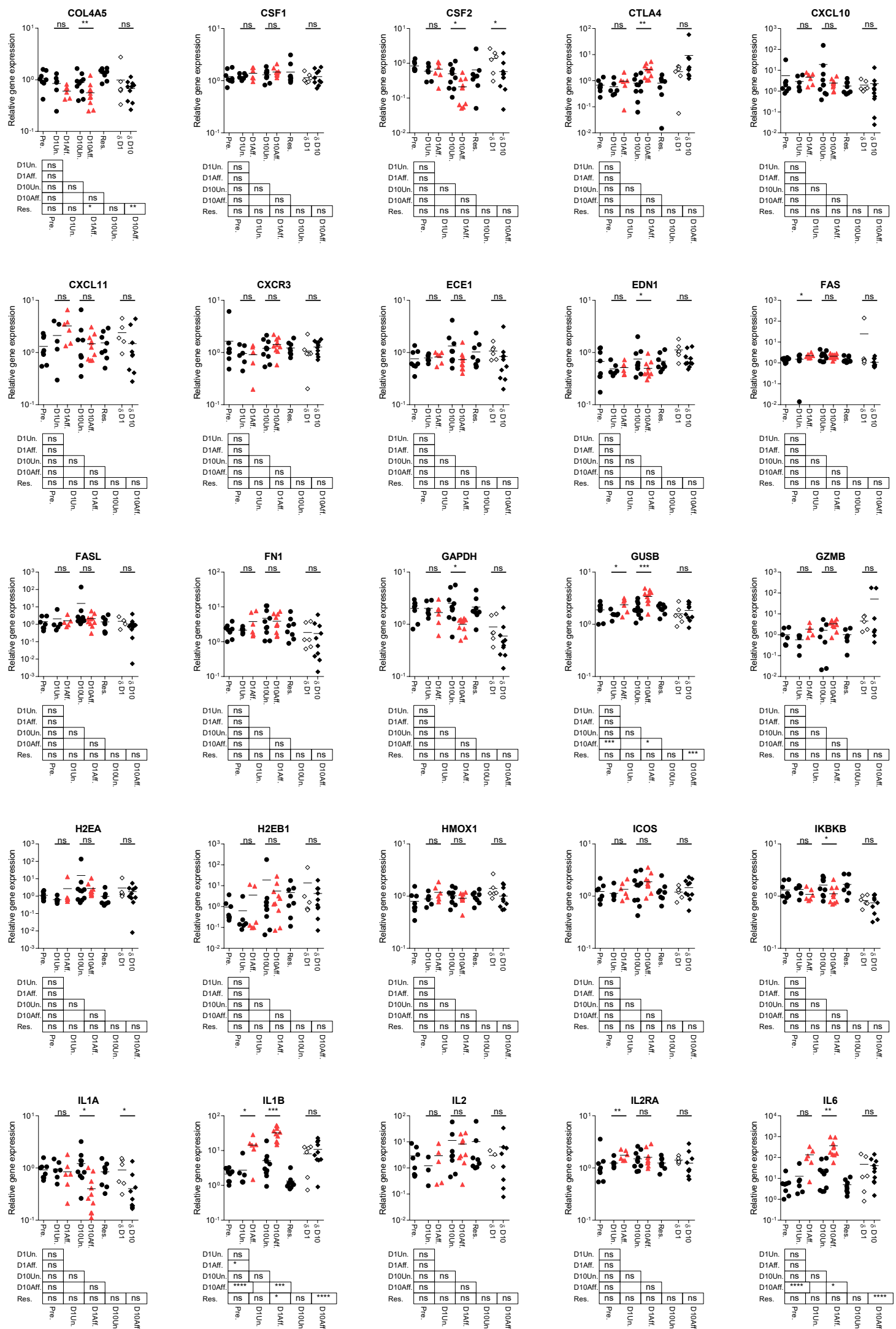
Figure 5

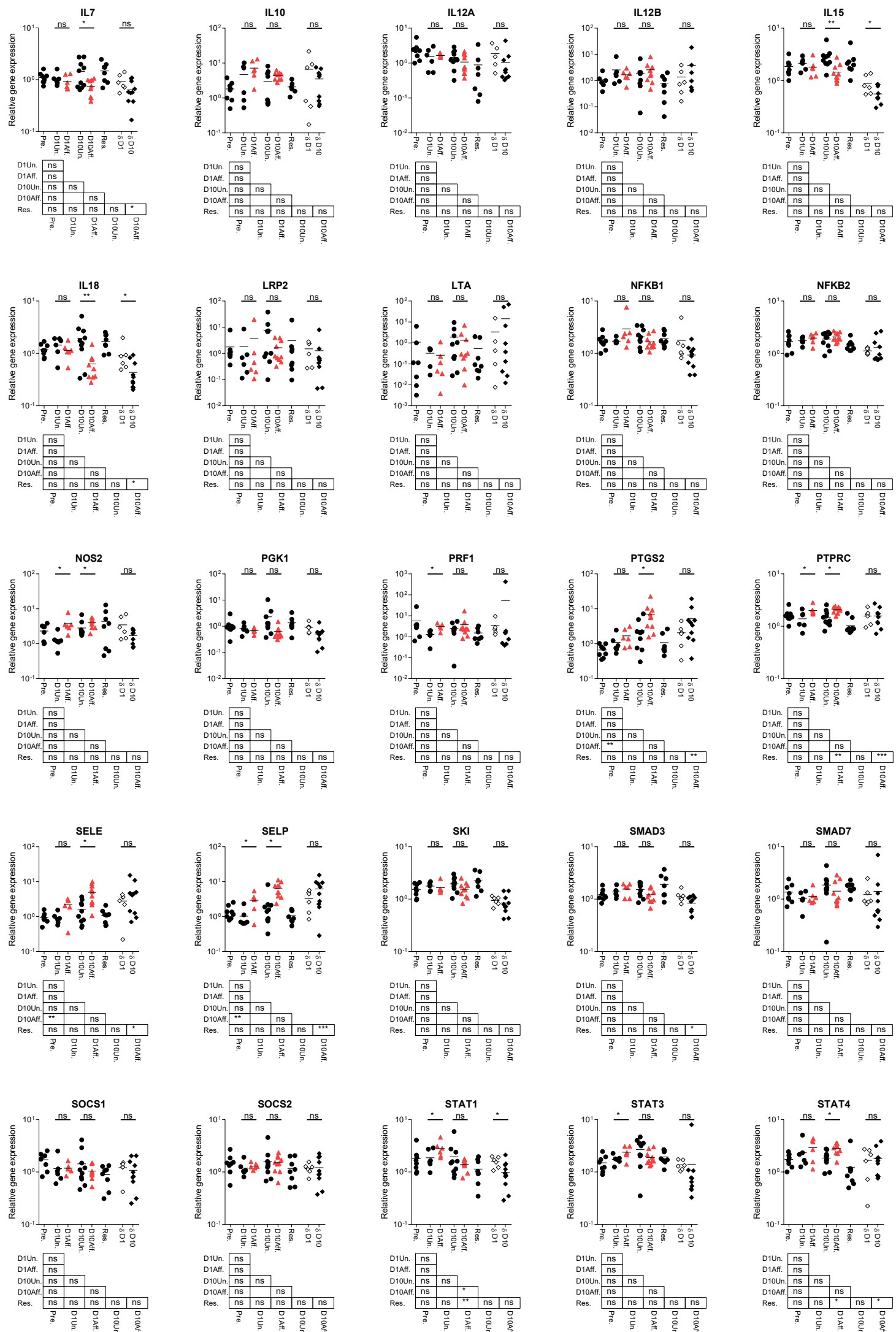


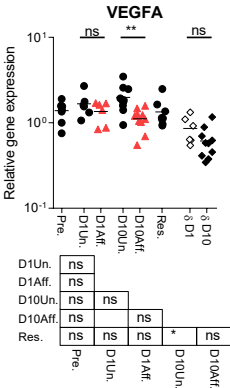
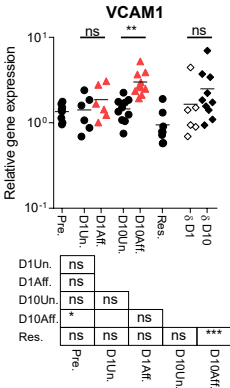
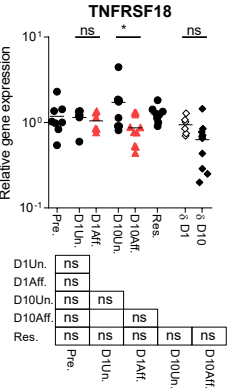
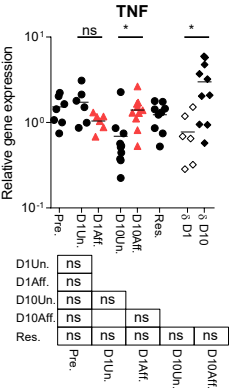
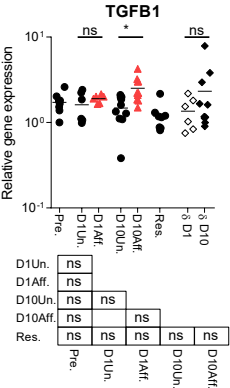
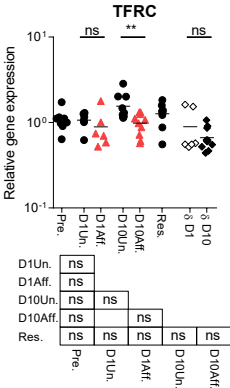
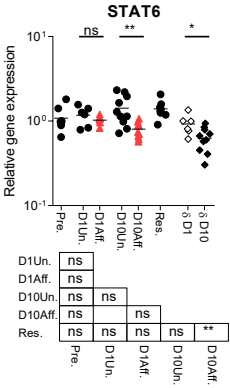
Supplementary Figure 1

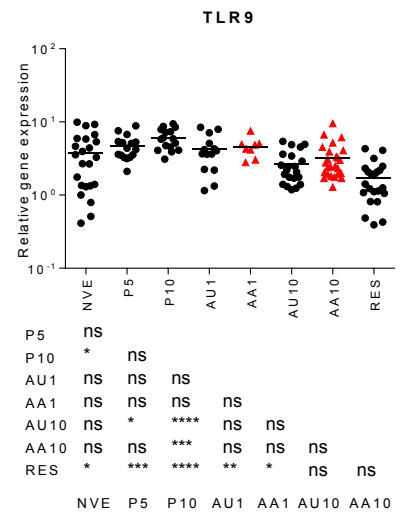
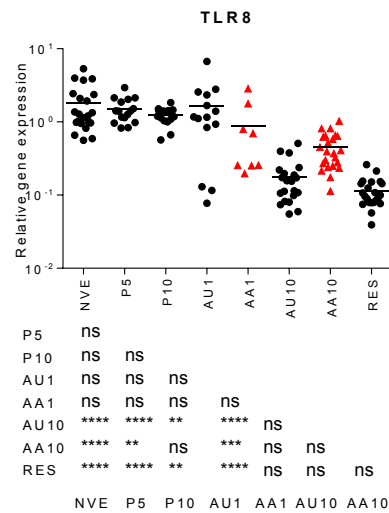
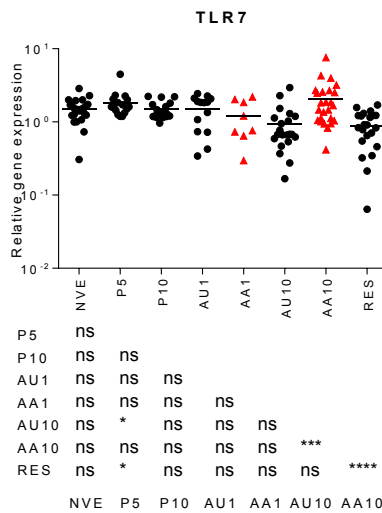
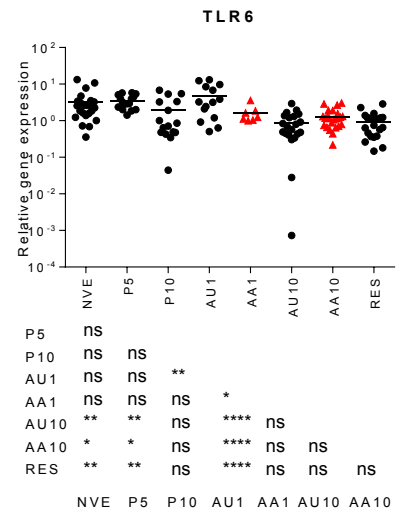
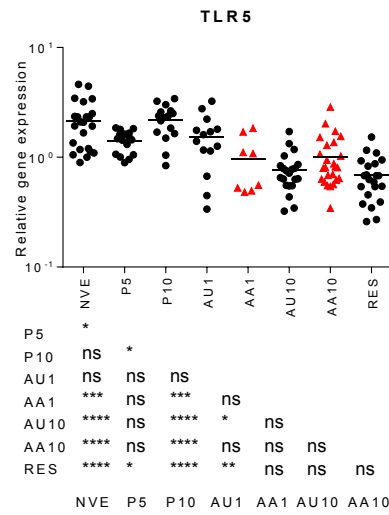
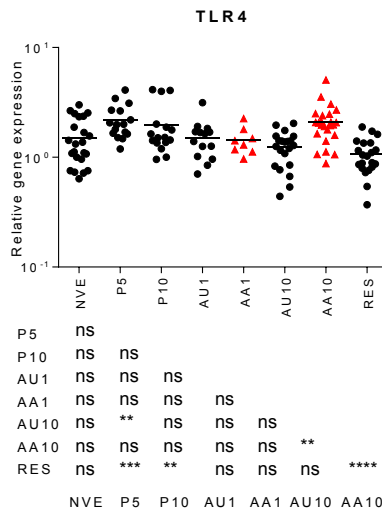
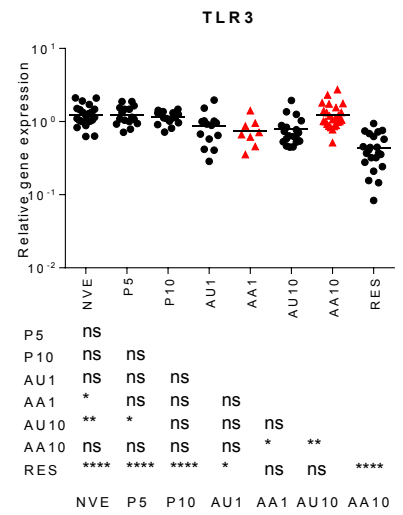
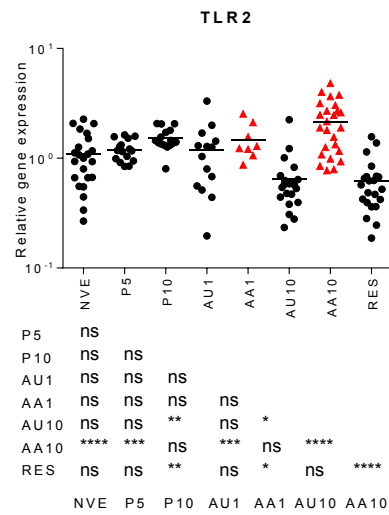
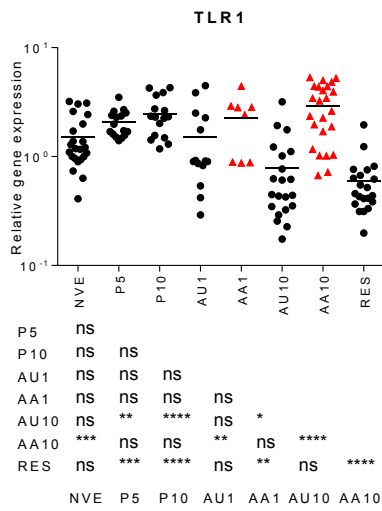




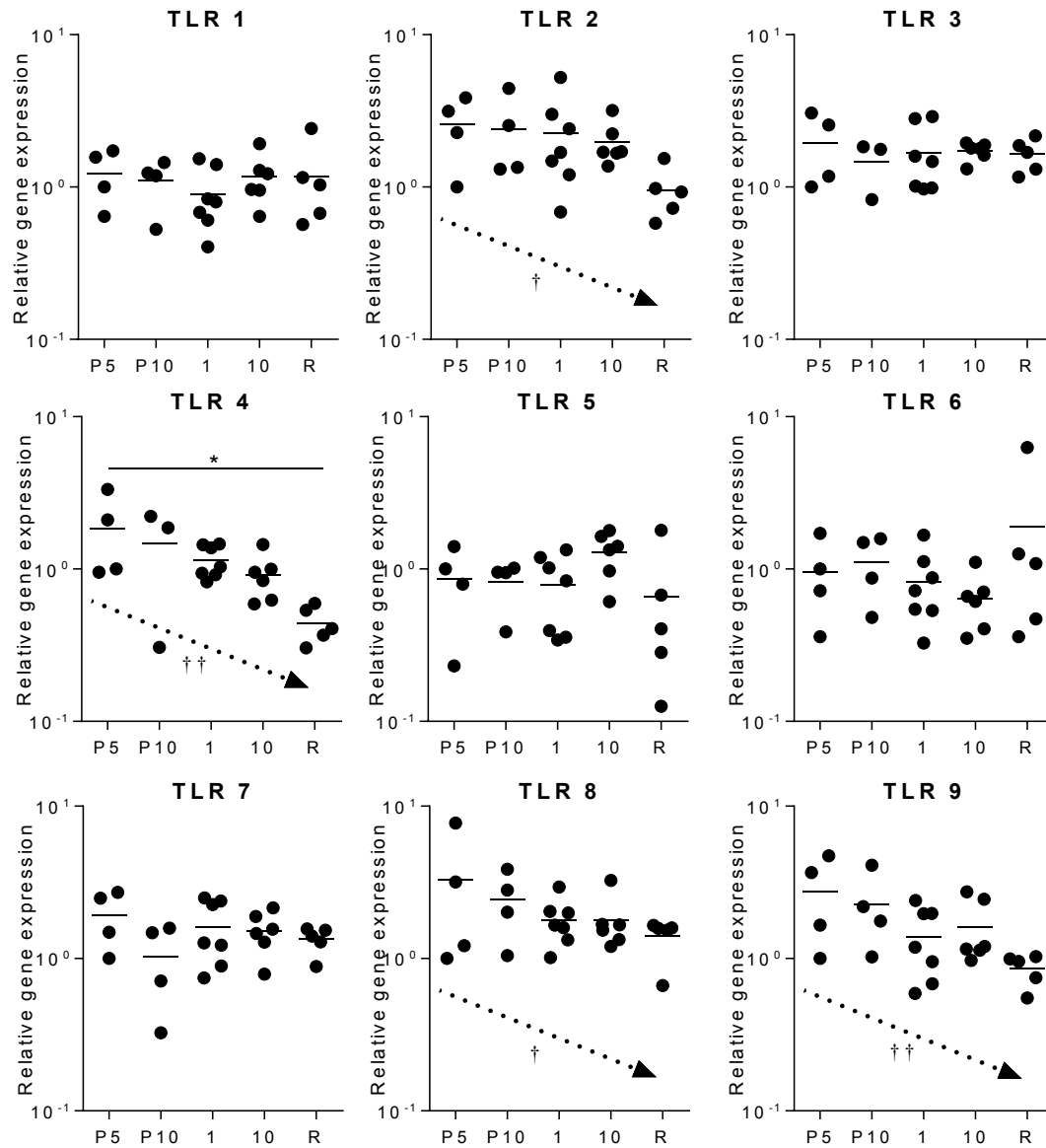
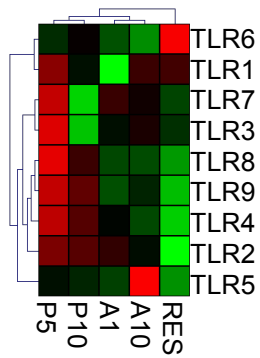




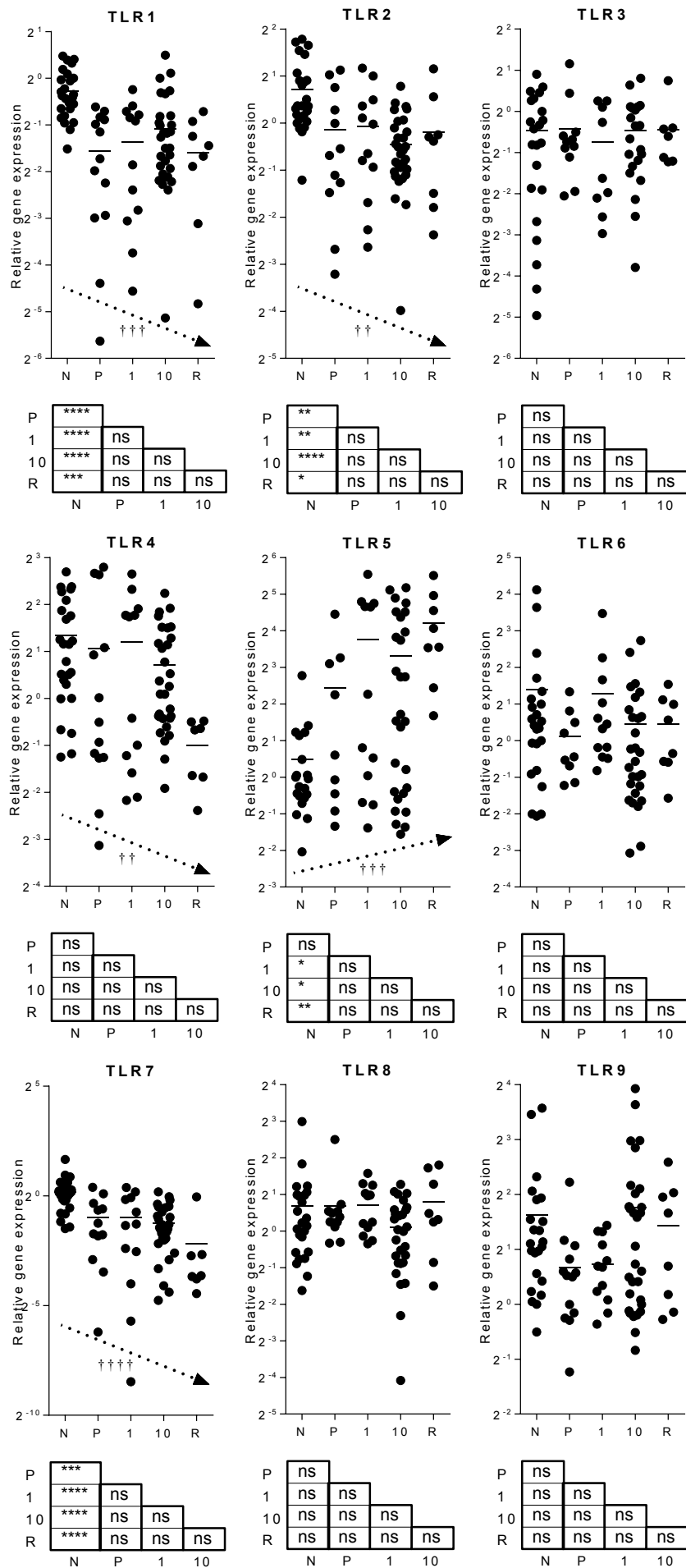




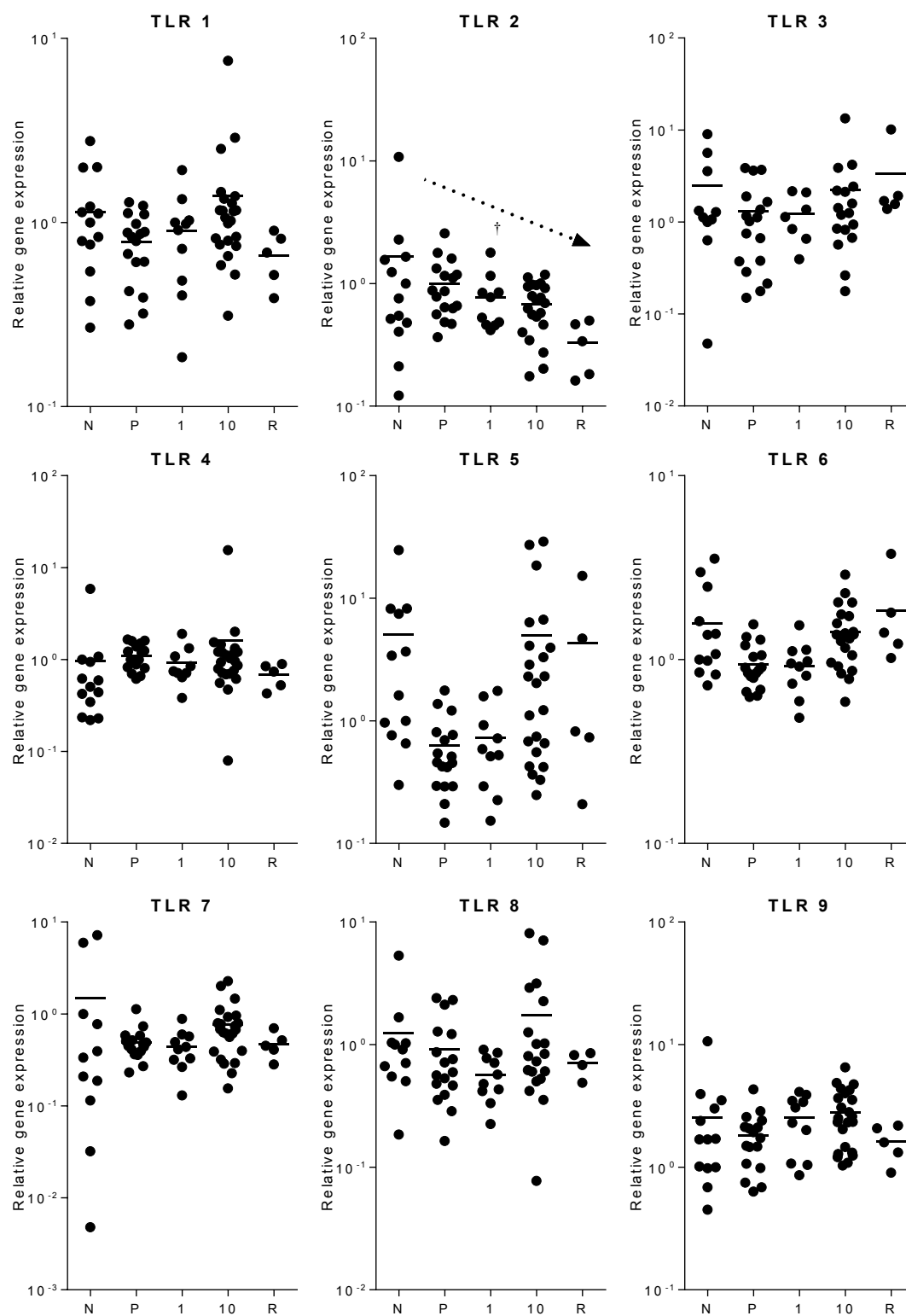
Supplementary Figure 3



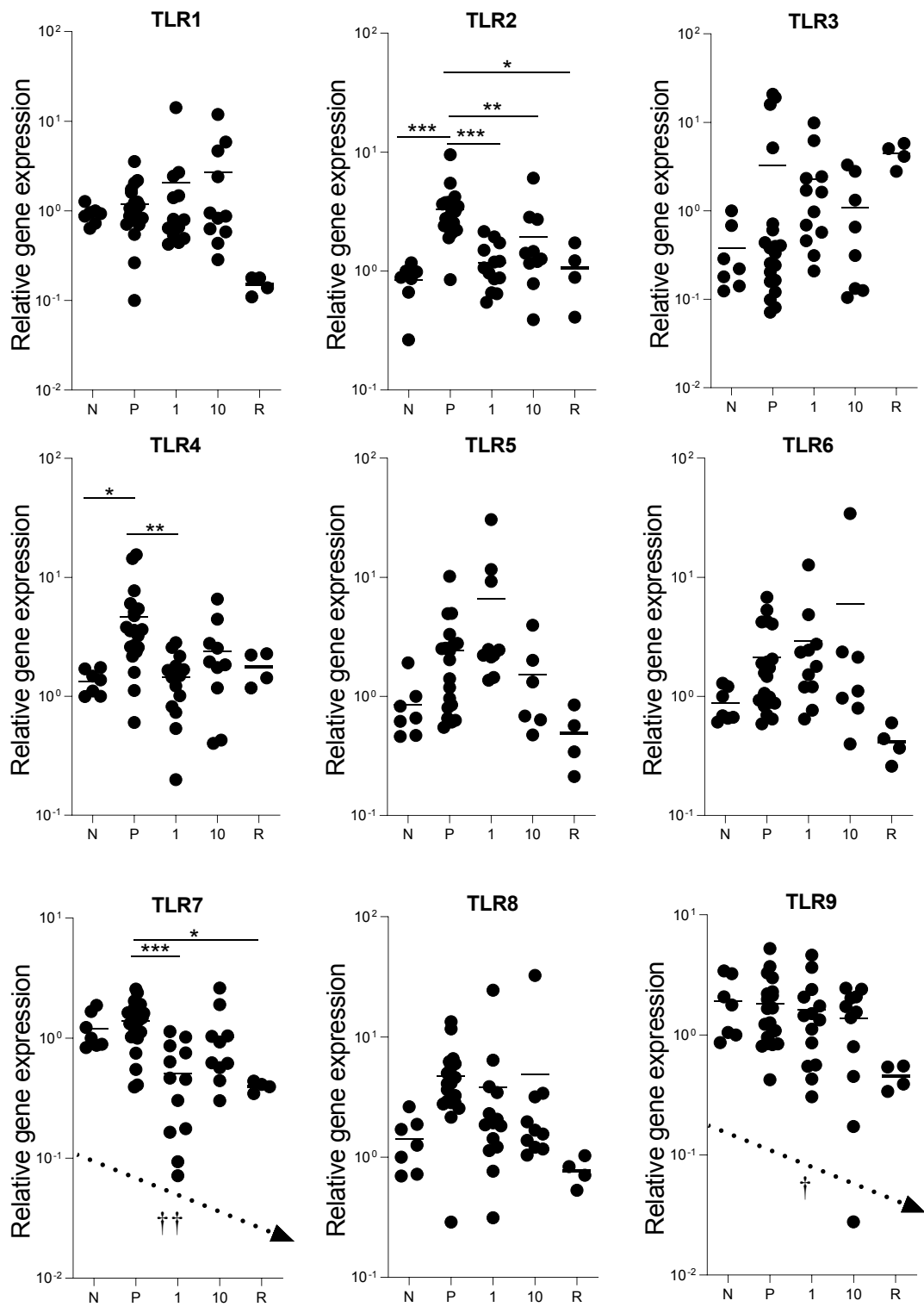
Supplementary Figure 4: Liver



Supplementary Figure 5: Spleens

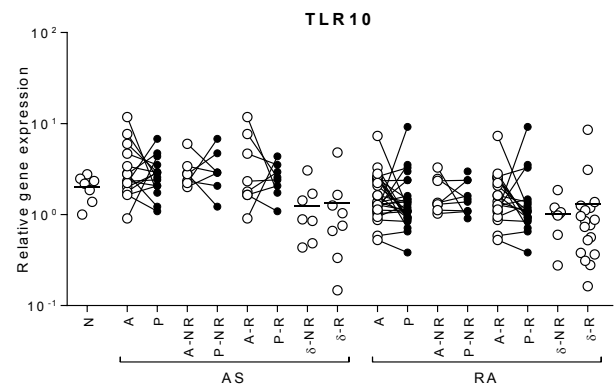
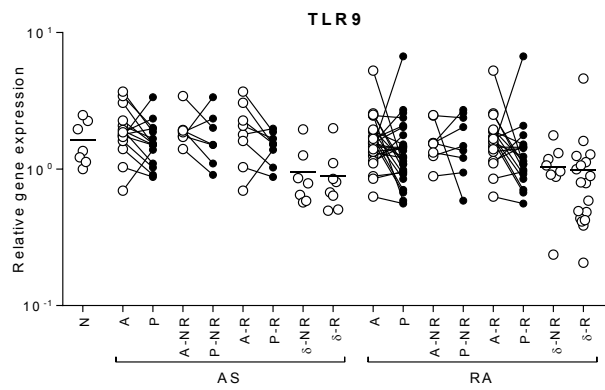
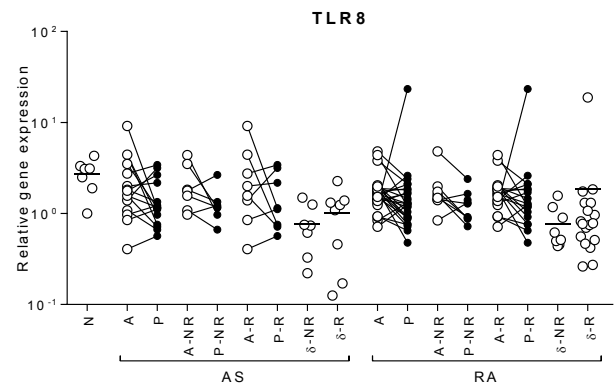
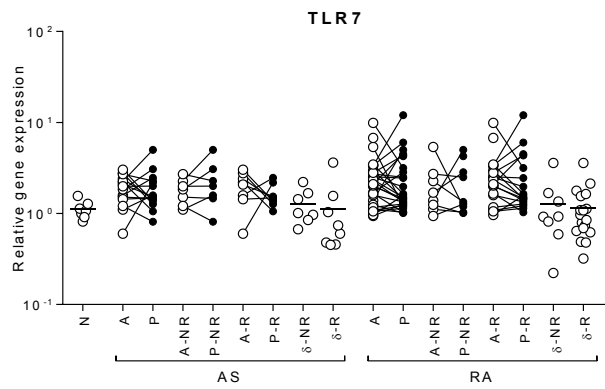
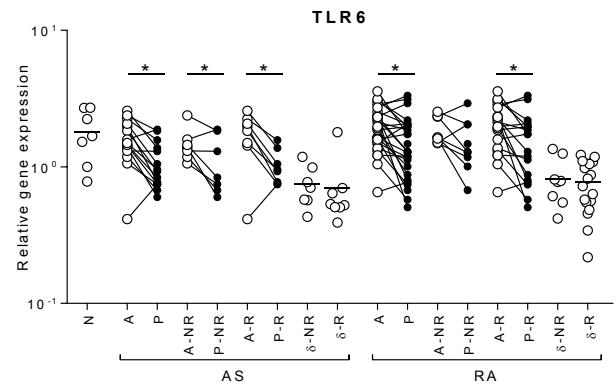
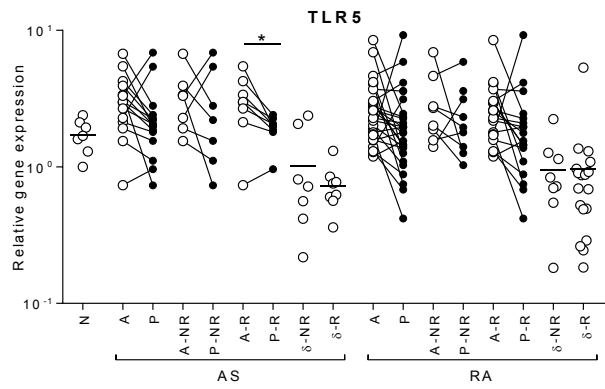
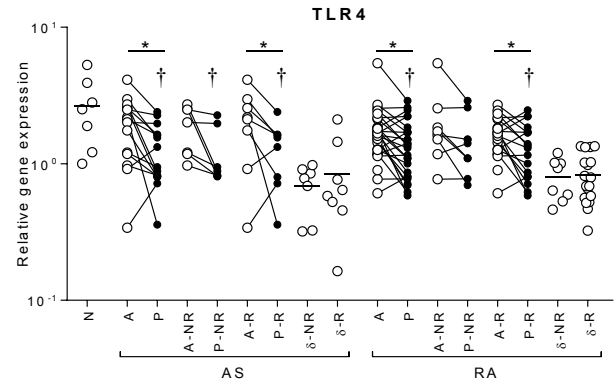
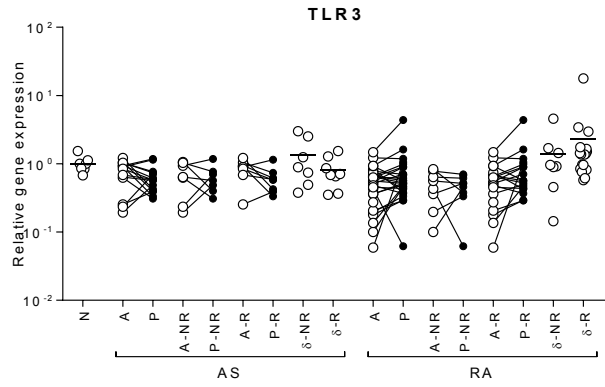
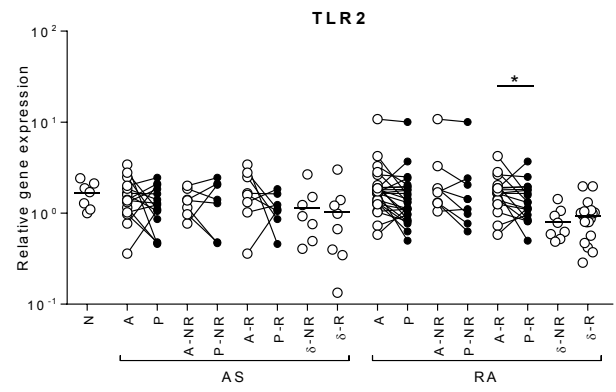
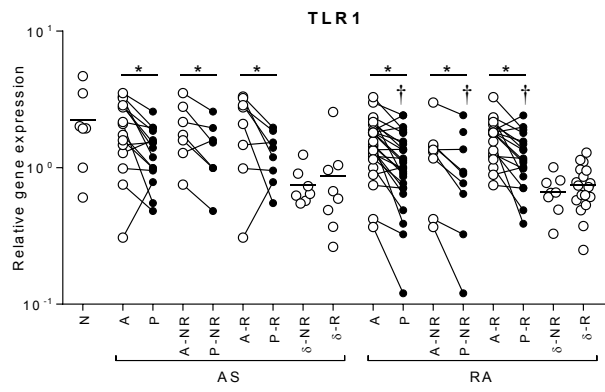


Supplementary Figure 6: Lymph nodes



Supplementary Figure 7: Blood





Supplementary Figure 8