Short title: DR3 regulates peritoneal inflammation

Death Receptor 3 promotes chemokine directed leukocyte recruitment in acute resolving inflammation and is essential for pathological development of mesothelial fibrosis in chronic disease

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No of pages: 25 (including References and Figure legends)**; 4 Figures**

Sources of support: WVP was supported by a PhD studentship from the I3-IRG, joint funded by the British Medical Research Council (MRC) and the School of Medicine, Cardiff University awarded to ECYW and SAJ. JPT was employed on MRC grant G0901119 awarded to ECYW and IRH. RKS was funded by BBSRC/GSK CASE studentship BB/H530589/1.

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ABSTRACT

Death Receptor 3 (DR3, TNFRSF25) and its TNF-like ligand TL1A (TNFSF15) control a number of processes in inflammatory diseases through the expansion of effector T cells and the induction of pro-inflammatory cytokines from myeloid and innate lymphoid cells. Here, using wildtype ($DR3^{+/+}$) and DR3 knockout ($DR3^{-/-}$) mice, we show that the DR3/TL1A pathway triggers the release of multiple chemokines following acute peritoneal inflammation initiated by a single application of *Staphylococcus epidermidis* supernatant (SES), correlating with the infiltration of multiple leukocyte subsets. In contrast, leukocyte infiltration was not DR3-dependent following viral challenge with murine cytomegalovirus (MCMV). DR3 expression was recorded on connective tissue stroma, which provided DR3-dependent release of CCL2 (MCP-1), CCL7 (MCP-3), CXCL1 (KC), and CXCL13 (BCA-1). CCL3 (MIP-1α), CCL4 (MIP-1β) and CXCL10 (IP10) production was also DR3-dependent, but qRT-PCR showed that their derivation was not stromal. *In vitro* cultures identified resident macrophages as a DR3-dependent source of CCL3. Whether DR3 signaling could contribute to a related peritoneal pathology was then tested using multiple applications of SES, the repetitive inflammatory episodes of which lead to peritoneal membrane thickening and collagen deposition. Unlike their $DR3^{+/+}$ counterparts, $DR3^{-/-}$ mice did not develop fibrosis of the mesothelial layer. Thus, this work describes both a novel function and essential requirement for the DR3/TL1A pathway in acute, resolving and chronic inflammation in the peritoneal cavity.

INTRODUCTION

Death Receptor 3 (DR3, TRAMP, LARD, WSL-1, Apo-3, TR3, TNFRSF25)[1-6], a member of the tumor necrosis factor receptor superfamily (TNFRSF), and its TNFSF ligand TL1A (TNFSF15) [7], have emerged as major regulators of inflammation and immunity. Genome-wide association studies (GWAS) have consistently linked polymorphisms around the *TNFSF15* gene as a risk factor in inflammatory bowel diseases (IBD) such as ulcerative colitis, Crohn's disease and/or irritable bowel syndrome [8-11], and in the arthritic disease ankylosing spondylitis [12], with associations also reported with primary billiary cirrhosis [13]. Furthermore, variation in the *TNFRSF25* gene locus has been suggested as a risk factor in rheumatoid arthritis (RA) [14], while other genetic studies have associated the *TNFSF15* gene with leprosy [15], implicating a further role for the DR3/TL1A axis in pathogenic immunity.

These wide-ranging links to multiple pathologies have been supported by *in vitro* and *in vivo* studies in both man and mouse. Levels of serum and tissue TL1A are increased in RA [16], IBD [17], psoriasis [18], primary billiary cirrhosis [19] and ankylosing spondylitis [20], while mice overexpressing TL1A develop colitis or ileitis [21-23]. In contrast, mice deficient for DR3 or TL1A, or those expressing dominant negative forms of DR3 are resistant to multiple models of inflammatory and autoimmune disease [24-31], or exhibit impaired immunity to bacterial or viral pathogens [32, 33] with a more debated impact on immunity to parasitic worms [34, 35]. Much of DR3 function has been attributed to its expression on T cells ($CD4^+$ regardless of lineage or $CD8^+$) and the capacity of TL1A, either directly or indirectly, to drive the proliferation of pathogenic effector T cell numbers [26, 27, 36, 37]. A single study also suggests a role in inhibiting proliferation of B cells [38]. This has defined significant roles for the DR3/TL1A pathway in adaptive immune responses.

However, DR3 expression is also found on innate immune cell and non-hematopoietic lineages. DR3 signaling on myeloid cells influences myeloid differentiation [30, 39], while on NKT and innate

lymphoid cells (ILCs), triggers effector cytokine release and expansion essential for the development of airway inflammation [25, 34]. Further, DR3$^{-/-}$ mice exhibit behavioural defects associated with impaired corticostriatal innervation controlled by DR3 expression on neurones [40] and its expression on tubular epithelial cells regulates renal inflammation and injury caused by reperfusion [41, 42]. On osteoblasts, DR3 can trigger apoptosis *in vitro* [43] or control bone mineral apposition *in vivo* [44]. These findings indicate that the DR3/TL1A pathway has intrinsic functions and also controls early events in immune processes in addition to the more established roles in acquired immunity.

In this report, we further investigated the *in vivo* role of the DR3/TL1A pathway using sterile challenge of *Staphylococcus epidermidis* supernatant (SES) in the peritoneal cavity [45, 46]. As a single application, this system mimics a resolving inflammatory response that returns to baseline levels [45], allowing scrutiny of infiltrating cells and soluble factors in peritoneal washouts within hours of the challenge. Using multiple applications, pathology develops in the form of peritoneal membrane fibrosis [47], mirroring the pathology associated with repeated bacterial infections in renal patients on peritoneal dialysis. Consequently, we show that the DR3/TL1A axis shapes the inflammatory response within the peritoneum and report a previously unappreciated role for DR3 in controlling inflammation via modulation of chemokine expression by both hematopoietic and stromal cells.

MATERIALS AND METHODS

Animals

Age-matched $DR3^{-/-}$ and $DR3^{+/+}$ littermates of 8-12 weeks of age on a C57BL/6 background were used in experiments, derived from a $DR3^{het}$ colony that was founded from mice provided by CRUK [48]. For peritoneal challenge with SES, animals of mixed sex were used as no significant differences in peritoneal leukocyte content of male and female mice were observed, either at baseline or during the course of experiments (data not shown). Irrespective, the male:female ratio in each $DR3^{+/+}$ and $DR3^{-/-}$ group for each timepoint were matched. For challenge with MCMV, all animals used were male. All procedures were approved by the Local Research Ethics Committee and performed in accordance with Home Office approved license PPLs 30/2401 (for SES), 30/2442 and 30/2580 (for MCMV).

Immunohistochemistry

Briefly, peritoneal membranes were harvested and fixed in neutral buffered formal saline and permeated with paraffin wax before 5µm sectioning. Sections were rehydrated and endogenous peroxidase activity was blocked. Antigen unmasking was achieved by incubating the sections in Proteinase K (20 µg/ml in TE buffer; Sigma) for 10 mins. Following blocking steps (20% goat serum and streptavidin/biotin blocking kit (Vector Laboratories), sections were incubated overnight with 4 µg/ml goat biotinylated anti-DR3 (R&D systems) or isotype controls diluted in PBS followed by streptavidin-HRPO secondary antibody (Vector Laboratories) as per manufacturer's instructions. Positive staining was visualized using a DAB chromogen (Vector Laboratories). Collagen was visualized using van Gieson stain. Sections were counterstained with hematoxylin, dehydrated and mounted in DPX. Images were captured using an Olympus N457 or Canon 100D digital camera and positive staining analyzed using Adobe Photoshop CS3.5. Randomly selected areas were used for analysis with the average from 5 being used to generate values for each section.

qRT-PCR

Total RNA was extracted following manufacturer's instructions using a RNAeasy mini kit (Qiagen, Crawley, UK) from snap frozen and powdered peritoneal membranes or pelleted macrophages. cDNA synthesis and PCR was performed using systems and primers generated through Primer Design (Southampton, UK) on an iCycler qPCR machine (Biorad, Hemel Hempstead, UK) with β-actin as the housekeeping control gene. Relative Quantity (RQ) was calculated after subtracting the negative control. Primers used (forward first, reverse second) were as follows:

CCL2 (5'-CCCAATGAGTAGGCTGGAGA-3' and 5'-TCTGGACCCATTCCTTCTTG-3');

CCL3 (5'-TTTTGAAACCAGCAGCCTTT-3' and 5'-CTGCCTCCAAGACTCTCAGG-3');

CCL4 (5'-CCCACTTCCTGCTGTTTCTC-3' and 5'-CTCACTGGGGTTAGCACAGA-3');

CCL5 (5'-GTGCCCACGTCAAGGAGTAT-3' and 5'-CCACTTCTTCTCTGGGTTGG-3');

CCL7 (5'-CCCAAGAGGAATCTCAAGAGC-3' and 5'-ATAGCCTCCTCGACCCACTT-3');

CXCL1 (5'-GCACCCAAACCGAAGTCATA-3' and 5'-AGGTGCCATCAGAGCAGTCT-3');

CXCL2 (5'-AAAGTTTGCCTTGACCCTGA-3' and 5'-CTTTGGTTCTTCCGTTGAGG-3');

CXCL5 (5'-GCCCTACGGTGGAAGTCATA-3' and 5'-GTGCATTCCGCTTAGCTTTC-3');

CXCL10 (5'-TGAATCCGGAATCTAAGACCA-3' and 5'-GAGGCTCTCTGCTGTCCATC-3');

CXCL13 (5'-TCGTGCCAAATGGTTACAAA-3' and 5'-GGTGCAGGTGTGTCTTTTGA-3');

TL1A (5'-CAGCAGAAGGATGGCAGA-3' and 5'-CTCTGGCCTGTGTCTACA-3').

Preparation of SES, determination of biological activity and the SES model of peritoneal challenge

SES was prepared and used in peritoneal challenge as described previously [46, 47]. Supernatants were filtered to remove live bacteria, lyophilized and stored at -70˚C. Biological activity was determined by its capacity to induce IL6 release from RAW 264 cells in a 24 hr culture, measured by ELISA (BD Pharmingen OpEIA; BD Biosciences, Oxford, UK).

Challenge with MCMV

Mice were challenged itraperitoneally (i.p.) with 5 x 10^4 plaque-forming units of MCMV Smith strain (American Type Culture Collection, Manassa, VA, USA), as previously described [49].

Flow cytometry

Following peritoneal lavage and centrifugation to isolate leukocytes, samples were treated with Fc block (BD Pharmingen) and then stained at 4˚C for 25 mins with combinations of the following pre-conjugated mAbs: CD19-FITC, CD8a-FITC, NK1.1-PE, Gr-1-PerCP-Cy5.5, CD4-PerCP-Cy5.5, CD11c-PE-Cy7, Ly6G-V450, CD44-V450, CD11b-APC-Cy7, CD3-APC-Cy7 (all BD Biosciences); B220-PE, F4/80-APC (Invitrogen); Ly6B.2-PE (AbD Serotec); TCRαβ-APC (Caltag). Samples were then washed, fixed in 1% paraformaldehyde before running and analysis on a CyAnTM ADP Flow Cytometer using Summit software (Beckman Coulter). Cell subsets were identified using the following criteria: neutrophils ($7/4^+$, $Ly6G^+$), resident macrophages ($F4/80^+CD11b^+$), inflammatory macrophages ($F4/80^{int}CD11b^{int}$), eosinophils ($F4/80^{int}CD11b^{int}SSC^{hi}$), NK cells ($NK1.1^+\alpha\beta TCR^-$), NKT cells ($NK1.1^+\alpha\beta TCR^+$), T cells ($CD3^+\alpha\beta TCR^+$) and B cells ($CD19^+B220^+$).

ELISAs and chemokine arrays

ELISAs were performed following manufacturers' instructions. For murine CCL2, CCL3, CCL5, CCL5, CXCL1. CXCL2, CXCL5, CXCL10 and CXCL13, ELISAs were obtained from R&D Systems (Abingdon, UK). ELISA for murine CCL7 was obtained from Antigenix America (NY 11746, USA). For determining concentration of chemokines in cultures of resident macrophages, the Legendplex^{TM} mouse proinflammatory chemokine panel was used following manufacturer's instructions (Biolegend).

Statistical analysis

Staining readouts were percentages, therefore, non-parametric Mann-Whitney U tests were used for statistical analysis. For protein concentrations from ELISAs and RQ from qRT-PCR, student's t-

Tests and ANOVAs were used. Analyses were performed on GraphPad Prizm - p values of <0.05 were considered significant and values of <0.01 were considered highly significant.

RESULTS

DR3 and TL1A expression in the peritoneal cavity following SES challenge

The DR3/TL1A pathway has been shown to be essential in the development of a number of models of disease involving intraperitoneal priming or challenge [25-27, 30, 32, 33], but little has been reported about DR3 and TL1A expression patterns in the peritoneal tissue and cavity during this process. DR3 expression in the mesothelial layer of the peritoneal membrane was first investigated, before and after SES challenge by staining of sections using a polyclonal antibody. Anti-DR3 showed minimal staining on $DR3^{-/-}$ peritoneal mesothelial layers (0.25 ± 0.08% positive pixels), but a significant signal was recorded from $DR3^{+/+}$ samples, in the absence of (6.5 ± 0.3%) and up to 12 hrs post-challenge (7.6 ± 1.6%) before becoming negative after 24 hrs (0.08 ± 0.04%) (Fig. 1A, B). DR3 was also found on $CD4^+$, $CD8^+$ and NKT cells within the peritoneal cavity, in agreement with previous reports studying DR3 expression in splenocytes ([25, 33] and data not shown). This data shows for the first time that DR3 is present on the stroma of the peritoneal cavity. In addition, expression of TL1A mRNA was measured using qRT-PCR on peritoneal membrane sections, as well as from resident peritoneal macrophages isolated by cell sorting ($F4/80^+CD11b^+$) and treated *in vitro* with SES. Whilst the mesothelial layers were negative for TL1A throughout the timecourse (Fig. 1C), TL1A mRNA was triggered from resident peritoneal macrophages within 1 hr of SES treatment, irrespective of their derivation from $DR3^{+/+}$ or $DR3^{-/-}$ mice (Fig. 1D). Thus, bacterial challenge in the peritoneal cavity primarily induces TL1A from resident macrophages, not stroma, but responses to TL1A can come from DR3 on both stroma and leukocytes.

Leukocyte accumulation is impaired in the absence of DR3

Leukocyte infiltration of the peritoneal cavity following SES challenge was next examined in the presence or absence of DR3. The composition of inflammatory cells in the peritoneal lavage was assessed using multiparameter flow cytometry and 2 panels of mAbs defining myeloid lineages and lymphoid populations (see Materials and Methods). Before challenge, there were no differences in

the leukocyte content of DR3$^{-/-}$ and DR3$^{+/+}$ peripheral blood or peritoneal lavage (Supplemental Table 1), showing that the absence of DR3 did not influence the baseline cellular constitution of the circulation or peritoneal cavity. The progression of this model from inflammatory insult to its resolution has been well-characterized, demonstrating a biphasic cellular infiltration predominantly consisting of neutrophils that peaks in the first 6 hrs and declines thereafter. Increasing numbers of other cell types follows, peaking after 48 hrs, before falling away over 96 hrs ([45, 46] and Fig. 2). In DR3$^{-/-}$ mice, significantly lower numbers of neutrophils in the early stage were observed following SES challenge compared to their DR3$^{+/+}$ counterparts. Interestingly, viral challenge with murine cytomegalovirus (MCMV) induced considerably less neutrophil accumulation and showed no differences between DR3$^{+/+}$ and DR3$^{-/-}$ mice (Fig 2A) , consistent with reports that neutrophil recruitment following MCMV challenge is IL-22 dependent [50]. During the second phase after SES challenge, eosinophils, inflammatory macrophages, T cells, B cells and NKT cells were decreased in number (Fig 2B). While the profile of resident macrophages and NK cell numbers were not significantly different between DR3$^{+/+}$ and DR3$^{-/-}$ mice throughout the time course, peritoneal B cell numbers remained significantly higher in DR3$^{+/+}$ animals by 96 hrs post-challenge (Fig. 2B). Overall, there was an intrinsic defect in the accumulation of leukocytes in DR3$^{-/-}$ mice after SES challenge in the peritoneal cavity.

Proliferation is unaltered in leukocytes from DR3$^{-/-}$ mice

Reduced numbers of effector cells, normally at the site of pathology, have been reported in the absence of DR3 in a variety of different pathological settings, primarily being ascribed to defects in proliferation [25-27, 32-34]. We, therefore, measured the levels of proliferation of cells from peritoneal lavage using detection of Ki67 at 48 hrs post-SES challenge, when there were peak numbers of infiltrating leukocytes. Surprisingly, there were no differences in Ki67 staining of any of the cell subsets when comparing DR3$^{+/+}$ and DR3$^{-/-}$ mice (Supplemental Fig. 1 and Supplemental Table 2), indicating that at this early stage of the inflammatory process, DR3 was regulating a distinct

mechanism of cellular accumulation. Because DR3 contains a death-inducing intracellular domain, apoptosis using Annexin-V staining was also measured, but consistent with other reports [33, 51], the absence of DR3 did not alter the levels of cell death in peritoneal leukocytes (data not shown). The DR3/TL1A pathway therefore regulated peritoneal leukocyte numbers early after bacterial challenge with a mechanism that was independent of proliferation and cell death.

Levels of multiple chemokines are impaired in DR3$^{-/-}$ mice following SES challenge

Another possible explanation for the defect in accumulation of cellular infiltrate is that DR3 regulates leukocyte recruitment. To ensure that the absence of DR3 did not adversely influence baseline levels, cell-free supernatant from peritoneal lavages of unchallenged DR3$^{+/+}$ and DR3$^{-/-}$ mice were tested for a range of chemokines capable of attracting virtually every subset of leukocytes between them. No significant differences were found at baseline, with detectable levels of CCL3, CCL4, CXCL5 (targeting neutrophils, monocytes), CXCL10 (T cells, NK cells) and CXCL13 (B cells), while CCL2, CCL5, CCL7 (dendritic cells, monocytes, T cells), CXCL1 and CXCL2 (neutrophils) were below levels of detection of their specific kits (Supplemental Fig. 2). The kinetics of chemokine release were then investigated in the peritoneal lavage of DR3$^{+/+}$ and DR3$^{-/-}$ mice challenged with SES. While some chemokines including CCL5, CXCL2 and CXCL5 showed no differences between genotypes, the expression of CCL2, CCL3, CCL4, CCL7, CXCL1, CXCL10, CXCL13 were all significantly impaired in DR3$^{-/-}$, as compared to DR3$^{+/+}$ mice, although not necessarily at the same timepoint as when peak concentrations were observed (Fig. 3A). In DR3$^{-/-}$ mice, CCL2, CCL7 and CXCL1 were significantly reduced at 1 hr; CXCL10 at 3 hrs; CCL3 and CCL4 at 6 hrs; and CXCL13 at 24 hrs.

Select chemokines are differentially produced from the stroma or resident macrophages in a DR3-dependent fashion

Chemokines can be derived from both stromal and hematopoietic cells. To delineate between these potential sources, qRT-PCR for the messages of all of the above chemokines were performed on RNA extracted from $DR3^{+/+}$ and $DR3^{-/-}$ mesothelial layers at timepoints between 1 and 24 hrs following SES challenge. Those showing no significant differences in protein levels between $DR3^{-/-}$ and $DR3^{+/+}$ mice (CCL5, CXCL2 and CXCL5) also showed no significant differences in message. In contrast, of the remaining 7, only CCL2, CCL7, CXCL1 and CXCL13 (attractants of dendritic cells, neutrophils, eosinophils, memory T and B cells) demonstrated a significant reduction in message from $DR3^{-/-}$ compared to $DR3^{+/+}$ mesothelial layers (Fig. 3B), implicating the stroma as a significant DR3-dependent source of these chemokines and suggesting non-stromal cells are the DR3-dependent source of the others. To further investigate this, resident macrophages (the largest innate immune leukocyte population in the unchallenged peritoneal cavity – see Supplemental Table 1) were extracted from $DR3^{+/+}$ and $DR3^{-/-}$ mice, pooled and challenged with SES *in vitro*. Of 13 chemokines studied, only CCL3 and CCL4 were recorded at levels above the detection limit of the array. $DR3^{+/+}$ resident macrophages produced significantly more CCL3, but not CCL4, compared to $DR3^{-/-}$ resident macrophages in response to SES (Fig. 3C). The overall conclusion is that DR3 signaling controls recruitment of multiple leukocyte subsets following bacterial challenge in the peritoneal cavity by a broad release of chemokines, some of which come from stroma, while others are derived from non-stromal cell types including resident macrophages within the peritoneal cavity.

DR3 deficiency protects against pathology of SES-induced chronic inflammation

To test whether DR3 signaling could contribute to pathology in a related model of disease, $DR3^{-/-}$ animals were challenged with multiple serial applications of SES, a system designed to mimic the fibrosis of the peritoneal membrane observed in peritoneal dialysis patients following persistent and regular bacterial infections in the peritoneal cavity [47]. Thickening of the mesothelial layer and increased deposition of collagen was observed in $DR3^{+/+}$ mice, but $DR3^{-/-}$ mice showed resistance to

this (Fig. 4), demonstrating an essential requirement for DR3 in the development of this fibrotic pathology.

DISCUSSION

In recent years, the DR3/TL1A pathway has emerged as a master regulator of inflammatory and autoimmune disease, as well as a co-ordinator of immune responses to a variety of pathogens. This is in the context of DR3$^{-/-}$ mice showing no impairments in the development of their immune systems, constitution of their immune (or other) organs [48] or intrinsic extrathymic T cell trafficking [26]. Much of the *in vivo* research in the field has used models of disease involving antigen priming or pathogen challenge in the peritoneal cavity, and have demonstrated that antigen-specific splenic responses following such challenges are relatively normal in the absence of DR3 indicating that immune priming is intact. Here, we show that the DR3/TL1A pathway controls the very earliest of immune processes during peritoneal challenge, regulating the production of chemokines that draws pro-inflammatory leukocytes into sites of inflammation. This effect is dependent on the type of challenge, with SES, but not MCMV, inducing DR3-dependent leukocyte infiltration. There is the possibility that the DR3/TL1A pathway interacts with specific TLR signaling pathways as *Staphyloccus epidermidis* is recognized through TLR2 [52], while MCMV triggers TLR3 and 9 [53] and induces neutrophil recruitment through IL-22 [50].

Prior to this report, DR3 function has primarily been attributed to the expression of DR3 on multiple cell types of the hematopoietic lineage, ranging from NKT, CD4$^+$, CD8$^+$ T and B cells to ILCs, macrophages and neutrophils. This is the first report of significant DR3 expression on a stromal layer in an *in vivo* setting and in the absence of active immune responses. Previous studies have indicated that DR3 is inducible on stroma by pro-inflammatory events, as has been described for tubular epithelial cells following ischemic injury [41] and possibly in joints undergoing antigen-induced arthritis, although the latter could be attributed to infiltrating cells [51]. More intriguingly, stromal DR3 expression seems to be closely regulated in the peritoneal cavity, with the signal disappearing 24 hrs after SES challenge. In contrast, although endothelial cells have been reported to release TL1A [7], its major source following SES challenge was resident macrophages, occurring within an hour of

stimulation and independent of DR3 expression (Fig. 1). This is in agreement with previous reports showing cells of the myeloid lineage respond to bacterial challenges by rapid TL1A release [26, 32].

DR3-dependent release of some chemokines has been previously reported, but not on the scale shown here. Thus, *in vitro* studies on the myeloid THP-1 cell line has demonstrated IL-8 release requiring TL1A and IFNγ priming [54], while CXCL1 is reduced in the joints of DR3$^{-/-}$ mice undergoing AIA [51]. Both these chemokines, in humans and mice respectively, are established neutrophil attractants. This report indicates that at least in the murine SES challenge system, the production of CXCL1, but not 2 other neutrophil chemoattractants (CXCL2/MIP-2 and CXCL5/LIX), is DR3-dependent. It also shows, for the first time, that there is a stromal contribution to this (Fig. 3 and 4). Whether this is the dominant DR3-dependent chemokine controlling neutrophil attraction remains an area of further study as there are multiple other chemokine/chemokine receptor interactions capable of this [55], many of which have not been tested in this study.

The stromal release of a number of other chemokines demonstrated DR3-dependency, namely CCL2, CCL7 and CXCL13. CCL2 and CXCL13 have primary chemokine receptors, CCR2 and CXCR5 respectively. CCR7 is more pleiotropic, binding CCR1, 2 and 3, together having the capacity to attract monocytes, eosinophils, DCs, T and B cells [55]. This correlated well with the subsets of leukocytes that showed impaired SES-induced accumulation in the peritoneal cavity of DR3$^{-/-}$ mice. Interestingly, however, there were other chemokines in DR3$^{-/-}$ peritoneal lavage that were reduced in concentration, namely CCL3, CCL4 and CXCL10. Their ligands include CCR1, CCR5 and CXCR3 found on monocytes, T, NK and mast cells. We further identified resident macrophages as a DR3-dependent source of CCL3 (Fig 3C). The assumption is that CCL4 and CXCL10 were derived from other as yet unidentified leukocytes as these chemokines were either not produced or did not show DR3-dependent production by resident macrophages, while their mRNA levels were unaltered in the DR3$^{-/-}$ mesothelium. Further studies would be required to identify which other cells are producing

DR3-dependent CCL4 and CXCL10, and determine the relative contribution of DR3 on stroma versus resident versus infiltrating leukocytes to recruitment in this system. The very rapid peak of production for many of the chemokines (less than 3 hrs – see Fig 3) would indicate either a stromal or resident leukocyte source for these, with the possibility of infiltrating leukocytes contributing to levels of chemokines at later time points. In this context, DR3 expression on innate immune cells is constitutive on NKT cells (which have been shown to produce IL-4 and IL-13 in models of allergic lung inflammation) [25], or can be induced on macrophages as well as neutrophils [51]. The latter can also produce multiple chemokines and is a major infiltrating cell type [56].

The leukocyte recruitment function of the DR3/TL1A pathway shows distinct differences to the role that has been characterized for IL-6 using intraperitoneal SES challenge. While both impact on T cell recruitment correlating with lower levels of CCL4 and CXCL10, IL-6$^{-/-}$ animals display a reduction in CCL5 [57], which was not observed in DR3$^{-/-}$ mice. Furthermore, unlike DR3$^{-/-}$ animals, IL-6$^{-/-}$ mice show normal early neutrophil [46] and later B cell [57] infiltration accompanied by unaltered levels of CXCL1 and CXCL13. This indicates that DR3 signaling is distinct and not directly upstream of IL-6. More recently, IL-6 has been shown to be essential for the development of peritoneal membrane fibrosis following multiple acute inflammatory episodes induced by serial applications of SES. This was attributed to IL-6-mediated Th1 effector commitment [47]. Our data indicate that this model is also dependent on DR3, but likely through its general regulation of effector T cells. DR3$^{-/-}$ mice are resistant to the induced increases in peritoneal membrane thickening and collagen deposition. This is consistent with the phenotype of transgenic mice overexpressing TL1A, which spontaneously develop intestinal and colonic fibrosis accompanied by goblet and Paneth cell hyperplasia [22, 23, 58]. One significant question is the relative contribution to pathology of DR3's role in leukocyte recruitment versus its function in driving effector cell expansion [22, 26, 27, 29, 33, 34, 59]. While this can be divided into early and late inflammatory processes in the SES challenge model, the two immunological events are not mutually exclusive and could explain some previously observed

phenomena, such as the altered localization of T cells and macrophages in DR3$^{-/-}$ mice undergoing OVA-induced lung inflammation [26].

It is evident from this work that DR3/TL1A signaling regulates primary inflammatory responses, with potential impact on downstream adjuvant and host defense activities. The understanding of these roles to pathological processes is an area of further research that will be important for developing the DR3/TL1A pathway as a therapeutic target in inflammatory disease, agonism or antagonism of which may prevent disease depending on the effector T cells being influenced.

Acknowledgements

Thanks to the Central Biotechnology Service, School of Medicine, Cardiff University for access to flow cytometers. WVP, GWJ, JPT and RKS performed experiments. ASW, IRH, PRT, SAJ and ECYW contributed through conceptual and experimental design, animal models and writing of the manuscript.

Conflict of Interest Disclosure

The authors declare no commercial or financial conflict of interest.

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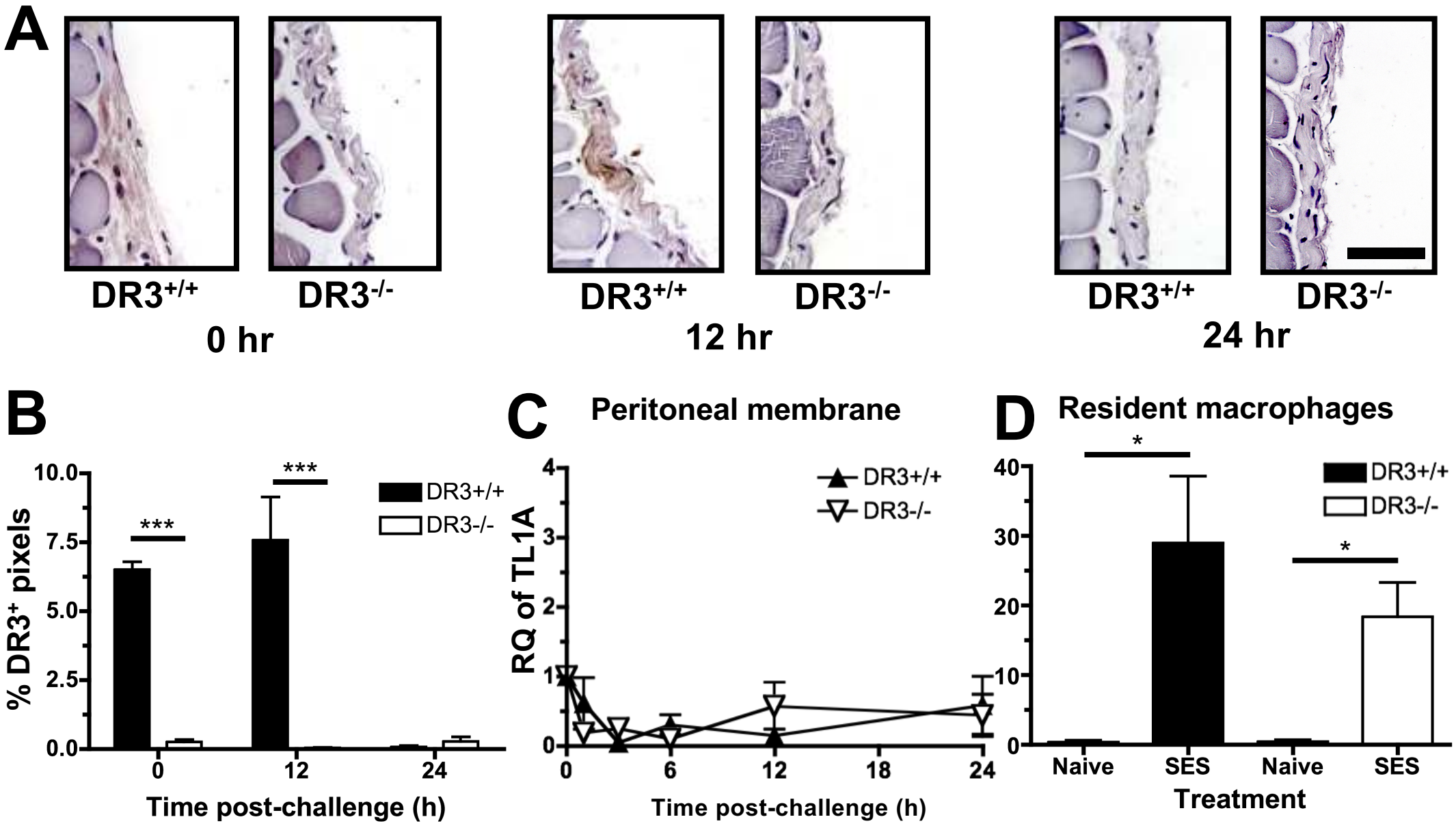
FIGURE LEGENDS

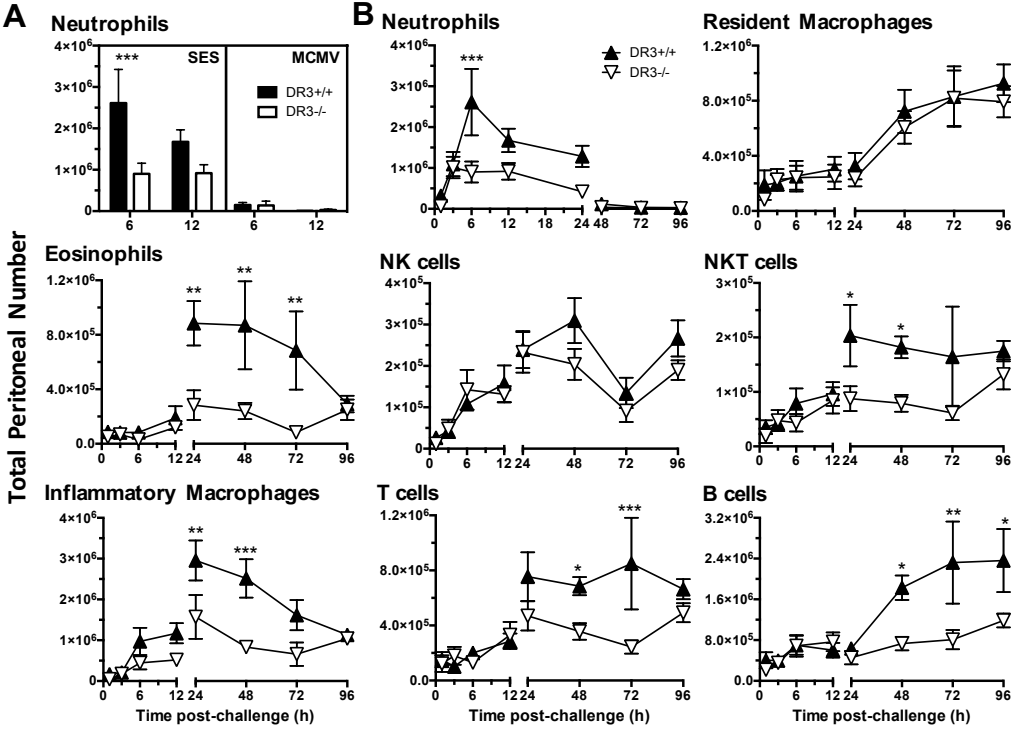
Figure 1 – DR3 expression and sources of TL1A in the peritoneal cavity. Inflammation in the cavity was induced via an i.p. injection of SES. Peritoneal membranes were harvested, fixed, sectioned and stained for DR3 expression or analyzed for the relative quantity (RQ) of TL1A mRNA by qRT-PCR. A) Representative pictures showing DR3 staining in the mesothelial layer of $DR3^{+/+}$ mice after 0 and 12 hours but absent after 24 of inflammation (bar = 25μm.) B) Summary of DR3 expression in the mesothelial layer. Mean % positive (brown) pixels within the mesothelial layer of the membrane shown ± SEM (n=3 to 6 $DR3^{+/+}$ mice and $DR3^{-/-}$ mice per timepoint), ***p<0.001 by one-way ANOVA. C) RQ of TL1A mRNA in the peritoneal membrane of $DR3^{+/+}$ and $DR3^{-/-}$ mice. Mean ± SEM from n=5 mice per timepoint; not significantly different by ANOVA. D) RQ of TL1A mRNA in $DR3^{+/+}$ and $DR3^{-/-}$ resident peritoneal macrophages 1 hour after stimulation using SES. Macrophages were sorted from other leukocytes found in the peritoneal cavity from 17 pooled $DR3^{+/+}$ and $DR3^{-/-}$ mice, challenged with SES and analyzed for RQ of TL1A mRNA. Mean ± SEM shown from 4 replicate experiments. *p<0.05 by t-Test assuming unequal variance.

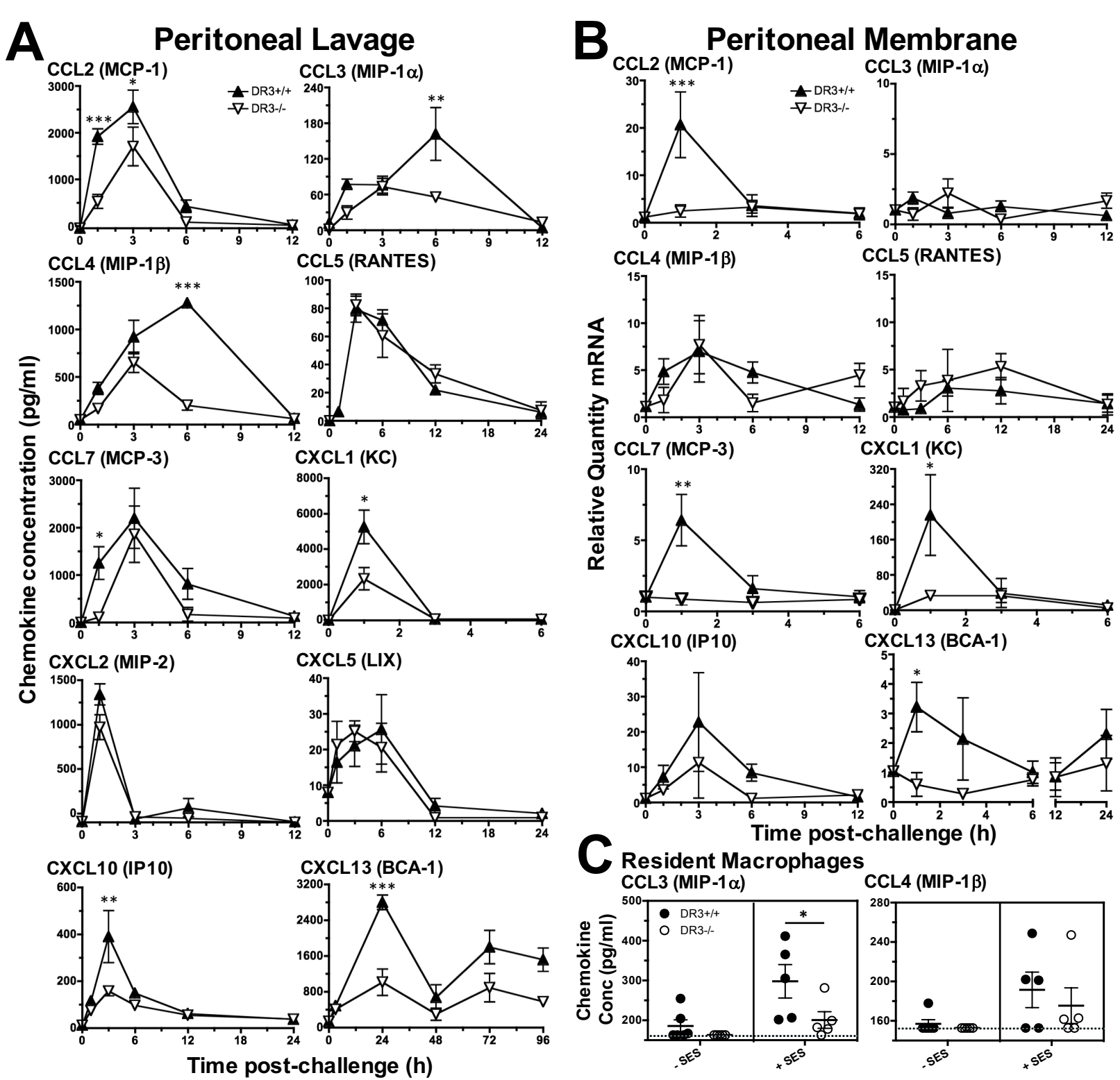
Figure 2 – Leukocyte subset numbers in the peritoneal cavity of $DR3^{+/+}$ and $DR3^{-/-}$ mice after SES and MCMV induced inflammation. Inflammation in the cavity was induced via an i.p. injection of SES or MCMV. (A) Numbers of neutrophils in the peritoneal cavity following the labeled challenges at the indicated times. (B) Peritoneal leukocytes were isolated from the cavity by lavage at the indicated times post-challenge and numbers of individual cell subsets calculated using cell counts and flow cytometry. Representative data from 1 of 2 expts. Each symbol represents mean of up to 6 mice per timepoint, error bars correspond to SEM; statistical analysis by ANOVA and Bonferroni post hoc test showed significant differences between $DR3^{+/+}$ and $DR3^{-/-}$ mice at ***p<0.001, **p<0.01, *p<0.05 for individual timepoints. Overall male:female ratio was 50:50 and matched for $DR3^{+/+}$ and $DR3^{-/-}$ groups.

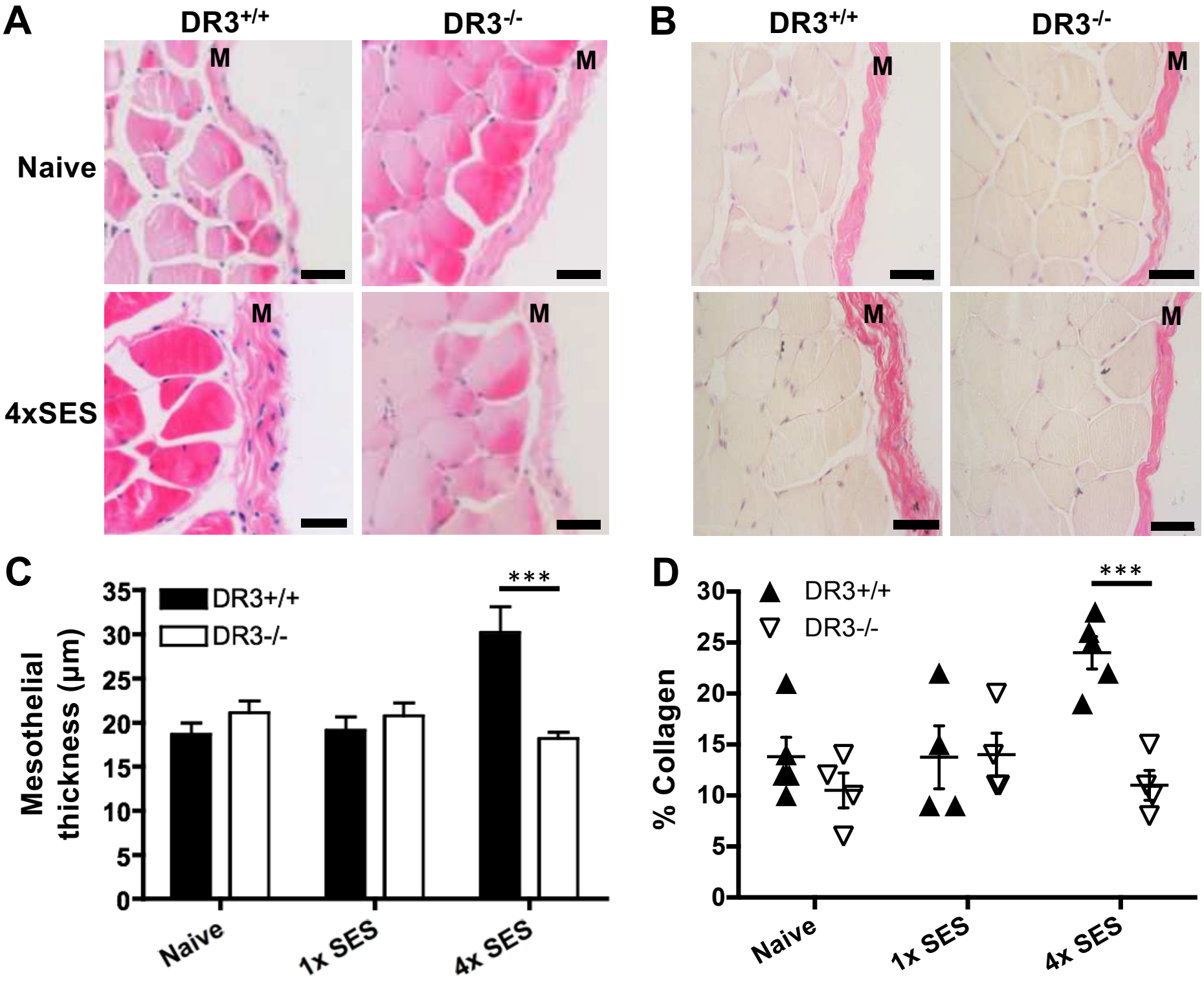
Figure 3 – Chemokine production in the peritoneal cavity of DR3$^{+/+}$ and DR3$^{-/-}$ mice after SES-induced inflammation. Inflammation in the cavity was induced via an i.p. injection of SES. levels of the indicated chemokines were measured in: **(**A) cell free supernatants in peritoneal lavage by ELISA and; (B) mRNA levels in the peritoneal membrane by qRT-PCR. Representative data from 1 of 2 expts. Each symbol and error bars represent mean ± SEM from up to 6 DR3$^{+/+}$ and DR3$^{-/-}$ mice per timepoint. Overall male:female ratio was 50:50 and matched for DR3$^{+/+}$ and DR3$^{-/-}$ groups. (C) Production of CCL3 and CCL4 by resident macrophages derived from DR3$^{+/+}$ and DR3$^{-/-}$ mice, stimulated with SES. Each point represents a culture; bars represent mean ± SEM. Values below the limit of the assay were assigned the lowest limit for generation of means and statistical testing. Dotted line shows lowest limit of assay. Statistical analysis by ANOVA and Bonferroni post hoc test showed significant differences between DR3$^{+/+}$ and DR3$^{-/-}$ mice at ***p<0.001, **p<0.01, *p<0.05.

Figure 4 – Thickness of the mesothelial layer and collagen deposition in DR3$^{+/+}$ and DR3$^{-/-}$ mice after inflammation induced by multiple applications of SES. Fibrosis of the mesothelial layer was induced by 4 i.p. injections of SES. Representative micrographs of the peritoneal membranes visualized with (A) hematoxylin and eosin, and (B) van Gieson stain, from mice treated as indicated. M = mesothelial layer; Bar = 50 µm. Summary of (C) mesothelial thickness and (D) collagen deposition. Bar and error bar represents mean ± SEM from up to 6 mice. Statistical analysis by ANOVA showed significant differences between DR3$^{+/+}$ and DR3$^{-/-}$ mice ***p<0.001 by Bonferroni post hoc test. Overall male:female ratio was 50:50 and matched for DR3$^{+/+}$ and DR3$^{-/-}$ groups.


A
DR3+/+
DR3-/-
0 hr
DR3+/+
DR3-/-
12 hr
DR3+/+
DR3-/-
24 hr
B
% DR3+ pixels
10.0
7.5
5.0
2.5
0.0
***
***
DR3+/+
DR3-/-
0
12
24
Time post-challenge (h)
C
Peritoneal membrane
RQ of TL1A
4
3
2
1
0
DR3+/+
DR3-/-
0
6
12
18
24
Time post-challenge (h)
D
Resident macrophages
40
30
20
10
0
*
*
DR3+/+
DR3-/-
Naive
SES
Naive
SES
Treatment


A
Neutrophils
SES
MCMV
DR3+/+
DR3-/-
***
B
Neutrophils
Resident Macrophages
Eosinophils
NK cells
NKT cells
Inflammatory Macrophages
T cells
B cells
Total Peritoneal Number
Time post-challenge (h)


A
Peritoneal Lavage
CCL2 (MCP-1)
CCL3 (MIP-1α)
CCL4 (MIP-1β)
CCL5 (RANTES)
CCL7 (MCP-3)
CXCL1 (KC)
CXCL2 (MIP-2)
CXCL5 (LIX)
CXCL10 (IP10)
CXCL13 (BCA-1)
DR3+/+
DR3-/-
Chemokine concentration (pg/ml)
Time post-challenge (h)
B
Peritoneal Membrane
Relative Quantity mRNA
Time post-challenge (h)
C
Resident Macrophages
CCL3 (MIP-1α)
CCL4 (MIP-1β)
Chemokine Conc (pg/ml)
- SES
+ SES


A
DR3+/+
DR3-/-
Naive
4xSES
M
B
DR3+/+
DR3-/-
M
C
Mesothelial thickness (µm)
DR3+/+
DR3-/-
***
Naive
1x SES
4x SES
D
% Collagen
DR3+/+
DR3-/-
***
Naive
1x SES
4x SES

Supplemental Table 1 – Counts for leukocyte subsets are not different in the unchallenged peritoneal cavity or peripheral blood of $DR3^{-/-}$ and $DR3^{+/+}$ mice

Cell subset	Peritoneal cavity [*]			Peripheral blood No/ml [†]		
	$DR3^{+/+}$	$DR3^{-/-}$	Significance [‡]	$DR3^{+/+}$	$DR3^{-/-}$	Significance [‡]
Macrophages/Monocytes	$7.2 \pm 1.8 \times 10^5$	$6.4 \pm 1.6 \times 10^5$	NS	$4.2 \pm 1.0 \times 10^5$	$4.7 \pm 1.6 \times 10^5$	NS
Dendritic cells	$5.1 \pm 1.6 \times 10^3$	$3.3 \pm 0.3 \times 10^3$	NS	N/A[§]	N/A[§]	N/A[§]
Neutrophils	N/A[§]	N/A[§]	N/A[§]	$2.3 \pm 0.6 \times 10^5$	$4.1 \pm 2.2 \times 10^5$	NS
Eosinophils	$1.9 \pm 0.7 \times 10^5$	$7.6 \pm 0.6 \times 10^4$	NS	$5.2 \pm 1.3 \times 10^4$	$3.5 \pm 0.8 \times 10^4$	NS
T Cells	$4.5 \pm 0.5 \times 10^5$	$4.6 \pm 1.1 \times 10^5$	NS	$4.0 \pm 0.5 \times 10^5$	$4.5 \pm 0.6 \times 10^5$	NS
B Cells	$8.6 \pm 1.0 \times 10^5$	$9.3 \pm 2.9 \times 10^5$	NS	$9.6 \pm 1.8 \times 10^5$	$1.0 \pm 0.2 \times 10^6$	NS
NK Cells	$6.8 \pm 1.7 \times 10^4$	$4.7 \pm 0.8 \times 10^4$	NS	$8.3 \pm 1.4 \times 10^4$	$7.2 \pm 1.0 \times 10^4$	NS
NKT Cells	$7.2 \pm 1.8 \times 10^4$	$6.9 \pm 0.6 \times 10^4$	NS	$1.3 \pm 0.2 \times 10^4$	$1.3 \pm 0.2 \times 10^4$	NS

[*] Values correspond to number of peritoneal leukocytes, mean ± SEM (n=5)
[†] Values correspond to number of peripheral blood leukocytes/ml, mean ± SEM (n= 4 $DR3^{+/+}$ or 5 $DR3^{-/-}$)
[‡] Significance between the two genotypes using t-Test, NS = no significant difference
[§] N/A corresponds to subsets either not present or not tested

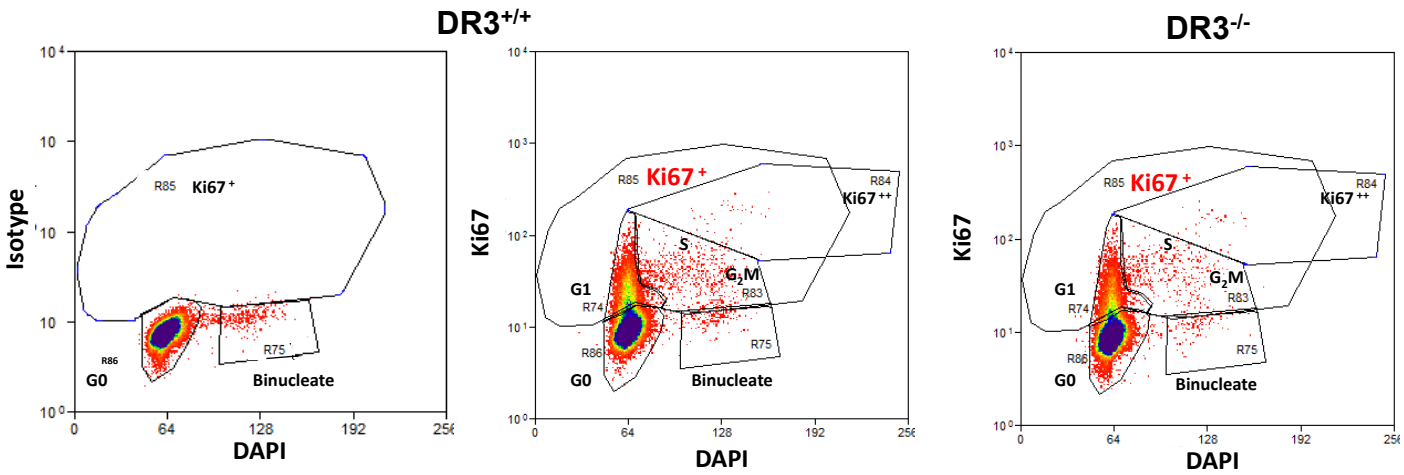
Supplemental Table 2 – Leukocyte proliferation in the peritoneal cavity following SES challenge

Cell Subset *	**Proportion of proliferating cells** †		
	DR3$^{+/+}$	**DR3$^{-/-}$**	**Significance** ‡
Resident macrophages	42.4 ± 13.2	44.5 ± 8.2	N.S.D
Inflammatory macrophages	43.7 ± 11.5	47.9 ± 7.7	N.S.D
T Cells	49.3 ± 2.3	50.5 ± 7.5	N.S.D
B Cells	36.9 ± 2.8	28.8 ± 8.2	N.S.D
NK Cells	35.2 ± 10.4	38.2 ± 3.8	N.S.D
NKT Cells	47.7 ± 3.8	51.8 ± 10.1	N.S.D

* Ki67^{+} cells were assessed in the indicated cell populations using the following phenotypic criteria: resident macrophages (F4/80^{+}CD11b^{+}); inflammatory macrophages (F4/80^{int}CD11b^{int}); NK cells (NK1.1^{+}αβTCR^{-}); NKT cells (NK1.1^{+}αβTCR^{+}); T cells (CD3^{+}αβTCR^{+}NK1.1^{-}); B cells (CD19^{+}B220^{+})

† Values = %Ki67^{+} of each subset 48 hours after SES challenge (mean ± SEM; n= 5 DR3$^{+/+}$ or 6 DR3$^{-/-}$). Gating as indicated in Supplemental Figure 1.

‡ Mann Whitney test showed N.S.D = no significant difference between DR3$^{+/+}$ and DR3$^{-/-}$.


DR3+/+
DR3-/-
Isotype
Ki67
DAPI
R85 Ki67+
R84 Ki67++
R86
R74
R83
R75
G0
G1
S
G2M
Binucleate

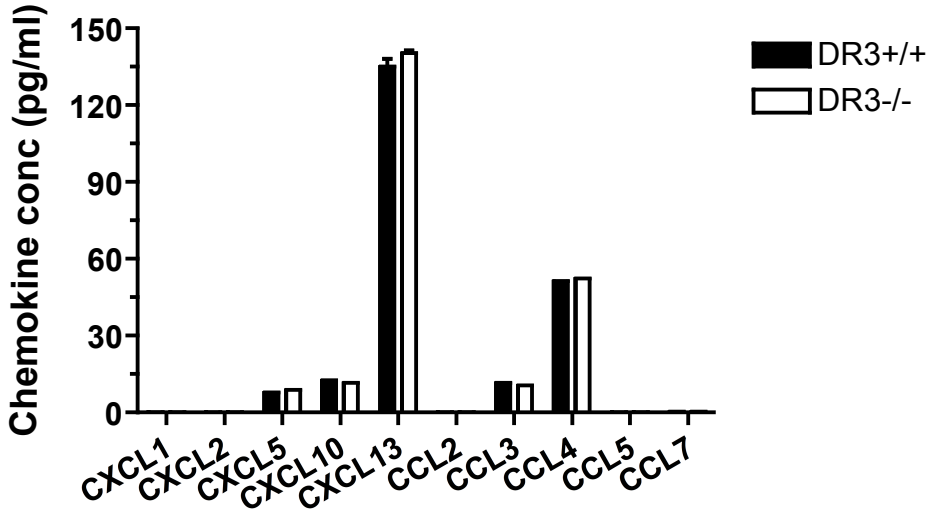

Chemokine conc (pg/ml)
150
120
90
60
30
0
CXCL1
CXCL2
CXCL5
CXCL10
CXCL13
CCL2
CCL3
CCL4
CCL5
CCL7
DR3+/+
DR3-/-

Figure Legends

Figure 1 – Flow cytometric gating for determining Ki67^{+} proliferating cells. Mice were challenged with SES and peritoneal leukocytes isolated from the cavity by lavage, stained for cell subsets, permeabilised and proliferation measured using the intracellular marker Ki67 and DAPI to differentiate binucleate non-proliferating cells. Representative plots of total peritoneal leukocytes showing isotype control and KI67 staining from DR3$^{+/+}$ and DR3$^{-/-}$ mice. Early proliferative G1 cells (Ki67^{+} DAPI^{lo}), S and G_2M phase (Ki67^{+} DAPI$^{int/hi}$) and non-proliferating binucleate cells (Ki67^{-} DAPI^{hi}) shown.

Figure 2 - Chemokine levels in the naïve peritoneal cavity are not different between DR3$^{+/+}$ and DR3$^{-/-}$ mice. The peritoneal cavity was lavaged, cells spun down and chemokine concentrations in the supernatant measured by ELISA. Data from n=3 DR3$^{+/+}$ and DR3$^{-/-}$ mice where available, but some values were below the manufacturer's detection threshold of the ELISA and therefore excluded from analysis. No significant differences were detected.