The role of Death Receptor 3 in allergic lung inflammation

A thesis submitted in candidature for the degree of Doctor of Philosophy

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Summary

Death Receptor 3 (DR3) is a death domain containing member of the TNF Receptor Superfamily (TNFRSF), refereeing a range of cellular responses from differentiation and proliferation to cell death, depending upon the context of receptor activation. DR3 has been reported to have a role in many inflammatory diseases, including inflammatory arthritis and inflammatory bowel disease. The aim of this study was to determine the contribution of DR3 in a mouse model of acute and chronic allergic lung inflammation.

Mice genetically deficient in the DR3 gene (DR3\textsuperscript{ko}) were resistant to cellular accumulation within the lungs and bronchoalveolar lavage following acute lung inflammation, induced by priming with ovalbumin (OVA) and the adjuvant aluminium hydroxide (Alum) prior to 2 OVA aerosol exposures. To discern the role of DR3 in a more physiologically relevant chronic model of allergic lung inflammation, mice underwent repeated inhalation challenges with OVA subsequent to priming with OVA and Alum. Whilst cellular accumulation did not differ, DR3\textsuperscript{ko} mice displayed reduced immunohistopathology, and goblet cell hyperplasia, hallmarks of the asthmatic phenotype. Intriguingly, DR3\textsuperscript{ko} mice exhibited reduced accumulation of various cell types into the spleen in both models. Early priming events were therefore investigated, prior to aerosolised antigenic challenge to decipher the effects of DR3. One sensitisation injection was sufficient to induce decreased DR3\textsuperscript{ko} splenocyte accumulation, though T and B cell responses were observed to be comparable between DR3\textsuperscript{ko} and DR3\textsuperscript{wt} controls. DR3\textsuperscript{ko} mice had depleted CXCL10 levels, suggesting cellular recruitment in response to inflammation is DR3 dependent.

The underlying DR3 dependent mechanisms concerning the DR3\textsuperscript{ko} splenic defects are under further investigation and may have impact on the use of the DR3/TL1A pathway as a therapeutic target, either as an anti-inflammatory or as a booster of the immune response to pathogens.
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Abbreviations

7AAD  7-Aminoactinomycin D
ADAMTS A disintegrin and metalloproteinase with thrombospondin motifs
AHR  Airway hyper-reactivity
AIA  Antigen induced arthritis
Alum  Aluminium hydroxide
ANOVA Analysis of variance
APC  Antigen presenting cell
BAFFR B cell-activating factor receptor
BAL  Bronchoalveolar lavage
BALF  Bronchoalveolar lavage fluid
BCR  B cell receptor
BSA  Bovine serum albumin
BrdU  Bromodeoxyuridine
BSA  Bovine serum albumin
CIA  Collagen induced arthritis
c-IAP  Cellular inhibitor of apoptosis
CRD  Cysteine-rich domain
DAB  3,3-Diaminobenzidine
DC  Dendritic cell
DcR3  Decoy receptor 3
DD  Death domain
DISC  Death inducing signalling complex
DR  Death receptor
DSS  Dextran sodium sulphate
EAE  Experimental autoimmune encephalitis
ECM  Extracellular matrix
EDTA  Ethylenediaminetetra-acetic (Ethanoic) acid
EGF  Epidermal growth factor
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbant assay</td>
</tr>
<tr>
<td>EST</td>
<td>Expressed sequence tag</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescent activated cell sorter</td>
</tr>
<tr>
<td>FADD</td>
<td>Fas associated death domain</td>
</tr>
<tr>
<td>FasL</td>
<td>Fas ligand</td>
</tr>
<tr>
<td>FC</td>
<td>Fragment crystallisable</td>
</tr>
<tr>
<td>FCγR</td>
<td>Fragment crystallisable gamma receptor</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>Flt3</td>
<td>FMS like tyrosine kinase 3</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescence in situ hybridisation</td>
</tr>
<tr>
<td>FSC</td>
<td>Forward scatter</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>HSS-HRP</td>
<td>High sensitivity streptavidin-horse radish peroxidise</td>
</tr>
<tr>
<td>IBD</td>
<td>Inflammatory bowel disease</td>
</tr>
<tr>
<td>IC</td>
<td>Immune complex</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IκB</td>
<td>Inhibitor of kappa B</td>
</tr>
<tr>
<td>IKK</td>
<td>IκB kinase complex</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>I.p.</td>
<td>Intra-peritoneal</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LT</td>
<td>Lymphotoxin</td>
</tr>
<tr>
<td>MAP3K</td>
<td>Mitogen-activated 3 kinases</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>mBSA</td>
<td>Methylated bovine serum albumin</td>
</tr>
<tr>
<td>MCMV</td>
<td>Mouse cytomegalovirus</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Macrophage colony stimulating factor</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean fluorescent intensity</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa B</td>
</tr>
<tr>
<td>NIK</td>
<td>NFκB inducing kinase</td>
</tr>
<tr>
<td>N.S.D</td>
<td>No significant difference</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>Ova</td>
<td>Ovalbumin</td>
</tr>
<tr>
<td>PAS</td>
<td>Periodic acid schiff</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>Penh</td>
<td>Enhanced pause</td>
</tr>
<tr>
<td>PG</td>
<td>Prostaglandin</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid arthritis</td>
</tr>
<tr>
<td>RANK</td>
<td>Receptor activator of NF-κB</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cell</td>
</tr>
<tr>
<td>RF</td>
<td>Relative fluorescence</td>
</tr>
<tr>
<td>RIP</td>
<td>Receptor interacting protein</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>SCF</td>
<td>Stem cell factor</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SSC</td>
<td>Side scatter</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris/Borate/EDTA</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris/Borate saline</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor β</td>
</tr>
<tr>
<td>THD</td>
<td>Tumour necrosis factor superfamily homology domain</td>
</tr>
<tr>
<td>TL1A</td>
<td>TNF-like protein 1A</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll like receptor</td>
</tr>
<tr>
<td>TMB</td>
<td>3, 3’, 5, 5’-Tetramethylbenzidine</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>TNFRSF</td>
<td>Tumour necrosis factor receptor superfamily</td>
</tr>
<tr>
<td>TRADD</td>
<td>TNF receptor associated death domain</td>
</tr>
<tr>
<td>TRAF</td>
<td>Tumour necrosis factor receptor associated factor</td>
</tr>
<tr>
<td>TRAP</td>
<td>Tartrate resistance acidic phosphatise</td>
</tr>
<tr>
<td>Treg</td>
<td>Regulatory T cells</td>
</tr>
<tr>
<td>TSLP</td>
<td>Thymic stromal lymphopoietin</td>
</tr>
<tr>
<td>TWEAKR</td>
<td>Tumour necrosis factor-like weak inducer of apoptosis receptor</td>
</tr>
<tr>
<td>VEGI</td>
<td>Vascular endothelial growth inhibitor</td>
</tr>
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</table>
Chapter 1:
General Introduction
1.1 The Tumour Necrosis Factor Superfamily (TNFSF)

1.1.1 History

The term TNF superfamily (TNFSF) was first coined following the discovery of TNF and lymphotoxin in 1975 and 1968 respectively (Granger and Kolb 1968; Carswell, Old et al. 1975). The proteins were found to be released by macrophages and lymphocytes and both were observed to cause tumour necrosis and regression, hence the title, tumour necrosis factor (TNF). TNF was first isolated in 1984, whereby its sequence and structure were determined and compared to that of lymphotoxin (Aggarwal, Eessalu et al. 1985). The two were found to bind to the same cell surface receptors as well as display 50% amino acid sequence homology (Aggarwal, Eessalu et al. 1985). To date the TNF superfamily consists of 19 members that bind to 29 receptors (Croft 2009). The TNF superfamily is now known to be involved in a number of cellular events and signalling pathways, which ultimately orchestrate the adaptive immune response and influence immune tissue development, homeostasis and organisation (Ware 2008).

1.1.2 Structure and binding of TNFSF members and their receptors

A recognised hallmark of any TNF receptor superfamily member is its cysteine rich domain (CRD), which lies within the extracellular region. Most receptors have between 1 and 4 CRDs, which typically comprise 6-8 cysteine residues, 3 pairs of which form disulphide bonds. These disulphide bonds act as a scaffold for the adaptation of an elongated receptor structure and receptor signalling (Locksley, Killeen et al. 2001; MacEwan and MacEwan 2002; Bossen, Ingold et al. 2006).
Of the 29 receptors identified, all but two are type I transmembrane proteins. Osteoprotegerin (OPG) and Decoy Receptor 3 (DcR3) lack a hydrophobic transmembrane region, thus forming soluble secreted proteins (Bossen, Ingold et al. 2006). Additionally, 8 of the 29 receptors display further homology within a cytoplasmic region known as the death domain (DD). This ~80 amino acid sequence contains 6 conserved alpha helices and its presence determines if a receptor is able to induce programmed cell death, these being known as death receptors (Locksley, Killeen et al. 2001; MacEwan and MacEwan 2002; Aggarwal 2003).

The 19 TNFSF members are type II transmembrane proteins, which can either be membrane bound, or cleaved and released into their soluble mature form (Idriss and Naismith 2000). TNFSF members, like their receptor counterparts, contain a region of homology within their C terminal, known as the TNF homology domain (THD). This conserved 150 amino acid sequence is rich in aromatic and hydrophobic residues, which assist in ligand binding to the CRD of its partnering receptor (Orlinick and Chao 1998; Bodmer, Schneider et al. 2002). There is thought to be 25-30% amino acid similarity between the different TNFSF members (Locksley, Killeen et al. 2001), although 15-35% of the THD is divergent to allow for specific binding to members of the TNFRSF (Orlinick and Chao 1998; Bodmer, Schneider et al. 2002).
Figure 1.1 Complete list of known TNFSF and TNFRSF members. TNFSF members are shown in purple and TNFRSF in yellow. The number of triangles correlates with the number of extracellular cysteine rich repeats. Death domain containing receptors are represented by a green star. Arrows represent TNFSF/TNFRSF interactions. Figure adapted from Ashkenazi (2002).
1.1.3 Signalling

The aromatic residues within the TNFSF members are predicted to be responsible for the assembly of ligand trimer complexes, which occur prior to receptor binding (Locksley, Killeen et al. 2001). The majority are non-covalently bound homotrimeric structures which can either be released from the membrane and exert their effects at a distance, or bind to their complimentary receptor via cell-cell contact (MacEwan and MacEwan 2002). After ligand binding, the cytoplasmic tails of the TNFRSF also oligomerise, to signal as trimeric complexes, ultimately forming a hexameric structure (Bodmer, Schneider et al. 2002). TNFR1 and 2 however, are an exception in that they pre-form receptor trimers before ligand binding via a pre-ligand binding assembly domain (PLAD) (Aggarwal 2003).

The binding of TNFSF members to their respective ligands can mediate one of two intracellular signalling cascades, dependent upon the adaptor proteins recruited (Ashkenazi and Dixit 1998). A proliferative response is initiated via the recruitment of the cytosolic 34kDa TNFR-associated death domain (TRADD), and receptor interacting protein (RIP) (MacEwan and MacEwan 2002). In death domain containing receptors, it is the motif itself which functions as the docking site, so encouraging homotypic binding. Following this, TNFR-associated factors (TRAFs) are recruited. These RING finger containing proteins act as adaptors which rapidly transduce the signal from the inert receptor to initiate downstream effects within the target cell (MacEwan and MacEwan 2002). There are 6 known TRAFs in humans, with different TRAFs being recruited by different receptors (Aggarwal 2003). In the case of TNFα-TNFR1, it is TRAF-2 which is recruited, leading to the association of the apoptotic adaptor cellular inhibitor of apoptosis proteins (c-IAP)1 and c-IAP2. These inhibitors of apoptosis
ensure that cell death does not occur, by blocking the action of caspase 8, thereby ensuring the NF-κB or AP-1 pathways are activated and cell proliferation is initiated, via the induction of immunomodulatory genes (Tartaglia and Goeddel 1992).

This ‘canonical’ NF-κB signalling pathway involves the ubiquitination of molecules centrally involved in the signalling complex, most notably RIP (Skaug, Jiang et al. 2009; Dynek, Goncharov et al. 2010; Verhelst, Verstrepen et al. 2011). Ubiquitination consists of a 3-enzyme post translational process whereby the 76 amino acid molecule ubiquitin, is joined to the target protein. An ATP dependent thiolester bond is formed between the C terminal of ubiquitin and the ubiquitin activating enzyme E1. Ubiquitin is then transported to the ubiquitin conjugating enzyme, E2, before being attached via its C terminal to the target protein, RIP, via an isopeptide bond with a lysine residue. This is performed by an ubiquitin protein ligase, known as E3, for which TRAF 2 and cIAP1/2 act in TNFR signalling (Varfolomeev, Goncharov et al. 2008). RIP1 is then poly-ubiquitinated with lysine ubiquitin chains, allowing further attachment of other ubiquitins formed by different lysine residues, allowing differential structural and downstream effects.

Following the poly-ubiquitination of RIP, a mitogen activated 3 kinase (MAP3K) molecule is recruited, known as TAK1 as well as the IκB kinase complex (IKK). Binding occurs via TAB2, an adaptor protein of TAK1, and IKKγ, a modulator subunit of IKK to specialised ubiquitin binding domains (UBAN), thus ensuring stabilisation of the signalling complex (Skaug, Jiang et al. 2009). IKKγ is then activated by direct transformation, TAK1 phosphorylation of IKK or close proximity of IKKγ subsections due to ubiquitination, thereby promoting trans-autophosphorylation (Skaug, Jiang et al.
Upon activation, IKK can then phosphorylate Inhibitor of kappa B (IκB) leading to its poly-ubiquitination and degradation, allowing NF-κB mediated gene transcription (Verhelst, Verstrepen et al. 2011).

As well as the canonical NF-κB pathway, particular members of TNFRSF are also able to utilise non-canonical signalling, including BAFFR (Morrison, Reiley et al. 2005), TWEAKR (Enwere, Holbrook et al. 2012), CD40 (Homig-Holzel, Hojer et al. 2008), LTβR (Dejardin, Droin et al. 2002), RANK (Novack, Yin et al. 2003), CD30 (Nishikori, Ohno et al. 2005), CD27 (Ramakrishnan, Wang et al. 2004) and TNFR2 (Rauert, Wicovsky et al. 2010). Rather than the degradation of the inhibitor of IκB, which the canonical pathway relies upon, non-canonical signalling depends on a mechanism involving NF-κB inducing kinase (NIK) (Dejardin, Droin et al. 2002). Following TNFSF-TNFRSF ligand binding, TRAFs are recruited into the TRAF binding motif, promoting NIK activation. NIK, via the kinase IKKα, phosphorylates p100, which functions as an NF-κB inhibitor. This leads to its poly-ubiquitination, allowing NF-κB to translocate into the nucleus and induce the transcription of target genes (Sun 2011).

As well as NF-κB activation, some DD containing members of the TNFRSF are also able to elicit an apoptotic response. The recruitment of TRADD leads to the association of Fas-associated death domain (FADD), leading to initiator caspase interaction and the formation of the death inducing signalling complex (DISC) (Hsu, Shu et al. 1996; Locksley, Killeen et al. 2001). The complex initiates a series of downstream events such as the cleaving of effector caspases (caspase 8 and 10), ultimately leading to programmed cell death (Bodmer, Holler et al. 2000; Wang, Chun et al. 2001).
The type of response induced is dependent upon cell type and configuration (MacEwan and MacEwan 2002), although apoptosis is commonly caused by the blocking of protein synthesis (Ashkenazi and Dixit 1998). The signalling mechanisms employed by the TNFSF ensure they have a wide range of biological functions that are dependent on context, from proliferation and differentiation to apoptosis.
1.2 Death Receptor 3 (DR3)

1.2.1 Human DR3

Discovered in 1996, Death Receptor 3, or TNFRSF25, immediately provoked interest due to its relatively high sequence homology to TNFR1, as well as its diverse effects when activated. DR3 however, was identified by more than one research laboratory, all of whom cloned the receptor at similar times, resulting in multiple names; TRAMP, LARD, Wsl-1, Apo-3 and TR3 (Chinnaiyan, Orourke et al. 1996; Kitson, Raven et al. 1996; Marsters, Sheridan et al. 1996; Bodmer, Burns et al. 1997; Screaton, Xu et al. 1997). The foremost approach to receptor discovery was screening cDNA databases using expressed sequence tags (EST) for sequence similarity to TNFR1 and/or Fas (Chinnaiyan, Orourke et al. 1996; Marsters, Sheridan et al. 1996; Bodmer, Burns et al. 1997; Screaton, Xu et al. 1997). Kitson et al however, took a different approach and expressed the death domain of TNFR1 to screen a yeast-two hybrid library (Kitson, Raven et al. 1996). Nevertheless, the end result was the same-DR3 had been discovered.

The human DR3 (hDR3) gene consists of 1665 base pairs in 10 exons. This encodes a 417 amino acid protein (Kitson, Raven et al. 1996; Screaton, Xu et al. 1997) with 4 CRDs within the extracellular region, as well as the characteristic death domain within its 193 amino acid cytoplasmic area (Bodmer, Burns et al. 1997). Moreover, 2 potential N-glycosylation sites were also found (Asn-X-Ser/Thr) (Chinnaiyan, Orourke et al. 1996) at amino acid positions 67 and 106 (Marsters, Sheridan et al. 1996) as well as a conserved peptide sequence at the N terminus (Screaton, Xu et al. 1997). This conserved sequence may act as a signal peptide, targeting proteins to the secretory pathway. The gene for DR3 was mapped to chromosome 1p36.2 using fluorescent in-
situ hybridisation (FISH) (Bodmer, Burns et al. 1997), in close proximity to other TNFRSF members including HVEM (Kwon, Tan et al. 1997), 4-IBBR (Kwon, Kozak et al. 1994), OX40-R (Birkeland, Copeland et al. 1995) and TNFR2 (Baker, Chen et al. 1991), suggesting TNFRSF clustering in the genome. DR3 displayed significant relation to TNFR1, equating to 47% amino acid homology in the death domain (Chinnaiyan, Oourke et al. 1996), compared to 29% overall (Marsters, Sheridan et al. 1996).

DR3 is thought to be alternatively spliced, with the number of predicted variants differing between individual scientific groups. Screaton et al, predicted at least 12 variants, suggesting they were generated by the splicing out of one or more complete exons. The majority of these splice variants had premature stop codons, prior to the transmembrane region, resulting in soluble proteins (Screaton, Xu et al. 1997). Two further variants were described that had retained introns due to 101 and 200 base pair insertions which matched to consensus splice sites at the 3’ and 5’ end (Warzocha, Ribeiro et al. 1998). This alternative splicing was thought to be important for DR3 up-regulation and function, much like TNFR1 which is cleaved from the membrane via the protease TNFα converting enzyme (TACE). The 13th DR3 splice variant was discovered relatively late and originally named DR3β. Insertions of 20 and 7 base pairs at positions 612 and 667, made this the longest of the splice variants at 426 amino acids compared to the 417 amino acids of the original gene. The 28 amino acid difference between the 2 variants was found to lie in the extracellular domain (Warzocha, Ribeiro et al. 1998).

TL1A is the only known ligand for DR3 and was discovered by Migone et al sometime after the finding of the Receptor itself (Migone, Zhang et al. 2002).
1.2.2 Decoy Receptor 3 (DcR3)

DcR3 is a soluble decoy receptor found in humans (Pitti, Marsters et al. 1998; Chen, Zhang et al. 2004), mapping to chromosome 20 region q13 (Ashkenazi 2002). Though structurally similar to DR3 in the extracellular region, DcR3 lacks a transmembrane domain, thus it can competitively bind DR3’s ligand TL1A without inducing cellular effects (Migone, Zhang et al. 2002). In addition to disrupting DR3/TL1A interactions, DcR3 can also bind other TNFSF members, namely Fas-L (Pitti, Marsters et al. 1998) and LIGHT (Yu, Kwon et al. 1999). Traditionally, this competitive binding has been viewed as anti-inflammatory, neutralising ligand effects, so dampening down the inflammatory response (Zhang, Salcedo et al. 2001). However, more recent studies in Crohn’s disease have suggested DcR3 can also promote inflammation by inhibiting apoptosis (Bai, Connolly et al. 2000; Macher-Goeppinger, Aulmann et al. 2008). As well as inflammatory disease, DcR3 is also over-expressed in cancerous tumours, suppressing T cell mediated immunity and preventing the apoptosis of malignant cells (Pitti, Marsters et al. 1998; Yu, Kwon et al. 1999; Zhang, Salcedo et al. 2001; Yang, Hsieh et al. 2004; Hsu, Wu et al. 2005; Han and Wu 2009). Indeed DcR3 has been cited as a prognostic marker in inflammatory disease and tumour progression, linked with severity (Roth, Isenmann et al. 2001; Chen, Yang et al. 2009).

Hsu et al developed a transgenic model of DcR3, whereby the receptor was systemically over-expressed using the 3-phosphoglycerate kinase (PGK) promoter, an enzyme necessary in every cell for glycolysis (McBurney, Staines et al. 1994; Hsu, Wu et al. 2005). When infected with *Listeria monocytogenes*, the mice displayed an attenuated Th1 response characterised by decreased IFNγ production. A Th2 based phenotype was instead observed, with the authors suggesting DcR3 assists tumour growth by
suppressing the cytotoxic Th1 response via down-regulation of the autocrine T cell growth factor IL-2 (Hsu, Wu et al. 2005). DcR3 transgenic mice demonstrated a tendency towards the Th2 response via the modulation of dendritic cell differentiation and maturation, inducing Th2 biased naive T cells (Hsu, Chang et al. 2002). This polarisation of the immune response has implicated DcR3 in lung inflammation (Cheng-Yu, Kuang-Yao et al.; Wortinger, Foley et al. 2003; Chen, Yang et al. 2009) and atopic disease (Chen, Yang et al. 2004).

1.2.3 Murine DR3
The murine homologue for hDR3 was isolated in 2001 by screening a 129Sv murine genomic library with full length hDR3 cDNA (Wang, Kitson et al. 2001). The 1619 base pair murine DR3 cDNA displayed 55% homology to human DR3, whilst at the protein level, similarity was higher at 63% (Wang, Kitson et al. 2001). Considerable homology was noted in the death domains of murine and human DR3, at an estimated 94%. In the extracellular domain this was just 52%. Like hDR3, the murine gene also consisted of 10 exons and retained 25 of the 28 cysteines present in hDR3 (Wang, Kitson et al. 2001). The chromosomal location of the murine DR3 gene was syntenic with human DR3 at chromosome 4 region E1 (Wang, Kitson et al. 2001). Murine DR3 also contained 2 putative N-linked glycosylation sites. Despite the significant homology and structural similarities, the third CRD of murine DR3 was found to be substantially different from not only the human DR3 protein, but also TNFR1. Two cysteine residues were missing, resulting in alternative positioning of the second disulphide bond compared to hDR3. Additionally, 2 amino acid substitutions were discovered, with cysteine being replaced with a threonine and phenylalanine with a cysteine, with this
new cysteine being the proposed site of the shifted disulphide bond (Wang, Kitson et al. 2001). Nevertheless, the ability to bind ligand was conserved, as the required regions of the 2nd CRD and first half of the 3rd CRD were not altered (Banner, D'Arcy et al. 1993).

Wang et al reported 3 murine DR3 splice variants; full length membrane bound variant 1, soluble variant 2 and truncated membrane bound variant 3 (Wang, Kitson et al. 2001). Investigations currently suggest DR3 mRNA may be differentially regulated following immune activation, indicating individual functions for each of the variants (Bamias, Mishina et al. 2006; Twohig, Marsden et al. 2012). However, the exact function of each isoform as well the factors determining their regulation remain unknown.

In contrast to humans, no decoy receptor for mouse TL1A has yet been identified, although it has been suggested that murine soluble DR3, or variant 2, may perform a similar role (Bamias, Mishina et al. 2006; Pappu, Borodovsky et al. 2008). Studies have been undertaken using DcR3 analogues, as before modification of the human decoy receptor via the replacement of an arginine residue for a glutamine, DcR3 was degraded following subcutaneous injection in mice and primates, resulting in the loss of Fas-L binding (Wroblewski, Witcher et al. 2003). However, the more stable analogue, known as FLINT, bound both Fas-L and LIGHT and prevented a Fas-L induced murine model of pulmonary inflammation (Wortinger, Foley et al. 2003). Soluble murine DR3, unlike DcR3, does not hold the capacity to bind Fas-L or LIGHT.
1.2.4 *DR3*<sup>ko</sup> mouse

*DR3*<sup>ko</sup> mice have played a major role in increasing our understanding of the gene and the effects it exerts *in vivo*. *DR3*<sup>ko</sup> mice were generated by replacing the whole coding region of the mouse DR3 gene, so eliminating the possibility of any splice variants. The coding region was replaced with a cassette containing an internal ribosome entry site (IRES), lacZ and neomycin resistance gene (Wang, Thern et al. 2001). The DR3 lacking construct was linearised and transfected into embryonic stem cells whereby clones were selected using the eukaryotic antibiotic G418, to which neomycin gives resistance. Selected recombinant clones were then microinjected into C57BL/6 blastocysts followed by implantation into pseudopregnant C57BL/6 mice (Wang, Thern et al. 2001). The resulting male chimaeric mice (*DR3*<sup>het</sup>) were bred to generate germline homozygote DR3 null mice (*DR3*<sup>ko</sup>). To ensure no gene product remained, PCR was carried out, to which no transcripts were detected (Wang, Thern et al. 2001). *DR3*<sup>ko</sup> mice displayed significantly enlarged thymuses compared to *DR3*<sup>wt</sup> and *DR3*<sup>het</sup> mice, particularly at 29-32 weeks of age whereby the increase was reported to be 30%, compared to 10% at 2-5 weeks. However, this was not attributed to an increase in thymocyte proliferation or turnover, as little difference was seen in BrdU incorporation between *DR3*<sup>wt</sup> and *DR3*<sup>ko</sup> mice. *DR3*<sup>ko</sup> mice instead suffered an impairment in thymus negative selection, though no autoimmune disease symptoms were detected (Wang, Thern et al. 2001). Except in the thymus, *DR3*<sup>ko</sup> development was akin to *DR3*<sup>wt</sup> mice, with no obvious differences observed in the spleen, brain, heart, kidney, lymph node or Peyers patches.

However, aged *DR3*<sup>ko</sup> mice display progressive loss of motor control compared to *DR3*<sup>wt</sup> and *DR3*<sup>het</sup> littermates (Twohig, Roberts et al. 2010). *DR3*<sup>ko</sup> behaviour was
characterised by abnormal gait, rapid head movement, disorientation and dyskinesia. Higher dopamine levels were also reported in the brains of DR3\textsuperscript{ko} mice, whilst serotonin levels decreased by 73\% (Twohig, Roberts et al. 2010).

1.2.5 DR3 signalling

Like TNFR1, DR3 is able to trigger canonical NF-\kappa B activation and apoptosis (Ashkenazi and Dixit 1998). NF-\kappa B, a key regulator of the cell survival decision, is activated by DR3 in much the same way as TNFR1, likely due to the high homology between these death domain containing molecules. DR3 was found to dose dependently induce reporter gene activity using a NF-\kappa B luciferase reporter gene assay (Bodmer, Burns et al. 1997). Dominant negative forms of TRAF-2 and RIP showed that upon ligand binding, DR3 was bound to the platform adaptor TRADD, leading to TRAF-2 and RIP association (Chinnaiyan, Orourke et al. 1996), which has serine/theorine kinase activity (Ashkenazi and Dixit 1998). The death domain was also shown to be essential for signalling, as truncation resulted in limited interaction between DR3 and TRADD (Chinnaiyan, Orourke et al. 1996). TRADD too, was found to be fundamental to the whole signalling process as its absence inhibited recruitment of adaptor proteins, independent of cell fate (Chinnaiyan, Orourke et al. 1996). CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells from TRADD\textsuperscript{ko} mice also failed to proliferate in response to TL1A, due to reduced NF-\kappa B signalling (Pobezinskaya, Choksi et al. 2011).

Similar to TNFR1, DR3 was also able to activate JNK, p38, and ERK1, 2 and MAPK, shifting cell fate towards survival (Wen, Zhuang et al. 2003). Supporting this, Wen et al found c-IAP2 to have a direct role in DR3 mediated signalling events within the CD34\textsuperscript{+}
progenitor cell line, TF-1, as inhibition sensitised cells to DR3 induced apoptosis. To prevent signalling in the absence of ligand, DR3 interacts with the silencer of death domains (SODD). Upon ligand binding, SODD is displaced by TRADD, commencing the proliferative response (Al-Lamki, Wang et al. 2008).

DR3, whilst favouring cell survival (Wen, Zhuang et al. 2003), also has the capacity to initiate apoptosis, particularly when protein synthesis and proliferation signals are inhibited (Migone, Zhang et al. 2002). DR3 over-expression on 293 T cells resulted in an 18% increase in cell death (Screaton, Xu et al. 1997), which was associated with the cytoplasmic death domain and the linker protein, FADD (Chinnaiyan, Oourke et al. 1996; Kitson, Raven et al. 1996).
**Figure 1.1.** DR3 and TNFR1 signalling cascades. Following TL1A or TNFα binding, the receptors recruit adaptor proteins to form a death inducing signalling complex (DISC). Dependent on the adaptor proteins, the receptors initiate an apoptotic response, via the caspase pathway and TRADD and FADD, or a proliferative response, via the NF-κB pathway and TRAF2. Figure adapted from Ashkenazi and Dixit (1998).
1.2.6 Human DR3 expression

Unlike TNFR1, which is ubiquitously expressed on all cell types, DR3 was found to be restricted to certain tissues and leukocyte subtypes. Original studies used northern blot analysis to identify 3.4-4kb hDR3 RNA transcripts in the brain and prostate, as well as those that are lymphocyte rich, including the thymus, spleen, small intestine and peripheral blood T cells (Chinnaiyan, Orourke et al. 1996; Kitson, Raven et al. 1996; Marsters, Sheridan et al. 1996; Bodmer, Burns et al. 1997; Screaton, Xu et al. 1997). Later studies also discovered DR3 to be expressed in the cerebellum (Newman, Bond et al. 2000), foam cells in atherosclerotic plaques from carotid endoarterectomy samples (Kang, Kim et al. 2005; Kim, Kang et al. 2008), vascular endothelial cells and tubular epithelial cells of rejecting or injured renal allografts (Al-Lamki, Wang et al. 2008), the epidermis and dermis of skin (Bamias, Evangelou et al. 2011), as well as on peripheral blood monocytes (Kang, Kim et al. 2005), activated B cells (Cavallini, Lovato et al. 2013), NK cells (Papadakis, Prehn et al. 2004) and bone marrow differentiated osteoblasts (Borysenko, Garcia-Palacios et al. 2006).

1.2.7 Murine DR3 expression

Similar to initial human studies, murine DR3 was detected by northern blot. A 7kb transcript was found in the murine brain, heart and kidney, whilst 2 smaller transcripts measuring 1.8kb and 2.8kb were identified in the spleen and thymus (Wang, Kitson et al. 2001). DR3 expression has also been confirmed on differentiated T cell subsets, including Th17 cells (Pappu, Borodovsky et al. 2008; Jones, Stumhofer et al. 2011), T_{regS} (Pappu, Borodovsky et al. 2008; Schreiber, Wolf et al. 2010), CD8^+ T cells (Fang,
Adkins et al. 2008; Twohig, Marsden et al. 2012) and NKT cells (Fang, Adkins et al. 2008; Twohig, Marsden et al. 2012).
1.3 TNF-like protein 1A (TL1A)

1.3.1 Discovery

The currently accepted ligand for DR3 is the type II transmembrane protein, TNF-like protein 1A (TL1A). Discovered in 2002 using ESTs, TL1A was found to be a longer variant of vascular endothelium growth inhibitor (VEGI) (Migone, Zhang et al. 2002), which had previously been shown to be produced by the endothelium, preventing tumour development, angiogenesis and endothelial expansion (Haridas, Shrivastava et al. 1999; Zhai, Ni et al. 1999; Tian, Liang et al. 2009). Unlike VEGI, TL1A contains a transmembrane domain, with the membrane bound form being cleaved to generate a soluble protein (Chew, Pan et al. 2002; Migone, Zhang et al. 2002). Both TL1A and VEGI were mapped to the TNFSF15 gene on the long arm of chromosome 9 (Chew, Pan et al. 2002), although TL1A is produced in much greater quantities than VEGI from endothelial cells (Migone, Zhang et al. 2002). Co-immunoprecipitation studies using FLAG tagged TL1A, established TL1A as the sole ligand for DR3, ensuring no interaction with other members of the TNFRSF, except DcR3 (Migone, Zhang et al. 2002; Bossen, Ingold et al. 2006).

1.3.2 Structure

The TL1A protein, standing at 251 amino acids in length was thought to be encoded by 4 exons (Migone, Zhang et al. 2002), with 2 potential N-linked glycosylation sites (Kim, Zhang et al. 2005). TL1A displayed homology to TNFα and Fas-L, at 24.6% and 22.9% respectively. It is predicted to circulate as a stable homotrimeric molecule, with each TL1A monomer measuring 21kDa (Kim, Zhang et al. 2005; Zhan, Yan et al.
2009), and holds high sequence similarity between humans and mice (63.7% cDNA) (Migone, Zhang et al. 2002).

1.3.3 Expression

TL1A expression was determined via RT-PCR, and was initially thought to be restricted to endothelial cells (Migone, Zhang et al. 2002), with significant levels also detected in the kidney, prostate, placenta, stomach, intestine, lung, spleen, thymus, skin, brain, renal allografts and artheromatous lesions (Migone, Zhang et al. 2002; Al-Lamki, Wang et al. 2008; Twohig, Roberts et al. 2010; Bamias, Evangelou et al. 2011; Bamias, Stamatelopoulos et al. 2013). Later studies revealed TL1A production from multiple cell types beyond endothelial cells, including monocytes (Bamias, Martin et al. 2003; Bamias, Mishina et al. 2006; Cassatella, da Silva et al. 2007; Prehn, Thomas et al. 2007), dendritic cells (Prehn, Thomas et al. 2007), T cells (Bamias, Martin et al. 2003; Meylan, Davidson et al. 2008), chondrocytes and fibroblasts (Zhang, Wang et al. 2009).

1.3.4 Function

TL1A exhibits several different functions, but was originally identified as a T cell co-stimulator, promoting IL-2 dependent proliferation of CD4+ T cells and the secretion of pro-inflammatory cytokines, including IFNγ and GM-CSF (Migone, Zhang et al. 2002) via NF-κB associated pathways (Zhan, Yan et al. 2009). However, IFNγ release from CD4+ T cells and TL1A release from monocytes could be ablated by R848, a TLR8 ligand (Saruta, Michelsen et al. 2009). The above cytokine profile suggested TL1A as a supporter of the Th1 phenotype. Together with IL-12 and IL-18, TL1A augments IFNγ
production by activated CD4<sup>+</sup> T cells and NK cells, increasing levels 9 fold compared to IL-12 and 18 alone in the former (Papadakis, Prehn et al. 2004; Prehn, Mehdizadeh et al. 2004). TL1A was also seen to increase the cytotoxicity of IL-12/IL-18 activated NK cells (Heidemann, Chavez et al. 2010). As well as Th1 cells, TL1A has been cited as a promoter of the Th2 response, accumulating effector T cells and promoting IL-13 release from NKT cells in a model of experimental acute allergic lung inflammation (Fang, Adkins et al. 2008). Effects were ablated upon the administration of an anti-TL1A antibody (Fang, Adkins et al. 2008). Further in vivo studies have found DR3 to expand effector T cell populations involved in bacterial (Buchan, Taraban et al. 2012) and viral host defence (Twohig, Marsden et al. 2012).

Studies have also implicated TL1A in the Th17 response, though reports describing conflicting underlying function exist. Pappu et al, using TL1A<sup>ko</sup> mice, reported TL1A as a supporter of Th17 differentiation and proliferation, as well as Th17 effector function (Pappu, Borodovsky et al. 2008). Meanwhile Jones et al, described TL1A as an inhibitor of Th17 differentiation from naive T cells, instead implicating TL1A as important in the maintenance of the Th17 phenotype (Jones, Stumhofer et al. 2011). TL1A has also been shown to co-stimulate T<sub>reg</sub> expansion, thereby rendering mice resistant to acute allergic lung inflammation (Schreiber, Wolf et al. 2010) and permitting cardiac allograft survival in a murine model of fully major histocompatibility complex mismatched ectopic heart transplant (Wolf, Schreiber et al. 2012). Despite expansion, the suppressive effects of T<sub>regS</sub> were overcome by the TL1A dependent CD4<sup>+</sup> T cell effects in a model of intestinal inflammation (Taraban, Slebioda et al. 2011), suggesting functional outcome may depend on the inflammatory context. TL1A has also been reported to expand CD8<sup>+</sup> T cells in vivo in an IL-2 independent fashion.
Naive OT-I T cells, which are MHC-I restricted OVA specific CD8$^+$ T cells (Clarke, Barnden et al. 2000), were transferred into mice and challenged with OVA. Transfer T cell numbers increased 12 fold in the peripheral blood following challenge. However, administration of OVA alongside soluble TL1A increased OT-I T cells by 81 fold in the peripheral blood, with a similar effect also noted in the spleens. This increase was abolished by concurrent injection of a neutralising TL1A antibody, demonstrating that DR3 is capable of enhancing antigen specific CD8$^+$ T cell expansion. Carboxyfluorescein succinimidyl ester (CFSE) studies, used to monitor lymphocyte proliferation, went on to show DR3 promoted OT-I T cell proliferation, whilst the generation and subsequent challenge of endogenous OVA specific memory CD8$^+$ T cells with OVA and TL1A, confirmed DR3 function as a co-stimulatory receptor for memory CD8$^+$ T cells. TL1A was also found to trigger splenic CD8$^+$ T cell differentiation into cytotoxic T cells, up-regulating the expression of granzyme B and perforin beyond that induced by OVA alone (Slebioda, Rowley et al. 2011). Consistent with this, DR3 was required for virus-specific CD8$^+$ T cell expansion in a CD4$^+$ and NK cell independent manner, which functioned to control viral replication (Twohig, Marsden et al. 2012). CD8$^+$ T cell numbers expanded early in response to both MCMV and Vaccinia virus infection, unlike the work done by Slebioda et al, DR3 had no effect on the secondary memory response (Twohig, Marsden et al. 2012). Thus the DR3/TL1A axis plays a major part in the modulation of the immune response.
Figure 1.2. The role of TL1A in propagating the inflammatory response. TL1A expression can be induced in myeloid cells, such as DCs or macrophages, T cells and structural cells such as endothelial cells. TL1A binds DR3, causing cytokine release and proliferation. The response is individual to each T cell subset.
1.4 DR3/TL1A in disease

Though the cellular pathways and expression profile of DR3 are fairly well understood, its role in disease initiation and progression are only just coming to light, largely thanks to experimentation in DR3<sup>ko</sup> mice, TL1A<sup>ko</sup> mice and DR3 over-expressing transgenic mice. DR3 has been implicated in several inflammatory diseases, some of which will be discussed below.

1.4.1 Rheumatoid arthritis

DR3 involvement in rheumatoid arthritis (RA) has been widely reported. A duplication of the DR3 gene was identified almost 200kb upstream of its original position using FISH in 78% of RA patients, compared to a 39% occurrence in healthy controls (Osawa, Takami et al. 2004). TL1A was also found to be elevated in the serum of patients suffering from RA (Bamias, Siakavellas et al. 2008) and could be induced from RA patient mononuclear phagocytes and fibroblast-like synoviocytes in response to insoluble immune complexes as well as TNFα and IL-1β (Cassatella, da Silva et al. 2007). This was associated with increased bone and cartilage destruction (Cassatella, da Silva et al. 2007; Bamias, Siakavellas et al. 2008; Zhang, Wang et al. 2009). Moreover, TL1A levels were suggested to correlate with disease activity and stage, as the cytokine was found more frequently in those with active disease as opposed to those in remission (Bamias, Siakavellas et al. 2008). In comparative modelling studies, DR3 mutations affecting ligand binding were linked to RA. DR3/TL1A interaction were predicted to destabilise, thereby conferring the aberrant response of bone cells to TL1A or T cell function, contributing to pathology and bone destruction in RA (Borysenko, Furey et al. 2005).
Different murine models of RA have been used to study DR3 function; antigen-induced arthritis (AIA), representing localised disease, and collagen-induced arthritis (CIA), simulating systemic disease. DR3\textsuperscript{wt} mice in both, displayed increased joint pathology compared to DR3\textsuperscript{ko} littermates (Bull, Williams et al. 2008; Zhang, Wang et al. 2009). In AIA, DR3\textsuperscript{ko} mice presented with reduced bone erosion and inflammatory cell infiltrate (Bull, Williams et al. 2008). In CIA, TL1A treatment of WT mice enhanced splenic germinal centre size and formation, as well as boosting serum anti-collagen titres (Zhang, Wang et al. 2009). Experimental manipulation of the DR3/TL1A pathway emphasised its therapeutic potential, as TL1A neutralisation ameliorated the inflammatory phenotype in both AIA and CIA (Bull, Williams et al. 2008; Zhang, Wang et al. 2009), whilst TL1A administration dose dependently exacerbated bone pathology in AIA (Bull, Williams et al. 2008). DR3 also promoted osteoclastogenesis in AIA, particularly in areas of bone erosion. The bone forming cells, osteoblasts, were also reported to express a soluble form of DR3 by cDNA based gene screening (Bu, Borysenko et al. 2003). TL1A has been proposed to enhance RA pathogenesis by acting as a Th1 inflammatory cytokine, increasing IL-17 and TNF\textgreek{a} production (Zhang, Wang et al. 2009).

DR3 may however, function differently depending on cell type and stage of disease. Thus Takami \textit{et al} reported CpG hyper-methylation within the DR3 promoter region in the synovial cells of RA patients, inhibiting DR3 expression, suggesting this as a mechanism for providing resistance to apoptosis and therefore maintaining disease (Takami, Osawa et al. 2004).
1.4.2 Atherosclerosis

TL1A is highly expressed in regions with abundant macrophages, from which foam cells derive, in carotid atherosclerotic plaques in humans (Kang, Kim et al. 2005; Kim, Kang et al. 2008). Furthermore, serum levels of TL1A correlated with the progression of carotid atheromotic plaque height, as well as disease progression as patients with low TL1A levels developed fewer new carotid plaques over the observation period (Bamias, Stamatelopoulos et al. 2013). Using the human macrophage like cell line THP-1, an anti-DR3 antibody was found to induce the matrix metalloproteinase (MMP)-1, MMP-9 and MMP-13, which are thought to contribute to plaque instability (Kim, Lee et al. 2001). Furthermore, addition of IFNγ concomitant with the anti-DR3 antibody led to the release of IL-8, CCL2 and TNFα. THP-1 treatment with TL1A induced MMP-9 in a dose dependent manner along with IL-8 (Kang, Kim et al. 2005). As well as MMPs, human monocyte derived macrophages treated with TL1A (in conjunction with IL-17), were shown to induce ADAMTS 1, 4 and 5, proteases which act on extracellular membrane substrates (Salter, Ashlin et al. 2010; Ashlin, Kwan et al. 2013). Collectively, these pro-atherogenic cytokines may help to destabilise the atherosclerotic plaque, leading to their rupture and consequently disease.

The DR3/TL1A axis has also been implicated in the differentiation of macrophages to foam cells, reflecting other members of the TNFSF (Mei, Chen et al. 2007; Persson, Nilsson et al. 2008). TL1A was shown to promote cholesterol uptake and incorporation, via the up-regulation of scavenger receptors such as SR-A, CD36 and SR-PSOX, as well as reducing cholesterol efflux and removal by decreasing ABC transporter expression, such as ABCA-1, ABCG-1 and apo-E (McLaren, Calder et al. 2010). These
data suggest DR3 has a complex role in the balance of atherogenesis and atherosclerosis.

1.4.3 Cancer

The role of DR3 in cancer is not well understood. However, the ability to induce both proliferative and apoptotic responses suggests that DR3 could be an obvious and potentially important target for manipulation by tumour cells. In neuroblastoma cell lines, the DR3 gene locus was tandemly duplicated on chromosome 1p36.2, and upon MYC-N over-expression, deleted or translocated to another chromosome. As a result, DR3 protein was substantially reduced or eliminated in approximately 40% of neuroblastoma cell lines (Grenet, Valentine et al. 1998). In contrast, DR3 was present in all lymphoma tumour samples tested by Warzocha et al (Warzocha, Ribeiro et al. 1998). TL1A has also been reported to enhance NK cell cytotoxicity against NK resistant tumour cell lines (Heidemann, Chavez et al. 2010; Park, Song et al. 2012) as well as colorectal adenocarcinoma epithelial derived cell lines by synergising with IL-12 and IL-18 (Heidemann, Chavez et al. 2010). Furthermore, in an in vivo tumour model, mice were injected with plasmacytoma cells expressing TL1A. Tumours were rejected in a CD8\(^+\) T cell dependent manner, as CD8\(^+\) T cell depletion rendered mice unable to eliminate malignant cells (Slebioda, Rowley et al. 2011). DR3 has also been reported to assist in cancer cell survival and dissemination. The chemoresistant human pancreatic cancer cell line AsPC-1, was found to augment invasion via constitutive DR3 mediated NF-κB activation (Ringel, Ibrahim et al. 1999). This was inhibited using the natural flavanoid, fistein, which instead triggered an apoptotic response (Murtaza, Adhami et al. 2009). A cohort of breast cancer patients revealed decreased DR3
expression compared to control patients which was coupled to a poor prognosis and a significantly shorter long term survival rate. However, targeting DR3 in breast cancer cell lines \textit{in vitro}, impaired migration rates, suggesting a complex role for the Receptor (Ge, Sanders et al. 2013).

DR3 has also been suggested to interact with the endothelial receptor E-selectin. Using the colon carcinoma cell line HT29, DR3 was reported to activate p38 and extracellular signal related kinase (ERK) mitogen activated protein kinase (MAPK) via E-selectin binding, conferring survival and metastatic advantages. DR3 knockdown using siRNA, decreased cancer cell adhesion to E-selectin, impairing HT29 transendothelial migration (Gout, Morin et al. 2006). Porquet \textit{et al} went on to say that DR3 activated the NF-κB pathway in colon carcinoma cell lines, as well as undergoing alternative splicing to generate a variant lacking the transmembrane and death domains, rendering the Receptor unable to induce apoptosis (Porquet, Poirier et al. 2011). However, using G-CSF mobilised human blood peripheral leukocytes to inflamed vascular endothelium, DR3 was not identified as an E-selectin ligand (Dagia, Gadhoum et al. 2006), despite using the same isolation techniques. Pull down assays were performed on membrane extracts of HT29 and LoVo cells (Gout, Morin et al. 2006) or human umbilical cord endothelial cells (HUVECs) (Dagia, Gadhoum et al. 2006) using E-selectin as a ligand. Gout \textit{et al} found 3 bands that migrated at 180kDa, 66kDa and <45kDa, which were presumed to be DR3 (Gout, Morin et al. 2006), whilst Dagia \textit{et al} observed glycoproteins migrating at 220kDa, 130kDa, 100kDa and 65kDa (Dagia, Gadhoum et al. 2006), none of which were identified as DR3, but included the known E-selectin ligands PSGL-1 and HCELL. Furthermore, Winkler \textit{et al} also used pull down experiments on haematopoietic stem/progenitor cell extracts, whereby no DR3 was
detected (Winkler, Barbier et al. 2012). This data suggests that the role of DR3 in cancer requires further investigation.

1.4.4 Inflammatory bowel disease (IBD)

TL1A levels are up-regulated in IBD, particularly in areas involved in Crohn’s disease, correlating with disease severity and progression (Bamias, Martin et al. 2003; Bamias, Kaltsa et al. 2012). TL1A expression was localised to intestinal lamina propria macrophages, CD4+ and CD8+ T cells from Crohn’s disease patients, as opposed to plasma cells from ulcerative colitis samples (Bamias, Martin et al. 2003; Prehn, Mehdizadeh et al. 2004) and is thought to be up-regulated through the transcriptional activation of the NF-κB pathway (Endo, Kinouchi et al. 2010). DR3 expression was also noted on intestinal lamina propria lymphocytes from IBD patients, co-stimulating IFN-γ secretion (Bamias, Martin et al. 2003). Small intestine isolated CD4+ T cells expressing CCR9, a gut homing receptor, displayed a 37 to 105 fold increase of IFNγ production when stimulated with a cytokine cocktail of TL1A, IL-12 and IL-18 (Papadakis, Zhu et al. 2005). Similarly, elevated TL1A and DR3 levels were observed in murine models of IBD. TL1A expression was associated with monocytes and CD11c+ dendritic cells (Prehn, Thomas et al. 2007) in the inflamed mucosa, whilst DR3 was preferentially expressed on activated CD4+ memory T cells (Bamias, Mishina et al. 2006). Akin to human data, TL1A was able to synergise with the IL-12/IL-18 pathway to induce IFNγ (Bamias, Mishina et al. 2006). DR3 has therefore been cited as a promoter of the Th1 effector phase in chronic intestinal inflammation (Papadakis, Zhu et al. 2005).
In addition to Th1 cells, the Th17 response is also believed to aid Crohn’s disease pathogenesis (Fuss, Neurath et al. 1996). IL-23 and TL1A were induced in lamina propria macrophages from Crohn’s disease patients in response to commensal bacteria stimulation, which in turn could synergistically enhance the production of IFNγ and IL-17 by lamina propria T cells (Kamada, Hisamatsu et al. 2010). Human CD4+ CD161+ T cells, typically expressed in Th17 mediated mucosal inflammation, were also found to release TNFα in response to TL1A stimulation, as well as IFNγ when cross-linked with DR3 and TL1A, suggesting DR3 works upstream of TNFα (Cohavy, Shih et al. 2011; Jin, Chin et al. 2012). CD4+CD161+TL1A+ T cells were also increased in gut biopsies from IBD patients, implying TL1A function during the Th17 response (Jin, Chin et al. 2012). TL1A, unlike IL-23, could also promote Th17 differentiation from naive lamina propria T cells (Kamada, Hisamatsu et al. 2010), a previously reported DR3 function (Pappu, Borodovsky et al. 2008), although DR3 involvement in Th17 differentiation has been questioned in other studies (Jones, Stumhofer et al. 2011). In a dextran sodium sulphate (DSS) model of chronic colitis, TL1A was observed to elicit dual responses; Th1, via the up-regulation of IFNγ, and Th17 via the release of IL-17, which themselves were induced by IL-12 and IL-23, respectively (Takedatsu, Michelsen et al. 2008). An anti-TL1A neutralising antibody prevented chronic colitis and attenuated disease when already established by down-regulating both Th1 and Th17 function. Similar results were found using G protein αi2-/- T cell transfer models, whereby G protein αi2-/- T cells were transferred into naive C57BL/6 mice, leading to spontaneous colitis (Takedatsu, Michelsen et al. 2008). T cells which lack the G protein subunit G αi2, located on chromosome 9, are able to induce colitis by skewing Th1 mediated immunity via increased release of cytokines such as IL-2, IFNγ and TNFα (Hörnquist, Lu et al. 1997).
The pathological effects of this have recently been discovered using constitutive TL1A expressing mice, which displayed spontaneous goblet cell hyperplasia, elevated IL-13 levels, paneth cell hyperplasia in the small intestine, increased collagen levels and increased IL-13 and IL-17 producing CCR9+ T cells (Meylan, Richard et al. 2011; Taraban, Slebioda et al. 2011; Zheng, Zhang et al. 2013). When constitutively expressed by T cells and DCs, TL1A also caused an increase in FoxP3+ T_{regS}, (Meylan, Richard et al. 2011) compared to expression by DCs, whereby mice displayed an increased T_{reg} turnover. Pro-inflammatory signals over rode suppressive effects, as TL1A attenuated the ability of T_{regS} to suppress conventional T cells (Taraban, Slebioda et al. 2011). TL1A over-expressing transgenic mice with DSS induced colitis displayed worsened inflammation, fibrostenosis, increased T cell activation markers and IL-17 expression. However, Rag1ko mice adoptively transferred with TL1A transgenic T cells displayed increased IFNγ but reduced IL-17 and Th17 cells (Shih, Barrett et al. 2011; Barrett, Zhang et al. 2012).

Crohn’s disease patients exhibiting high levels of TL1A, also suffered intestinal fibrostenosis and worsened inflammation in the small intestine, suggesting TL1A as a prognostic marker (Shih, Barrett et al. 2011). Genetic polymorphisms of TL1A have been shown to correlate with the development of Crohn’s disease as well as risk. The TL1A gene has been associated with increased risk in Jewish patients (Michelsen, Thomas et al. 2009), whilst genome wide association studies have revealed TL1A to be a susceptibility gene in Japanese and European populations (Yamazaki, McGovern et al. 2005; Barrett, Hansoul et al. 2008; Thiebaut, Kotti et al. 2009). Therefore TL1A has been nominated as a candidate therapeutic target for IBD development.
1.4.5 Neurological disease

TL1A and DR3 are constitutively expressed in the brains of normal humans, rat and mice (Wang, Kitson et al. 2001; Twohig, Roberts et al. 2010), implicating the Receptor in the maintenance of normal neuronal function. DR3 was also shown to be elevated in the brains of patients suffering with Alzheimer’s disease, suggesting it was related to neuronal disease cell loss (Newman, Bond et al. 2000). Using an anti-DR3 peptide polyclonal antibody, Newman et al studied the localisation of DR3, finding that expression was neuron specific and within the same population of cells affected by Alzheimer’s (Newman, Bond et al. 2000). Increased DR3 expression was also detailed by Harrison et al in the human cerebellum, as well as in many regions of the rat brain (Harrison, Roberts et al. 2000). An in vivo model of ischaemia, which occurs as a consequence of stroke, demonstrated DR3 expression to be biphasic, peaking early and decreasing by 6 hours. DR3 was therefore proposed to be involved in neuronal elimination (Harrison, Roberts et al. 2000). DR3 function in neuropathology was further supported by Twohig et al, who suggested DR3 had a non-redundant role in maintaining normal motor control function during ageing in mice and that the basal levels found in the normal brain were essential for preventing the development of behavioural defects (Twohig, Roberts et al. 2010). DR3 also preserved the balanced expression of striatal neurotransmitters, as its absence saw an increase in dopamine levels and decreased serotonin production, indicating DR3 as an important mediator in the maintenance of the normal brain phenotype.
1.4.6 Anti microbial immunity

The DR3/TL1A pathway has been linked to both anti-bacterial (Shih, Kwan et al. 2009; Buchan, Taraban et al. 2012) and anti-viral immunity (J Reddy, Schreiber et al. 2012; Twohig, Marsden et al. 2012). Shih et al reported TL1A expression in human antigen presenting cells (APC), including monocytes and monocyte-derived DCs, in response to multiple bacteria, including gram negative organisms (E.coli, Salmonella typhimurium), gram positive bacteria (Listeria monocytogenes, Staphylococcus epidermis), partial anaerobes (Campylobacter jejuni) and obligate anaerobes (Bacteroides thetaiotaomicron, Bifidobacterium breve, Clostridium A4). TL1A expression peaked at 4 hours in monocytes, compared to 8 hours in DCs and was dependent on p38 MAPK and NF-κB activation. When induced, TL1A potentiated CD4+ T cell effector function by enhancing IFNγ production (Shih, Kwan et al. 2009). In an in vivo model of bacterial stimulation, mice were infected with Salmonella enterica Typhimurium, where TL1A was found to be up-regulated on macrophages after 4 hours, peaking again at day 14 concurrent with CD4+ T cell proliferation. In DR3ko mice, reduced IFNγ producing CD4+ T cells were seen, as cells retained a more immature phenotype, resulting in defective bacterial clearance (Buchan, Taraban et al. 2012).

DR3 has also been shown to regulate virus specific T cell function in vivo following murine cytomegalovirus (MCMV) or Vaccinia virus infection. DR3ko mice presented with increased viral loads associated with a lack of CD4+ and CD8+ T cell expansion. This, like Salmonella enterica infection, resulted in reduced numbers of activated IFNγ producing T cells and faulty viral clearance, which could be lethal (Twohig, Marsden et al. 2012). In a model of herpes simplex virus 1 (HSV-1), an anti-DR3 antibody was found to expand DR3 expressing cells, which were observed to predominantly consist
of T_{regS}, thus causing reduced cellular infiltrates into the inflammatory lesion, including CD4^{+} Th1 cells (J Reddy, Schreiber et al. 2012). The antibody was proved effective in disease prevention, when administered at the time of or before infection, as well as therapeutically, when administered with galectin-9 to limit effector T cell function and thereby orchestration of the immune response (J Reddy, Schreiber et al. 2012).

**1.4.7 Allergic lung inflammation**

Until recently, literature on DR3 had centred on its role in Th1/Th17 function and consequently, diseases that induce such a response. However allergic lung inflammation, though classically considered a Th2 mediated disease, was also found to be DR3 dependent (Fang, Adkins et al. 2008; Meylan, Davidson et al. 2008). Systemic priming with ovalbumin (OVA) and aluminium hydroxide (Alum) or aluminium potassium sulphate, followed by local challenge with OVA in DR3^{ko} and dominant negative (DN) DR3 mice, rendered mice resistant to the development of acute allergic lung inflammation compared to WT littermates. Mice lacking DR3 function displayed fewer lung and bronchoalveolar lavage cell infiltrates, including CD4^{+} T cells, invariant NKT cells and eosinophils. As well as infiltration, mice also exhibited reduced mucin production and peribronchial inflammation, characterised by fewer T cells and macrophages localised in the interstitial and peribronchial airways. Expression of mRNA for IL-5 and IL-13, prominent Th2 cytokines, also decreased in comparison to WT mice, although IFNγ levels were unchanged (Fang, Adkins et al. 2008; Meylan, Richard et al. 2011). Similar results were achieved using a TL1A blocking antibody, which was capable of attenuating lung inflammation in mice already primed and exposed to antigen (Fang, Adkins et al. 2008). Using DR3^{ko} mice, splenocyte
proliferation, cytokine production and antibody production were deemed to be normal following lung inflammation (Meylan, Davidson et al. 2008), whilst OVA IgE production was reduced in DN DR3 mice (Fang, Adkins et al. 2008). Meylan et al., proposed that DR3 was required for the recruitment of Th2 effector cells at the site of inflammation rather than systemically, suggesting that the gene’s absence resulted in defective Th2 cell accumulation and function (Meylan, Davidson et al. 2008).

To determine whether the requirement for DR3 was T cell intrinsic, transfer experiments were performed using OT-II cells, defined as MHC-II restricted OVA specific CD4+ T cells (Barnden, Allison et al. 1998). DR3ko OT-II OVA specific T cells and OT-II controls were activated in vitro under Th2 cell differentiation conditions and subsequently transferred into naive host mice. Following respiratory challenge with OVA, DR3ko OT-II T cell recipient mice displayed defective OVA-specific T cell accumulation and proliferation in the lung, as well as the draining mediastinal lymph nodes. This corresponded with reduced inflammatory infiltrates in the BAL, implying DR3 expression upon T cells was necessary to initiate an appropriate and effective local response to invading antigen (Meylan, Davidson et al. 2008).

Fang et al meanwhile, suggested DR3 was essential for IL-13 release from NKT cells, amplifying the Th2 response and driving allergic lung inflammation (Fang, Adkins et al. 2008). OVA primed NKT cell deficient mice, which are resistant to allergic lung inflammation (Akbari, Stock et al. 2003), were adoptively transferred with DN DR3 NKT cells and aerosol challenged with OVA. After 3 days, DN DR3 NKT cells transferred mice displayed no signs of lung inflammation, BALF eosinophilia or Th2
cytokine production, in comparison to WT NKT cells, which restored the features of
allergic lung inflammation (Fang, Adkins et al. 2008).

Furthermore, T_{regS} have also been cited as important in acute allergic lung inflammation. Both an anti-DR3 antibody (Schreiber, Wolf et al. 2010) and TL1A-IgG fusion protein (Khan, Tsai et al. 2013) were found to expand T_{regS} following acute allergic lung inflammation, reversing the ratio of conventional T cells to T_{regS}. T_{regS} made up 55% of CD4^{+} T cells compared to 22% in control mice using an anti-DR3 antibody, compared to a 66% and 25% using a soluble TL1A-IgG protein, suggesting the kinetics of expansion are similar between the two. This T_{reg} expansion protected against lung inflammation, blocking cell infiltration, BALF eosinophilia and mucin production (Schreiber, Wolf et al. 2010; Khan, Tsai et al. 2013). These findings suggest the DR3/TL1A pathway is central to the development of acute allergic lung inflammation.
1.5 Asthma

1.5.1 Definition
The World Health Organisation (WHO) define asthma as a disease characterised by recurrent attacks of breathlessness and wheezing, varying in frequency and severity between individuals. Asthma is increasing in prevalence and severity worldwide with an estimated 5.4 million people in Britain receiving treatment (Pawankar, Canonica et al. 2012). This presents a huge economic burden in terms of direct (medication, hospitalisation) and indirect costs (work and school loss) (Bahadori, Doyle-Waters et al. 2009). Clinical symptoms used to diagnose asthma include episodic wheezing, chest tightness, shortness of breath and coughing, leading to variable or reversible airflow obstruction (Hegele 2000), although several patients have shown poor reversibility (Bousquet, Jeffery et al. 2000).

1.5.2 Pathophysiology
Asthma is considered a chronic disease of the airways typified by lung inflammation, bronchial hyper-responsiveness, airway obstruction and airway remodelling in response to normally inert antigen (Hessel, Van Oosterhout et al. 1995; Kay 1996; Wills-Karp 1999). The chronic nature of the disease is mediated by the infiltration of multiple inflammatory cell types into the airway submucosa, including eosinophils, mast cells and activated Th2 lymphocytes (Hamid and Tulic 2009). Originally thought to be exclusively Th2 mediated, the immune regulation and response to innocuous allergen is now considered to be highly heterogeneous (Moore, Meyers et al. 2010). As well as inappropriate immune activation, dysregulated endogenous immune regulatory
processes are also thought to contribute to the development of disease (Holt, Strickland et al. 2008; Lloyd and Hawrylowicz 2009; Robinson 2009). Chronic airway inflammation is thought to be contained to the large conducting airways, with the severity and chronicity of the disease determining migration to the small airways and in some cases, alveoli (Kraft 1999; Diamant and van der Molen 2005).

Chronic inflammation is often accompanied by structural changes, which when referring to the asthmatic response, is known as airway remodelling. Alterations in airway architecture following chronic allergic lung inflammation encompass a range of changes, such as thickening of the airway walls, dysregulated extracellular matrix protein deposition and mucus gland hyperplasia (Hogaboam, Blease et al. 2000; Tattersfield, Knox et al. 2002; Lloyd and Robinson 2007). This complex and dynamic process is considered fundamental to the chronicity of the asthmatic disorder, as well as contributing to airway obstruction (Holgate 2007). Despite this, current therapies for asthma are directed towards the inflammatory response rather than structural remodelling (Di Valentin, Crahay et al. 2009), so whilst halting disease progression, they act to cause disease remission, thereby prolonging the asthmatic response (Jarman and Lamb 2005).

Asthma can be classified as either intrinsic or extrinsic (Wardlaw, Brightling et al. 2002). Intrinsic, or non-atopic, asthma does not rely on allergens to trigger a response but rather internal signals, such as respiratory infections, an upper airway reflex or even exercise. As it is not hyper-sensitivity induced, levels of IgE remain comparable to those of non-asthmatics during a non-atopic response (Rackemann 1940; Walter and Holtzman 2005). Extrinsic, or atopic, asthma on the otherhand, is IgE mediated and
occurs as a result of hyper-sensitivity to an inhaled antigen which is otherwise considered harmless. Individuals become sensitised to allergen, and upon re-exposure initiate an inflammatory response (Walter and Holtzman 2005). As a result, extrinsic asthma is loosely considered to be composed of 2 phases, the sensitisation and effector phase.

1.5.3 Sensitisation

A fundamental feature of allergen sensitisation is uptake and processing. DCs, situated in the airway epithelium and submucosa, are generally considered the most potent APCs (von Garnier, Filgueira et al. 2005; Lambrecht and Hammad 2009). Allergen recognition often occurs by pattern recognition receptors (PRR), although uptake is enhanced by IgE binding to FCγR on DCs (de Heer, Hammad et al. 2005; Kitamura, Takeda et al. 2007; Kool, Hammad et al. 2012). Allergen recognition causes the up-regulation of co-stimulatory molecules and migration to the lymph node via the chemokine receptor CCR7 and its ligands CCL19 and CCL21, and to a lesser extent CXCR4 and CXCL12 (Sallusto, Schaarli et al. 1998; Humrich, Humrich et al. 2006). However lymphotoxic ko mice, which lack peripheral lymph nodes, were able to generate antigen specific T cell responses and clear influenza infection (Lund, Partida-Sanchez et al. 2002). Furthermore, CCR7 ko mice, though unable to traffic DCs from the lung to the lymph node, exhibited proliferation of antigen specific CD4+ T cells in response to Mycobacterium tuberculosis infection (Olmos, Stukes et al. 2010). These data suggest that T cell activation in the lung can occur outside of lymph nodes, as resident lung antigen presenting cells hold the capacity to promote T cell differentiation (Constant, Brogdon et al. 2002). Once allergen is internalised, it is processed by
Cathepsin S and presented on the surface of the cell via MHCII, enabling interaction, and therefore priming to form an antigen-specific effector T cell. However, anergy can also occur depending on the nature as well as efficiency of co-stimulatory molecule interaction between DCs (CD80/86) and T cells (CD28). The co-stimulatory molecule, cytotoxic T lymphocyte antigen 4 (CTLA 4), has a high affinity for CD28, preventing CD80/CD86 binding and thereby rendering T cells anergic. This is the basis for the RA therapy drug, abatacept (Weinblatt, Combe et al. 2006). However, in more severe asthma, alternative co-stimulatory molecules are engaged, including ICOS as well as the TNFSF members OX40/OX40L (Salek-Ardakani, Song et al. 2003; Burgess, Blake et al. 2005). In asthmatic patients a trend is seen towards Th2 differentiation by DCs, leading to the production of the Th2 signature cytokines IL-4, IL-5, IL-9 and IL-13, the majority of which are expressed on the long arm of chromosome 5 (Ryu, Jung et al. 2006). This cytokine milieu acts to promote the Th2 response, initiating class switching and affinity maturation to produce IgE, recruiting secondary effector cells into the inflammatory zone and up-regulating FcεRI expression on the surface of mast cells and basophils. Basophils themselves have been implicated in the initiation of the Th2 response, as early producers of IL-4. They have been shown to enhance allergen sensitisation in vivo, and polarise towards a Th2 response in vitro in the absence of DCs (Perrigoue, Saenz et al. 2009; Sokol, Chu et al. 2009; Yoshimoto, Yasuda et al. 2009). Others argue however, that they are dispensable for Th2 priming, only partially affecting sensitisation upon depletion, instead suggesting inflammatory DCs were necessary (Hammad, Plantinga et al. 2010). CCR9+ invariant NKT cells have also been linked to the inflammatory response in chronic asthma (Sen, Yongyi et al. 2005; Akbari, Faul et al. 2006), although it is the CCR4+ T cells which dominate the allergic immune response, correlating with disease severity (Ishida, Ishii et al. 2006).
1.5.4 Effector phase

Following re-exposure to allergen, bronchoconstriction occurs due to allergen specific IgE cross linking upon the surface of mast cells as well as basophils via FcεRI binding, and leukocytes via the lower affinity FcεRII (Gould, Sutton et al. 2003). This binding leads to de-granulation, the release of multiple inflammatory mediators and continuation of the asthma pathogenesis.

1.6 Characteristics of asthma

1.6.1 Airway inflammation

Airway inflammation is a cardinal feature of asthma, occurring in even mild forms of the disease (Laitinen, Laitinen et al. 1996). The extent of inflammation often correlates with disease severity, inducing the common symptoms of asthma (Walter and Holtzman 2005). Despite not being naturally prone to allergic disease, mouse models have been shown to demonstrate this key symptom observed in asthmatic patients (Williams and Galli 2000; Kips, Anderson et al. 2003; Koerner-Rettberg, Doths et al. 2008). It is a multi-cellular process which due to the complex nature of asthma, cannot be attributable to a single cell type.

1.6.1.1 Mast cells

Due to their ability to release pro-inflammatory mediators, high affinity for IgE and proximity to blood vessels, smooth muscle and mucosal surfaces, mast cells are
considered a key contributor to the asthmatic response. Located in the airway epithelium, submucosa, deep airway wall and peripheral airways (Yu, Tsai et al. 2006), mast cells interact with smooth muscle through leukotriene D₄, prostaglandin D₂ and histamine, contributing to fibrogenesis, microvascular permeability and airway remodelling (Kaur, Saunders et al. 2006; Plante, Semlali et al. 2006). Mast cells, after cross linking by IgE, provide an early source of inflammatory mediators, such as IL-4, influencing effector T cells, and IL-5, promoting eosinophil recruitment and activation (Lorentz, Schwengberg et al. 1999). De-granulation also sees the release of preformed mediators, such as histamine, heparin and tryptase, as well as rapidly synthesised mediators, including lipid mediators and other cytokines and chemokines, including TNFα (Okayama, Tkaczyk et al. 2003; Bradding, Walls et al. 2006). This release helps to prolong the inflammatory response via the infiltration of further cell types. Mast cell deficient mice which underwent sensitisation (without adjuvant) and repeated aerosol challenges, displayed reduced numbers of eosinophils, neutrophils and lymphocytes and impaired goblet cell hyperplasia (Yu, Tsai et al. 2006), which could not be restored using FcεR1⁻ko cells (Yu, Eckart et al. 2011), TLR4⁻ko cells (Nigo, Yamashita et al. 2006) or TNFα⁻ko cells (Suto, Nakae et al. 2006), supporting the idea that mast cells contribute towards airway inflammation.

1.6.1.2 Eosinophils

Eosinophils were originally described as the signature cell type of atopic asthma, dominating the infiltrate of asthma patients as well as correlating with disease severity in some patients (Bousquet, Jeffery et al. 2000; Kay 2005). Found within the BAL as well as the sputum (Kay 2005; Lemiere, Ernst et al. 2006), eosinophils are recruited as
CD34$^+$ precursors from the bone marrow in response to IL-3, GM-CSF, CCL2 and CCL5 amongst other soluble factors (Robinson, North et al. 1999; Sehmi, Dorman et al. 2003). IL-5 however, is considered particularly important for eosinophil recruitment, differentiation, proliferation and maturation (Foster, Mould et al. 2001). However, its blockade, though reducing eosinophil numbers, did not cause the abolition of airway inflammation (Flood-Page, Menzies-Gow et al. 2003), despite beliefs it was centrally involved in asthma development and inflammation. Eosinophil depletion was instead found to affect extracellular matrix protein deposition (Flood-Page, Menzies-Gow et al. 2003), reinforcing the complex nature of asthma pathogenesis. Upon activation, eosinophils release cationic proteins, such as major basic protein (MBP) and eosinophil peroxidase (EPO), as well as inflammatory mediators such as Th2 cytokines, prostaglandins and leukotrienes (Kariyawasam and Robinson 2006), amplifying the immune response. Eosinophil deficient mouse studies have shown a conflicting role for eosinophils in the allergic lung response. Whilst those on a C57BL/6 background displayed reduced airway hyper-reactivity (Lee, Dimina et al. 2004) and Th2 cytokines, correlating with a reduced ability to recruit effector T cells (Jacobsen, Ochkur et al. 2008), BALB/c mice failed to replicate this phenotype. They instead displayed reduced remodelling (Humbles, Lloyd et al. 2004), although this has since been disputed (Fattouh, Al-Garawi et al. 2011). Nonetheless, when crossed onto a C57BL/6 background, BALB/c mice too displayed airway hyper-reactivity attenuation and a failure to recruit CD4$^+$ T cells via CCL17 and CCL22 (Walsh, Sahu et al. 2008).
1.6.1.3 Macrophages

The precise role of macrophages in mediating tissue damage and pathology is yet to be defined. Macrophages have been cited as a regulator of allergic lung inflammation via the release of IL-10 and TGFβ (Soroosh, Doherty et al. 2013), as well as a promoter of eosinophilic inflammation (Moon, Kim et al. 2007) and airway remodelling (Mautino, Henriquet et al. 1999). Alveolar macrophages (AMs), which some suggest originate from interstitial macrophages (Landsman and Jung 2007), release CCL2 to attract monocytes to inflamed tissue (Brieland, Jones et al. 1992). Th2 cytokines skew macrophages towards an M2 phenotype, causing the release of inflammatory factors, which although intended to maintain pulmonary homeostasis in asthma, actually lead to aggravated lung injury (Moreira and Hogaboam 2011). Macrophages have also been reported to inhibit T cell proliferation and DC induced T cell activation (Holt, Oliver et al. 1993; Bedoret, Wallemacq et al. 2009) as transfer of un-sensitised AMs to sensitised mice initiated protection and suppressed pro-inflammatory functions (Careau, Proulx et al. 2006). In contrast, there are also reports of alveolar macrophages promoting inflammation by releasing IL-17 in an OVA mouse model of allergic lung inflammation (Song, Luo et al. 2008), controlling the proteolytic activity of macrophages (Sergejeva, Ivanov et al. 2005). These contrasting data suggests further work needs to be done to explore the role of macrophages in the allergic response.

1.6.1.4 CD4+ T cells

Th2 cells, through the production of cytokines, can initiate and maintain the cardinal features of allergic lung disease, though epithelial derived cytokines such as IL-25 (Tamachi, Maezawa et al. 2006), IL-33 (Kurowska-Stolarska, Kewin et al. 2008) and
Thymic stromal lymphopoietin (TSLP) (Zhou, Comeau et al. 2005) recently emerged with a role. The number of Th2 cells in patient asthmatic airways correlates with disease severity (Walker, Kaegi et al. 1991). IL-4 and IL-13 promote IgE class switching, mucus secretion and fibrosis, and together with IL-9, cell recruitment, whilst IL-5 supports eosinophil activation. Th2 cells have therefore been established as a central cell type in the asthma paradigm.

1.6.1.5 CD8\(^+\) T cells

The role of CD8\(^+\) T cells in asthma remains controversial, as they have been reported to exhibit both beneficial and deleterious effects. Depletion of CD8\(^+\) T cells in rats led to an enhancement of airway inflammation and remodelling (Laberge, Wu et al. 1996; Tsuchiya, Isogai et al. 2009), thought to be mediated by IFN\(\gamma\) and IL-12 (Wells, Cowled et al. 2007; Takeda, Dow et al. 2009). In contrast, CD8\(^+\) T cells have also been observed to enhance the asthmatic phenotype by collaborating with sensitised CD4\(^+\) T cells (Miyahara, Swanson et al. 2004; Koya, Miyahara et al. 2007). In these latter studies, CD8\(^+\) T cell deficient mice were less susceptible to allergic lung inflammation, measured by decreased airway eosinophilia, cytokine production and goblet cell metaplasia, suggesting CD8\(^+\) T cells were required for chronic disease. Phenotype could be restored upon transfer of \textit{in vitro} primed WT effector memory CD8\(^+\) T cells, but not those from IL-13\(^{-}\)mice, suggesting CD8\(^+\) T cells were activated in the airways and produce IL-13 locally (Miyahara, Swanson et al. 2004).
1.6.1.6 Innate like T cells

Aside from αβ T cells, innate like lymphocytes have recently been reported to have a role in allergic lung inflammation. NK cells are present in the naive lung, expressing a variety of receptors so enabling immune surveillance (Grégoire, Chasson et al. 2007). Increased numbers of NK cells were observed in asthma patients (Weissler, Nicod et al. 1987). Under steady state conditions, resident lung NK cells expressed fewer activating receptors, adhesion/migration receptors and co-stimulatory molecules compared to splenic NK cells, instead displaying a higher frequency of inhibitory receptors. It was thus suggested that NK cells were quiescent, with activation being tightly regulated by the pulmonary environment (Wang, Li et al. 2012). Mouse models however support a role for NK cells in allergic lung inflammation, as their depletio n before OVA sensitisation or during challenge inhibited eosinophilia, T cell infiltration and inflammatory mediator release, suggesting NK cells contributed to both the initiation and maintenance of the Th2 inflammatory response (Korsgren, Persson et al. 1999).

NKT cells, which recognise glycolipids through CD1d on APCs, have also been implicated in the asthmatic response using mouse studies. NKT deficient mice retained eosinophilic inflammation but failed to develop airway hyper-reactivity, though this was restored upon the transfer of purified WT NKT cells, though not IL-4\(^{-}\) or IL-13\(^{-}\) cells (Akbari, Stock et al. 2003). The glycolipid NKT ligand, α-galactosylceramide (α-GalCer), promoted IL-13 and TSLP release from NKT cells, as well as NKT mediated allergen sensitisation when administered with antigen (Kim, Kim et al. 2004; Nagata, Kamijuku et al. 2007). An increase in CD4\(^{+}\) α-GalCer loaded CD1d tetramer cells was also noted in the BAL of asthmatic children compared to controls, suggesting a role for NKT cells in disease (Pham-Thi, de Blic et al. 2006).
An increase in pulmonary γδ T cells has also been reported in asthmatic patients, particularly during asthma exacerbations (Molfino, Doherty et al. 1996; Spinozzi, Agea et al. 1996). In allergic lung inflammation mouse models, γδ T cells were shown to act as regulators of lung inflammation, as increased airway hyper-reactivity was observed upon depletion (Lahn, Kanehiro et al. 1999; Hahn, Taube et al. 2003), particularly when co-infected with rhinovirus (Glanville, Message et al. 2013). Others however, suggest γδ T cells are pro-inflammatory, potentiating the activation of the Th2 response (Schramm, Puddington et al. 2000; Svensson, Lilliehook et al. 2003).

ILC2 cells, a relatively new population involved in innate defence, have also been found in the lung (Monticelli, Sonnenberg et al. 2011). They are functionally characterised by the production of IL-5 and IL-13 in response to the stimulating cytokines IL-25 and IL-33 (Neill, Wong et al. 2010; Price, Liang et al. 2010). Both IL-5 and IL-13 have already distinguished roles in allergic lung inflammation, promoting eosinophil proliferation and maturation, as well as goblet cell hyperplasia and antibody class switching. Roles have also emerged for both IL-25 and IL-33 in allergic lung inflammation. IL-25, a member of the IL-17 family, is thought to promote Th2 immunity via the induction of IL-4, IL-5 and IL-13 (Fort, Cheung et al. 2001). Similarly, IL-33, a member of the IL-1 cytokine family, has been shown to induce eosinophilia and increased IgE production in vivo (Schmitz, Owyang et al. 2005), as well as reduce symptom severity when blocked (Oboki, Nakae et al. 2011). IL-33 is thought to act as a danger signal, released by epithelial cells in response to allergen (Pichery, Mirey et al. 2012), in turn leading to the accumulation of ILC2’s and Th2 mediated inflammation. Adoptive transfer of ILC2’s to IL-13ko mice restored airway hypereactivity (Chang, Kim et al. 2011), whilst intranasal administration of IL-25 and IL-33 in mice, initiated an asthmatic phenotype via
increased accumulation of ILC2’s in the lung and BAL, even in the absence of T cells (Wolterink, KleinJan et al. 2012).
Figure 1.3. Inflammatory and immune cells involved in asthma. Inhaled allergen is presented by DCs causing Th2 differentiation and the release of multiple cytokines. IL-4 and IL-13 initiate antibody class switching, IL-13 contributes to airway remodelling, IL-5 causes eosinophil maturation and IL-9 enhances mast cell proliferation. IgE cross links on the mast cell surface, releasing bronchoconstrictors such as histamine, leukotrienes and prostaglandins. These collective events lead to bronchoconstriction, airway remodelling and airway inflammation.
1.6.2 Airway hyper-responsiveness (AHR)

AHR is a clinical feature of asthma, though intensity often varies between patients (Busse 2010). It is defined as a greater degree of airway closure via increased sensitivity to stimuli (O'Connor, Crowther et al. 1999). AHR occurs in response to a range of direct stimuli, including histamine and methacholine, which act directly on specific smooth muscle receptors to cause constriction. Histamine acts on H1, whilst methacholine exerts its effects through muscarinic receptors, increasing in the eosinophil mediated absence of the regulatory muscarinic receptor 2 (Elbon, Jacoby et al. 1995; Castro, Resende et al. 2013). Methacholine is often used in a diagnostic test for asthma, as a lesser concentration is required to achieve airway contraction. Indirect stimuli, such as cold air, exercise and adenosine monophosphate (AMP) also induce AHR, though they do not act directly upon smooth muscle. AMP is thought to stimulate mast cells, causing the release of mediators independent of IgE (O’Byrne, Gauvreau et al. 2009). The developmental process of AHR remains unknown, though structural changes have been implicated, as epithelial damage exposes nerve endings, thereby increasing response to stimuli, whilst airway thickening results in narrower airways and so greater constriction when stimulated (Jeffery, Wardlaw et al. 1989; Oliver, Fabry et al. 2007). However, it is not clear whether there is a change in airway smooth muscle mechanically, increasing its contractile ability, or structurally, increasing in mass and thickness (An, Bai et al. 2007).
1.6.3 Airway remodelling

Chronic airway inflammation is often accompanied by structural changes, termed airway remodelling in asthmatic patients. These changes include collagen and fibronectin deposition, thickening of the sub-epithelial basement membrane, goblet cell hyperplasia, increased smooth muscle mass and size and fibrosis, all of which contribute to a thickening of the airway wall, obstructing airflow via a reduction in airway diameter (Bousquet, Jeffery et al. 2000; Hamid and Tulic 2009).

1.6.3.1 Goblet cell hyperplasia

Goblet cell hyperplasia and increases in mucin secretion have long been associated with asthma, as increased levels were observed in asthmatic airways compared to controls (Aikawa, Shimura et al. 1992), while the levels of the predominant mucin gene, MUC5AC are raised in asthmatic disease (Ordonez, Khashayar et al. 2001). MUC5AC has previously been linked to mucus hyper-secretion and is the most abundant gel forming mucin present at the airway surfaces (Young, Williams et al. 2007). This increase in goblet cells, which parallels with a reduction in ciliated and Clara cells (Reader, Tepper et al. 2003), leads to increased bronchial obstruction due to mucus plugging in both the central and peripheral airways (Andoh, Aikawa et al. 1992; Ordonez, Khashayar et al. 2001; Kuyper, Pare et al. 2003). Inflammation is thought to stimulate the activation of epidermal growth factor (EGF) and IL-13, inducing Clara and ciliated cells to differentiate into goblet cells via the transcription factors FoxA2, thyroid transcription factor 1 (TTF-1) and SAM pointed domain ETS transcription factor (SPDEF) (Evans, Williams et al. 2004; Wan, Kaestner et al. 2004; Tyner, Kim et
al. 2006; Chen, Korfhagen et al. 2009; Cras, Acciani et al. 2011). This leads to the up-regulation of MUC5AC and increased mucin granules, which fuse to the plasma membrane through the myristoylated alanine rich C kinase substrate (MARCKS) (Li, Martin et al. 2001; Singer, Martin et al. 2004), soluble N-ethylmaleimide sensitive factor attachment receptors (SNAREs) and the accessory Munc proteins, allowing plasma membrane fusion to occur and mucin release into the airway lumen (Curran and Cohn 2010).

IL-13 is essential for goblet cell hyperplasia, regulating the chloride channel GOB5, involved in mucin regulation, composition and secretion from goblet cells (Thai, Chen et al. 2005; Long, Sypek et al. 2006), as well as acting directly on the airway epithelium in a STAT6 dependent manner (Kuperman, Huang et al. 2002; Kuperman, Huang et al. 2005; Fujisawa, Ide et al. 2008). IL-9 induced expression of MUC5AC is also IL-13 dependent (Steenwinckel, Louahed et al. 2007). Irrespective of the mechanism, the over production of mucus is a hallmark of the asthmatic lung, contributing to airway obstruction, and in extreme cases, mortality (Sheehan, Richardson et al. 1995).

1.6.3.2 Fibrosis

Sub-epithelial fibrosis is associated with thickening of the basement membrane caused by deposition of ECM proteins (Roche, Beasley et al. 1989), particularly collagen types I and III, fibronectin and proteoglycans (Freyer, Billington et al. 2004). Type I collagen lends support to the alveolar and bronchial walls, whilst type III is essential for type I fibrillogenesis (Bradley, McConnell-Breul et al. 1974; Bienkowski, Ripley et al. 1990). The thickness of the sub-epithelial layer was reported to increase with the severity of
disease (Boulet, Laviolette et al. 1997; Chetta, Foresi et al. 1997), though others found no correlation (Chu, Halliday et al. 1998). Sub-epithelial fibroblasts and myofibroblasts, normally responsible for tissue repair, are increased in asthma, where they are thought to contribute to increased basement membrane thickening and ECM deposition, including collagen (Brewster, Howarth et al. 1990; Morishima, Nomura et al. 2001; Matsumoto, Niimi et al. 2005). TGFβ promotes the differentiation of fibroblasts to contractile myofibroblasts, triggering proliferation and the release of fibroblast growth factor (FGF) and connective tissue growth factor (CTGF) (Khalil, Xu et al. 2005; Le, Cho et al. 2007; Michalik, Pierzchalska et al. 2009). Eosinophils are thought to be a major source of TGFβ, as their depletion in the asthmatic airways led to reduced levels (Flood-Page, Menzies-Gow et al. 2003). The loss of epithelial characteristics and subsequent acquisition of mesenchymal features, giving rise to fibroblasts and myofibroblasts has also been cited as a mechanism behind the alterations in lung architecture. This emerging concept, known as epithelial mesenchymal trophic unit, is also thought to be mediated in part by TGFβ (Jill, Paul-Andre et al.; Hackett, Warner et al. 2009; Holgate, Roberts et al. 2009; Heijink, Postma et al. 2010; Grainge, Lau et al. 2011; Johnson, Roos et al. 2011) in response to allergen induced epithelial damage and inflammatory insults (Holgate, Lackie et al. 2001). In vivo, TGFβ blockade led to a reduction in peribronchiolar ECM deposition during established allergic lung disease (McMillan, Xanthou et al. 2005), whilst over-expression of the cytokine induced severe fibrosis and increased ECM deposition (Kolb, Bonniaud et al. 2002).

In addition, the dysregulated function of MMPs, particularly MMP-9, has been reported in the asthmatic airway (Mattos, Lim et al. 2002; McMillan, Kearley et al. 2004; Lim, Cho et al. 2006), altering ECM remodelling and degradation, as well as platelet derived

1.6.3.3 Smooth muscle and vascularity

Increased smooth muscle mass, due to hyperplasia and hypertrophy, is a major determinant of airflow obstruction together with fibrosis (Benayoun, Druilhe et al. 2003; Woodruff, Dolganov et al. 2004). However, difficulties have arisen in distinguishing hyperplasia and hypertrophy in asthmatic patients, as Ebina et al noted hyperplasia in the large airways in some patients, whereas hypertrophy in the large and small airways of others (Ebina, Takahashi et al. 1993). In the case of hyperplasia, it is unknown if smooth muscle cells proliferate and receive sustained survival signals (Trian, Benard et al. 2007; Kaur, Hollins et al. 2010), or cells, such as fibrocytes, are recruited, transforming into smooth muscle cells (Kaur, Saunders et al. 2006; Saunders, Siddiqui et al. 2009). Therefore, although smooth muscle levels have been recognised to increase in asthma, by 200-400% in fatal cases and 50-200% in non fatal asthma (Bai, Cooper et al. 2000), the mechanisms remain unknown. Similarly, published data suggests angiogenesis and vascular remodelling occurs in asthma via vascular endothelial growth factor (VEGF), although it is unclear whether this is due to an increase in blood vessel formation or just size (Carroll, Cooke et al. 1997; Baluk, Lee et al. 2004; Bhandari, Choo-Wing et al. 2006).
Figure 1.4. A schematic diagram of normal and asthmatic airways. Inhaled allergen initiates the release of multiple cytokines and growth factors from immune cells as well as components of the airway wall. This leads to dysregulation of the pulmonary airways including goblet cell hyperplasia, smooth muscle hyperplasia and hypertrophy, sub-epithelial fibrosis and angiogenesis.
1.7 Mouse models of asthma

Unlike cats (Venema and Patterson 2010) and horses (Herszberg, Ramos-Barbon et al. 2006), which display particular features, mice do not spontaneously develop asthma. Consequently, it must be induced using allergen sensitisation and challenge. Several different allergens can be used to elicit an asthmatic response, including house dust mite (Cates, Fattouh et al. 2004), short ragweed extracts (Chapoval, Nabozny et al. 1999) and ovalbumin (Smith and Broadley 2007). OVA is the most commonly used allergen and is often injected alongside the adjuvant Alum to induce sensitisation.

Acute models of allergic lung disease focus on inflammatory mechanisms linked to manifestations of asthma in humans, as inflammation is predominantly restricted to the proximal airways (Wegmann, Fehrenbach et al. 2005). Whilst useful for investigating targets directly relating to mast cells, acute models remain problematic in their ability to assess other disease processes, such as airway wall changes, and novel therapies targeting the evolution of asthma in established disease. Chronic models of allergic lung inflammation experience allergen exposure over a longer period, simulating the chronic nature of human asthma. These models allow treatments to be tested in a therapeutic rather than a preventative setting. However, limitations still exist with regards to human asthma, as remodelling shows a tendency towards fibrosis rather than smooth muscle thickening as occurs in humans (Holmes, Solari et al. 2011) and inflammation occurs in the lung parenchyma, particularly perivascular and peribronchiolar areas, rather than within the airway wall as seen in humans (Kumar and Foster 2002).
Mice do however provide several advantages over their larger counterparts, such as lower costs, a well characterised immune system and the availability of KO mice. As such, murine models of asthma are becoming ever more prevalent (Kumar and Foster 2002; McMillan and Lloyd 2004). However, despite being instrumental in understanding the mechanistic and underlying pathways of disease, debate continues as to the suitability of mice modelling what is predominantly considered a human disease (Gelfand 2002; Persson 2002; Holmes, Solari et al. 2011).

1.8 Treatments

Inhaled corticosteroids and β2-adrenoreceptor agonists, both short acting (SABA) and long acting (LABA), are currently utilised for asthma treatment. Corticosteroids suppress Th2 mediated inflammation by targeting cytokines, chemokines and adhesion molecules. They diffuse across the cell membrane and interact with cytoplasmic glucocorticoid receptors, causing activation, translocation to the nucleus and either the transactivation or repression of target genes. However, corticosteroids, though effective in suppressing airway inflammation, do not influence disease progress (Holgate and Polosa 2008). Inhaled corticosteroids are absorbed from the lungs and thus hold potential for systemic side effects. This has resulted in a search for safer treatments with reduced adsorption from the lungs and inactivation in the circulation, allowing the administration of higher doses in severe cases of asthma. Dissociated steroids attempt to separate these side effects mediated by transactivation from the anti-inflammatory effects conducted by transrepression (Heike, Arndt et al. 2004). Bronchodilators, the most predominant being β2-agonists, assist in the management of asthma via the
prevention of bronchoconstriction. LABAs, the most common being formoterol and salmeterol, are often used in conjunction with inhaled corticosteroids to prevent further exacerbations (McIvor, Pizzichini et al. 1998; Jaeschke, O'Byrne et al. 2008), inducing bronchodilation for up to 12 hours (Usmani, Ito et al. 2005). Inhaled SABAs, such as salbutamol and terbutaline, are currently used for the rapid relief of asthma. Once bound to $\beta_2$-adrenoreceptor, adenylate cyclase is induced, converting adenosine triphosphate (ATP) into cyclic adenosine monophosphate (cAMP), thereby activating protein kinase A. This then leads to smooth muscle relaxation, phosphorylation of myosin light chain kinase, opening of the Ca$^{2+}$ dependent K$^+$ channel, ultimately relieving bronchoconstriction in asthma.

Although widely used, severe asthmatics are often unable to adhere to treatments such as corticosteroids, resulting in a lack of effectiveness (Chapman, Boulet et al. 2008). Corticosteroids are unable to decrease the levels of TNF$\alpha$, which are often increased in severe cases (Truyen, Coteur et al. 2006), thus the TNF receptor fusion protein etanercept was trialled in small studies with reported efficacy (Howarth, Babu et al. 2005; Berry, Hargadon et al. 2006). Omalizumab has also been shown to inhibit allergen induced inflammatory responses in mice and humans by blocking IgE binding to Fc$\varepsilon$RI and Fc$\varepsilon$RII (Corne, Djukanovic et al. 1997; Busse, Morgan et al. 2011; Hanania, Alpan et al. 2011), whilst mast cell stabilising drugs, such as masitinib, have been reported to limit mast cell survival via the inhibition of c-kit and thereby stem cell factor (SCF) (Humbert, De Blay et al. 2009). Cytokine therapy has also been volunteered as a potential asthma treatment, although due to the redundancy that exists between them, results have sometimes proved disappointing (Hart, Blackburn et al.
2002; Linhart, Bigenzahn et al. 2007). IL-13 however, is still being considered as a therapeutic target (Spahn, Szeffler et al. 1996; Wenzel, Wilbraham et al. 2007).
1.9 Aims and Hypothesis

Hypothesis: DR3 exerts differential effects in acute versus chronic allergic lung inflammation.

In order to test this hypothesis, the residing aims of this thesis are to use OVA/Alum induced \textit{in vivo} experimental models of lung inflammation to investigate the effects of the DR3/TL1A pathway during homeostasis, acute allergic lung inflammation and chronic allergic lung inflammation, thus exploring its potential as a therapeutic target in asthmatic disease. More specifically, this thesis intends to:

- Determine DR3 expression on leukocytes and within the lung using both flow cytometry and immunohistochemistry and a newly commercially available DR3 antibody. DR3 expression will be examined in the naive and inflammatory states.

- Characterise leukocyte populations from the lung, peripheral blood and spleens of naive DR3\textsuperscript{wt} and DR3\textsuperscript{ko} mice to investigate whether DR3 influences leukocyte homeostasis.

- Investigate the role of DR3 in the accumulation of leukocyte populations into the lung, BALF and spleen following acute allergic lung inflammation, as well as allergic antibody levels and airway remodelling.
Investigate the role of DR3 in the accumulation of leukocyte populations into the lung, BALF and spleen following chronic allergic lung inflammation, as well as allergic antibody levels and airway remodelling.
Chapter 2: Materials and Methods
2.1 Reagents

2.1.1 Chemicals

All chemicals were purchased from Sigma Aldrich Ltd (Poole, Dorset, UK) unless otherwise stated.

2.1.2 Solutions

**Distilled Water (dH$_2$O)**

dH$_2$O was generated by a reverse osmosis system with filtration through two ion residue columns using a Millipore-Q System.

**RPMI 10:**

RPMI 61 with 2mM L-glutamine, 10 units ml$^{-1}$ Penicillin Streptomycin, 10% heat inactivated Foetal Calf Serum (FCS).

**Dulbecco’s Modified Eagle’s Media (DMEM)-10:**

DMEM with 2mM L-glutamine, 10 units ml-1 Penicillin Streptomycin, 10% FCS.

**MACS buffer:**

PBS containing 0.5% FCS and 5mM EDTA.

**DNA Lysis Buffer:**

25mM Tris-HCL (pH 8.0), 10% SDS, 1M EDTA, 1M NaCl, 2mg Proteinase K (Roche Diagnostics), 5ml dH$_2$O.
Protein Lysis Buffer:

50mM Tris, 150mM NaCl, 1% Triton X, 1mM EDTA, 100mM PMSF, Protease inhibitor cocktail EDTA free (Roche).

DNA Loading Buffer Orange G:

2.5mg/ml Orange G loading dye in 2X TBE containing 30% glycerol.

Freezing Media:

90% FCS with 10% Dimethyl sulfoxide (DMSO) (v/v).

FACS buffer:

PBS containing 1% FCS and 2mM EDTA.

Wash Buffer:

PBS containing 0.05% Tween-20.

Eosin Solution:

10mg/ml Eosin dissolved in dH₂O.

Celestine Blue

400ml dH₂O, 20g Ammonium Ferric Sulphate (III), 2g Celestine Blue, 14ml Glycerol.
2.2 Animals and *in vivo* models

2.2.1 Housing and Home Office approval

Animals were housed in the Joint Biological Services Unit of Cardiff University. Animals were maintained at a constant temperature and humidity on a 12 hour light/dark cycle within filter top cages, until experiments commenced, whereby mice were transferred to conventional caging. Food and water were available to animals *ad libitum*. DR3 mice were bred on a C57BL/6 background within the facility, with genotype being confirmed via ear snip digests and PCR (Section 2.2.2). All procedures performed were Home Office regulated and within the license 30/2580 held by Dr E.C.Y. Wang.

2.2.2 Genotyping

2.2.2.1 Genomic DNA isolation

Earsnip samples were resuspended in 750μl of lysis buffer and incubated overnight at 56°C in a waterbath. A DNA salt extraction was performed by adding 310μl of 5M NaCl, whereby the sample was vortexed and left at room temperature for 30 minutes before being spun at 16060g for 25 minutes. The aqueous phase (800μl) was then removed and added to 500μl of ice cold isopropanol, before the sample was again vortexed and the precipitated DNA spun down at 16060g for 10 minutes. The supernatant was removed and 500μl of 70% ethanol was added before leaving at room
temperature for 30 minutes. The DNA was once again spun and as much liquid removed as possible, followed by a 37°C incubation for 1 hour or until all ethanol had evaporated. The remaining pellet was then resuspended in 50μl of distilled water and left overnight at 37°C to allow the DNA to dissolve, ready for PCR amplification.

### 2.2.2.2 Genotyping PCR

To thin walled PCR tubes, 20μl of dH₂O was added, along with 8μl of genomic DNA. A master mix was made up consisting of:

- 5μl 4mM dNTP
- 4μl 10X PCR buffer
- 1.2μl 50mM MgCl₂
- 0.2μl 100μM AV1 primer (CAT CGC CTT CTA TCG CCT TC)
- 0.4μl 100μM 4F primer (AGA AGG AGA AAG TCA GTA GGA CCG)
- 0.8μl 100μM 2R primer (GAA AGG ATG AAA CTT GCC TGT TGG)
- 0.5μl Taq polymerase

12.1 μl of master mix was added to each reaction tube and the PCR programme begun using a Thermocycler (Hangzhou Long Gene Scientific Instruments Co, Zhejiang, China) with the program below:
Denaturation: 94˚C 5 minutes
Annealing: 62.5˚C 30 seconds 1 Cycle
Extension: 72˚C 40 seconds

Denaturation: 94˚C 30 seconds
Annealing: 62.5˚C 30 seconds 33 Cycles
Extension: 72˚C 40 seconds

Denaturation: 95˚C 30 seconds
Annealing: 62.5˚C 40 seconds 1 Cycle
Extension: 72˚C 5 minutes

Once thermocycling was complete, 4μl of Orange G was added to the samples which were then loaded onto a 1.6% agarose gel and run for 90 minutes at 100V. The gel was visualised under UV light (Ultra-Violet Products Ltd, Cambridge, UK), with the wildtype band lying at 280bp and the knockout band at 320bp.
2.2.2.3 Agarose gel

Powdered agarose (2.4g) was added to 150ml of 1X TBE and microwaved for 95 seconds with regular shaking. Ethidium Bromide (7.5μl of 10mg/ml) was then added to the mixture and cooled under cold tap water for 90 seconds. This was then poured into a gel tank, where combs were inserted to allow for the addition of sample. The gel was left to set for at least 20 minutes, whereby the combs were removed and 20μl of sample added to each well or 5μl of 1KB DNA ladder (Life Technologies Ltd, Paisley, UK).

2.2.3 Measuring lung function in mice

2.2.3.1 Whole body plethysmography

Enhanced Pause (Penh) is an established method for measuring lung function in conscious mice. Mice are un-restrained during the technique resulting in decreased stress for the animal and ideally ‘normal’ breathing patterns. However, mice still need to be conditioned to sit within the airtight chamber, for a minimum of 2 weeks 5 minutes/day before any readings can be taken. The process relies on an exchange of air between the body and the chamber, so causing pressure changes within the chamber (Mo, Chung et al. 2011). A single chamber whole body plethysmograph was used (Buxco Electronics Inc, Wilmington, NC, USA). Mice were exposed to nebulised PBS within the chamber for 3 minutes to establish baseline Penh readings. Values were then averaged for each mouse.
Figure 2.1. The box pressure wave in inspiration (down) and expiration (up) from which Penh can be derived. Diagram taken from Hamelmann et al (1997).

Waveform from flow whole body plethysmography can be divided into 2 regions, Ti or inspiratory time, the time from the start of inspiration to the end, and Te the expiratory time, or the time from the end of inspiration till the start of the next inspiration. Tr, or relaxation time, is the seconds taken for the pressure to decay to 36% of total expiratory pressure. Levels of peak inspiration (PIP) and peak expiration (PEP) are used to calculate Penh, a dimensionless value, which reflects changes in the waveform of the box pressure signal, indicative of pulmonary resistance and airway responsiveness. A positive percentage change in Penh values signifies bronchoconstriction, and negative values bronchorelaxation.

2.2.3.2 Hyper-responsiveness in mice

Hyper-responsiveness in naive mice was measured using aerosolised Methacholine (Sigma Aldrich, Dorset, UK) at 30mg/ml. Mice were exposed for 3 minutes to nebulised Methacholine, and recordings averaged for each mouse.
2.2.3.3 Analysis of mouse activity

Mice were habituated to the whole body plethysmography chamber for 2 weeks, during which time a basic assessment was made of their activity in the chamber and ability to remain still. Scores of 0, for no activity, and 1 for movement, were assigned depending on the behaviour of the mice. Video recordings were also made of this habituation period.

2.2.4 Separation of murine serum

2.2.4.1 Murine serum seperation

Serum was collected to allow the identification of Immunoglobin antibodies following the induction of inflammation. Up to 1ml of obtained blood was placed into specially designed serum separator tubes (Becton Dickinson Ltd, Oxford, UK), inverted and then left at room temperature for at least 30 minutes. The microtainers were then centrifuged at 9503g for 2 minutes at RT and the top layer of serum removed. Serum was frozen at -70°C for later use.
2.2.5 Harvesting of the lung, bronchoalveolar lavage, peripheral blood and splenic cell populations

2.2.5.1 Collagenase lung digestion

Once animals were sacrificed, lungs were exposed by removing the ribcage and cutting the diaphragm. Lungs were perfused with 5ml of PBS via injection into the heart and immediately harvested from the mouse, whereby they were stored on ice in PBS. They were then chopped into small fragments using dissection scissors and placed into 2ml of 1mg/ml collagenase D (Roche Diagnostics Ltd, West Sussex, UK) in serum free media. Lungs were placed in a 37°C waterbath for 45 minutes with occasional shaking. Digested lungs were then passed though a 70μm cell strainer and flushed with media several times. The flow through was then spun at 425g for 3 minutes at 4°C, and a RBC lysis performed if necessary (Section 2.2.6.1). Cells were then suspended in 1ml of FACS buffer.

2.2.5.2 Bronchoalveolar lavage (BAL) of mice

Once animals were sacrificed, the peritoneal cavity was opened and the diaphragm carefully cut away from the thorax, so allowing for inflation of the lungs. The trachea was exposed and a 23g catheter needle inserted. PBS/2mM EDTA (1ml) was then slowly injected via the catheter, inflating the lungs. Lavage fluid was carefully withdrawn 10-15 seconds later and kept on ice. The process was repeated with fresh lavage fluid to enhance cell recovery. Fluid was then centrifuged at 370g for 4 minutes at 4°C and cells resuspended in 500μl of FACS buffer.
2.2.5.3 Spleen harvest

Once mice were sacrificed, the peritoneal cavity of the mouse was opened and the intestines pushed to one side. The spleen was then carefully removed and placed into 5ml of FACS buffer and kept on ice. It was then mashed through a 70μm cell strainer which was flushed with media several times. The flow through was centrifuged at 425g for 3 minutes at 4°C and a RBC lysis performed (Section 2.2.6.1). After washing (x2) with 5ml of FACS buffer, the spleen cell pellet was resuspended in 5ml FACS buffer.

2.2.5.4 Cardiac puncture

Once mice were sacrificed, the peritoneal cavity of the mouse was cut and the ribcage removed to expose the heart. A 21g needle containing 1μl of heparin was carefully inserted into the right ventricle and the blood slowly extracted to avoid collapse of the heart. Acquired blood was kept at room temperature. A RBC lysis was performed (Section 2.2.6.1), cells washed in 1ml of FACS buffer at 272g for 4 minutes at 4°C and later resuspended in 1ml of FACS buffer.
2.2.6 Determining lung, bronchoalveolar lavage, peripheral blood and splenic total cell number

2.2.6.1 Red blood cell lysis

Following cell isolation, red blood cells were lysed to ensure they did not interfere with later analysis or skew total cell number. For the lung, BAL and spleen, cells were resuspended in 2ml of 1X RBC lysis buffer (Biolegend Co, Cambridge, UK) and left for 2 minutes. FACS buffer was then added (3ml) to halt the lysis, and the cells centrifuged at 425g for 3 minutes at 4˚C. Cells were then resuspended in an appropriate volume of FACS buffer. RBC lysis of the peripheral blood required 10ml of 1X RBC lysis buffer. Cells were gently vortexed and left for 15 minutes at RT. FACS buffer (15ml) was then added to stop the lysis reaction and cells centrifuged at 325g for 3 minutes, whereby the supernatant was removed and the procedure repeated if necessary. Cells were then washed in FACS buffer and resuspended.

2.2.6.2 Total cell counts

All cell counts were performed using a Neubauer Haemocytometer and the viability dye, trypan blue (Sigma Aldrich Ltd, Dorset, UK). Live cells actively exclude the dye due to a functioning membrane, compared to dead or dying cells which feature a compromised membrane, resulting in stained cells. The coverslip of the haemocytometer was misted and then immediately placed over the haemocytometer counting chamber. A 1:1 dilution of cell suspension was prepared with trypan blue and used to load the haemocytometer. Cells were then viewed under a microscope at 10x
magnification and the number of viable cells within each of the 4 1mm² areas determined. Cell concentration was calculated using the following formula:

\[
\text{Total Cell Number/ml} = \left( \frac{\text{total No. of cells}}{\text{No. of squares counted}} \right) \times \text{(dilution factor)} \times (\text{chamber volume})
\]

Chamber volume = 1x10⁴

Sample dilution = 2

Squares counted = 4

2.2.6.3 Cryopreservation of cells

Cells to be frozen were centrifuged at 370g for 4 minutes at 4°C. Once counted, they were again centrifuged and resuspended in freezing mix to ensure approximately 1x10⁶ cells/ml. Each 1ml was aliquoted into cryogenic freezing vials and then immediately placed into a Nalgene 5100 Cryo 1°C freezing container to allow for a slow reproducible freezing rate to ensure good cell recovery. This was placed into the -70°C freezer overnight with vials then being transferred to a liquid nitrogen tank. If vials were to be thawed, they were placed into a waterbath at 37°C and gently agitated until completely thawed. Cells were transferred to a 15ml falcon and 10ml of warm RPMI-10 slowly added. Cells were spun and counted to calculate recovery following freezing.
2.2.7 Allergic lung inflammation

2.2.7.1 Sensitisation

The allergen used throughout the study was Ovalbumin (OVA) (VWR International Ltd, Leicestershire, UK), a major protein found in avian egg whites, which has previously been shown to induce sensitisation and allergic pulmonary inflammation. Mice were sensitised via an intra-peritoneal injection with a mix containing OVA (100μg) and aluminium hydroxide (50mg) (Fisher Scientific UK Ltd, Loughborough, UK) in PBS (Life Technologies). The mixture was stirred for at least 2 hours prior to injection to ensure the aluminium hydroxide had completely dissolved and to assist with homogenisation. Mice were injected intra-peritoneally with 0.5ml of the sensitisation mix using a 25g needle on days 0 and 5 unless otherwise stated. All procedures commenced on day 15 for allergic lung inflammation experiments, a method developed by Rodriguez et al (Fernandez-Rodriguez, Ford et al. 2008). Mice were observed following injection, in line with Home Office License 30/2580 regulations.

2.2.7.2 Sensitisation process for aluminium hydroxide dosing

Mice were immunised via an intra-peritoneal injection of 100μg OVA with or without different concentrations of adjuvant, aluminium hydroxide. Naive mice were untreated, whilst the control group were injected with PBS. Aluminium hydroxide concentrations were tested at 0.1mg, 0.4mg, 1mg, 2mg, 10mg and 50mg. The mixtures were stirred for at least 2 hours prior to a single injection, which occurred on day 0. Mice were culled on day 7, weighed, and the necessary organs harvested.
2.2.7.3 Acute allergic lung inflammation

Following sensitisation on days 0 and 5, mice were challenged via inhalation with a 0.5% (w/v) solution of OVA, or in the case of the control groups PBS, on day 15. Mice were challenged twice, 4 hours apart, with each challenge lasting 1 hour. Two challenges were necessary as literature had previously stated that 1 OVA challenge was not sufficient to provoke a pulmonary inflammatory response in mice (Ohkawara, Lei et al. 1997). Exposure was carried out in a Perspex box measuring 38cm x 20cm x 20 cm (Buxco Electronics Inc, Wilmington, NC, USA) attached to a Wright nebuliser (Pulmostar, Devillbiss Healthcare Ltd, Stourbridge, UK), which delivered the OVA or saline at an air pressure of 20lb p.s.i and a rate of 0.3ml/min. Mice were sacrificed 24 or 72 hours post challenge, whereby the lungs and spleen were removed, bronchoalveolar lavage performed and blood extracted to allow for the isolation of serum.

Figure 2.2. The sensitisation and challenge protocol used to induce acute allergic lung inflammation in DR3\textsuperscript{wt} and DR3\textsuperscript{ko} mice. Mice were injected via the peritoneal cavity on days 0 and 5. On day 15, mice underwent two 1 hour inhalation challenges with OVA or PBS, 4 hours apart. Mice were sacrificed either 24 or 72 hr post inhalation challenge.
2.2.7.4 Chronic allergic lung inflammation

Mice were sensitised using the same sensitisation procedure (Section 2.2.7.1), as well as the same timescale (days 0 and 5) as the acute allergic lung inflammation protocol (Section 2.2.7.3). However, the challenge process was different, with mice being exposed to nebulised OVA or saline aerosol 3 days/week for 6 weeks from day 15. The final challenge occurred on day 57, resulting in a total of 18 challenges. Each challenge lasted for 30 minutes, excluding the final challenge which lasted for 1 hour. The chronic protocol used a 2% OVA aerosol, as it was known that tolerance could develop with such repeated exposures (Fernandez-Rodriguez, Ford et al. 2008). Chronic exposures utilised the same equipment as that in the acute protocol. Mice were sacrificed 24 and 72 hours post final inhalation challenge, whereby the lungs and spleen were harvested, bronchoalveolar lavage performed and blood extracted to allow for serum isolation.

Figure 2.3. The sensitisation and challenge protocol used to induce chronic allergic lung inflammation in DR3<sup>wt</sup> and DR3<sup>ko</sup> mice. Mice were injected via the peritoneal cavity on days 0 and 5. On day 15, inhalation challenges began with either OVA or PBS. Challenges occurred 3 days/week for 6 weeks for 30 minutes. The 18<sup>th</sup> and final challenge lasted for 1 hour inhalation. Mice were sacrificed either 24 or 72 hr post final inhalation challenge.
2.2.8 Phenotypic analysis of cells using flow cytometry

2.2.8.1 Preparation of cells for flow cytometry

Cells were processed, resuspended in an appropriate volume of FACS buffer (Section 2.2.5) and total cell number determined (Section 2.2.6.2). Cells (200μl) were then added to the assigned well of a U bottomed 96 well plate. Plates were centrifuged at 483g for 2 minutes at 4°C, whereby the supernatant was removed without disturbing the cell pellet. An FC block (CD16/32) was then applied to all wells at a volume of 50μl/ well, so ensuring that any staining seen was due to specific antibody/cell surface antigen interaction. Plates were left for 15 minutes at 4°C and then spun at 483g for 2 minutes at 4°C. Following this, 50μl of primary conjugated antibodies were added to the designated wells, at predetermined concentrations. Antibodies were incubated at 4°C for 25 minutes to allow for antibody binding, and later washed twice with FACS buffer. Isotype controls and compensation controls were prepared in the same way as test antibodies. Optimum antibody concentrations were determined by earlier titration experiments. If a secondary biotinylated antibody was required, this was added after washing and incubated for 25 minutes at 4°C, followed by 2 washes with FACS buffer. Lastly, cells were resuspended into a 200μl volume and immediately processed using the Beckman Coulter CyAN Flow Cytometer machine (Beckman Coulter Ltd, High Wycombe, UK). For each experiment, an unstained sample was required to set channel voltages, as well as positive single stains to allow for compensation of samples during analysis.
2.2.8.2 DR3 staining for analysis by flow cytometry

DR3 staining was achieved following the steps outlined in Section 2.2.8.1, up to the addition of 50μl of FC Block (CD16/CD32) in diluted FACS buffer. Following this, cells were resuspended in 200μl of FACS buffer containing 2% (v/v) normal goat serum and incubated for 30 minutes at 4°C. Cells were then centrifuged at 483g for 2 minutes at 4°C and the supernatant discarded. The polyclonal biotinylated DR3 antibody (or biotinylated Goat IgG isotype control) along with any other primary antibodies were then added at their optimum concentration in FACS buffer containing 0.5% (v/v) normal goat serum and incubated for 30 minutes at 4°C. The remainder of the steps outlined in Section 2.2.8.1, including the addition of a fluorochrome conjugated secondary antibody, were then followed.

2.2.8.3 Murine antibodies used for flow cytometry

Different antibody panels were used to classify specific cell types within the lung, BAL, peripheral blood and spleen (Table 2.1). Combinations of antibody markers meant that a range of cells from both the myeloid and lymphocytic lineage could be identified and therefore populations defined (Table 2.3).
<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Antibody Dilution</th>
<th>Supplier</th>
<th>Clone</th>
<th>Raised in</th>
<th>Isotype Control</th>
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</thead>
<tbody>
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<td>BD Pharmingen</td>
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<td>1/200</td>
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<td>M1/70</td>
<td>Rat</td>
<td>IgG2b, κ</td>
</tr>
<tr>
<td>NK1.1- FITC</td>
<td>1/200</td>
<td>BD Pharmingen</td>
<td>PK136</td>
<td>Mouse</td>
<td>IgG2a, κ</td>
</tr>
<tr>
<td>CD4 PE</td>
<td>1/200</td>
<td>Invitrogen</td>
<td>RM 4-5</td>
<td>Rat</td>
<td>IgG2a</td>
</tr>
<tr>
<td>CD4 PE Cy5.5</td>
<td>1/200</td>
<td>BD Pharmingen</td>
<td>RM 4-5</td>
<td>Rat</td>
<td>IgG2A, κ</td>
</tr>
<tr>
<td>TCRγδ PE Cy7</td>
<td>1/200</td>
<td>Biolegend</td>
<td>GL3</td>
<td>Hamster</td>
<td>IgG</td>
</tr>
<tr>
<td>CD8 PE Cy7</td>
<td>1/200</td>
<td>Invitrogen</td>
<td>5H10</td>
<td>Rat</td>
<td>Not given</td>
</tr>
<tr>
<td>CD44 V450</td>
<td>1/200</td>
<td>BD Pharmingen</td>
<td>IM7</td>
<td>Rat</td>
<td>IgG2b</td>
</tr>
<tr>
<td>CD4 PE Pacific Orange</td>
<td>1/200</td>
<td>Invitrogen</td>
<td>RM 4-5</td>
<td>Rat</td>
<td>IgG2a</td>
</tr>
<tr>
<td>CD8 PE Pacific Orange</td>
<td>1/200</td>
<td>Invitrogen</td>
<td>5H10</td>
<td>Rat</td>
<td>IgG2b</td>
</tr>
<tr>
<td>TCRB- APC</td>
<td>1/200</td>
<td>BD Pharmingen</td>
<td>H57-597</td>
<td>Hamster</td>
<td>IgG2, λ</td>
</tr>
<tr>
<td>CD3 APC Cy7</td>
<td>1/200</td>
<td>BD Pharmingen</td>
<td>17A2</td>
<td>Rat</td>
<td>IgG2b, κ</td>
</tr>
<tr>
<td>DR3 Biotin</td>
<td>1/100</td>
<td>R&amp;D</td>
<td>polyclonal</td>
<td>Goat</td>
<td>IgG</td>
</tr>
<tr>
<td>Streptavidin PE</td>
<td>1/500</td>
<td>Invitrogen</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>CD16/32</td>
<td>1/200</td>
<td>BD Pharmingen</td>
<td>2.4.G2</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
2.2.8.4 Flow cytometry acquisition

Once stained, cells (including the unstained, isotype and compensation controls) were run through a Beckman Coulter CyAn™ ADP Analyzer Flow Cytometer (Beckman Coulter Ltd, High Wycombe, UK). All analysis and compensation was carried out offline using Summit software v4.2 (Beckman Coulter Ltd, High Wycombe, UK).

2.2.8.5 Multi-parameter flow cytometry compensation

The Beckman Coulter CyAn™ ADP Analyzer Flow Cytometer allows 9 fluorescence markers to be analysed at once. Single stained compensation controls helped ensure that any signal seen was not a result of spectral overlap, or fluorescence emission into a neighbouring channel, resulting in false positives in other detectors. Compensation accounts for a fluorochrome being detected in a channel that was not allocated to measure it, which can be resolved using the compensation function in the summit software, as outlined in the manufacturer’s instructions. Table 2.2 shows the emission spectra for each of the fluorochromes used throughout this study.
Table 2.2. Lasers, Excitation Wavelength and Emission Spectra of fluorochromes used for flow cytometry.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Fluorochrome</th>
<th>Emission Peak (nm)</th>
<th>Laser Light Source (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FL1</td>
<td>FITC</td>
<td>521</td>
<td>488</td>
</tr>
<tr>
<td>FL2</td>
<td>PE</td>
<td>578</td>
<td>488</td>
</tr>
<tr>
<td>FL3</td>
<td>PE Texas Red</td>
<td>615</td>
<td>488</td>
</tr>
<tr>
<td>FL4</td>
<td>PE Cy5/.5</td>
<td>695</td>
<td>488</td>
</tr>
<tr>
<td>FL5</td>
<td>PE Cy7</td>
<td>767</td>
<td>488</td>
</tr>
<tr>
<td>FL6</td>
<td>V450</td>
<td>448</td>
<td>405</td>
</tr>
<tr>
<td>FL7</td>
<td>Pacific Orange</td>
<td>551</td>
<td>405</td>
</tr>
<tr>
<td>FL8</td>
<td>APC</td>
<td>660</td>
<td>660</td>
</tr>
<tr>
<td>FL9</td>
<td>APC Cy7</td>
<td>767</td>
<td>660</td>
</tr>
</tbody>
</table>

2.2.8.6 Defining cell populations using flow cytometry

Once compensated, a live cell FSC/SSC gate was applied to samples, as well as a doublet exclusion gate (Figure 2.5). Cell populations could then be recognised using extracellular cell markers and appropriately drawn ‘gates’ (Table 2.3) (Figure 2.4) (Figure 2.5) (Figure 2.6). Once identified, cell numbers and percentages were calculated relative to the previously determined total cell number.
Figure 2.4. Flow cytometry gating pattern for the identification of myeloid cell subsets. Cells were passed through a flow cytometer and analysed accordingly using monoclonal antibodies to known myeloid cellular markers. (A) Neutrophils (7/4+ Ly6G+ CD11b+), (B) Eosinophils (F4/80+ SSCchi CD11b+), (C) 7/4 Monocytes (F4/80low 7/4 SSClow CD11b+), (D) 7/4 Monocytes (F4/80low 7/4 SSClow CD11b+), (E) Splenic Macrophages (F4/80+ CD11b+), (F) Myeloid Dendritic Cells (CD11b+ CD11c+ MHCII+), (G) Plasmacytoid Dendritic Cells (CD11c+ B220+ CD11b-).
Figure 2.5. Flow cytometry gating pattern for the identification of T and NK cell subsets. Cells were passed through a flow cytometer and analysed accordingly using monoclonal antibodies to known T lymphocyte cellular markers. (A) CD3+ T cells (CD3+ TCRβ+), (B) CD4+ T cells (CD3+ TCRβ+ CD4+), (C) CD8+ T cells (CD3+ TCRβ+ CD8+), (D) NKT cells (NK1.1+ CD3+ TCRβ+), (E) γδ T cells (CD3+ TCRγδ+), (F) NK cells (NK1.1+ CD3+ TCRβ-).
Figure 2.6. Flow cytometry gating pattern for the identification of B cells. Cells were passed through a flow cytometer and analysed accordingly using monoclonal antibodies to known lymphocyte cellular markers. B cells (CD11b<sup>low</sup> B220<sup>+</sup> MHCII<sup>+</sup>).
Table 2.3. Cell markers used to phenotype the stated cells within the lung, BAL, spleen and peripheral blood samples.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Cell Markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alveolar Macrophages</td>
<td>CD11b$^{low}$ CD11c$^+$</td>
</tr>
<tr>
<td>Splenic Macrophages</td>
<td>F4/80$^+$ CD11b$^{low}$</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>7/4$^+$ Ly6G$^+$ CD11b$^+$</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>F4/80$^+$ SSC$^{hi}$ CD11b$^+$</td>
</tr>
<tr>
<td>7/4$^+$ Monocytes</td>
<td>F4/80$^{low}$ 7/4$^+$ SSC$^{low}$ CD11b$^+$</td>
</tr>
<tr>
<td>7/4$^-$ Monocytes</td>
<td>F4/80$^{low}$ 7/4$^-$ SSC$^{low}$ CD11b$^+$</td>
</tr>
<tr>
<td>Myeloid Dendritic Cells</td>
<td>CD11b$^+$ CD11c$^+$ MHCII$^+$</td>
</tr>
<tr>
<td>Plasmacytoid Dendritic Cells</td>
<td>CD11c$^-$ B220$^-$ CD11b$^+$</td>
</tr>
<tr>
<td>B cells</td>
<td>CD11b$^-$ B220$^+$ MHCII$^+$</td>
</tr>
<tr>
<td>CD4$^+$ T cells</td>
<td>CD3$^+$ TCRβ$^+$ CD4$^+$</td>
</tr>
<tr>
<td>CD8$^+$ T cells</td>
<td>CD3$^+$ TCRβ$^+$ CD8$^+$</td>
</tr>
<tr>
<td>NK Cells</td>
<td>NK1.1$^+$ CD3$^+$ TCRβ$^+$</td>
</tr>
<tr>
<td>NKT Cells</td>
<td>NK1.1$^+$ CD3$^+$ TCRβ$^+$</td>
</tr>
<tr>
<td>γδ T cells</td>
<td>CD3$^+$ γδTCR$^+$</td>
</tr>
</tbody>
</table>
2.2.9 Enzyme Linked Immunosorbent Assay (ELISA)

A murine Total IgE ELISA kit was purchased from Biolegend (Biolegend Inc, Cambridge, UK). Human TL1A ELISA was purchased from Peprotech (Peprotech EC Ltd, London UK). All other ELISAs were purchased from R&D systems unless otherwise stated and were carried out in accordance with manufacturer’s instructions (Table 2.4). The OVA specific IgG ELISA was developed in-house using individual antibodies.

2.2.9.1 Kit ELISAs

ELISAs were performed according to the manufacturer’s instructions. Briefly a 96 well plate was coated with a 100μl of capture antibody in PBS at a specified concentration. This was left overnight at 4°C. Plates were washed the following day with Wash Buffer (x4), before the addition of 200μl of Blocking Reagent (1% BSA PBS). Block was left for at least 1 hour at RT, at which point plates were once again washed (x4) and diluted sample and standard were added to the appropriate wells in duplicate. Samples were left for 2 hours at RT, followed by another washing cycle and the addition of 100μl of biotinylated detection antibody, which was left for 2 hours at RT. Plates were once again washed to remove any unbound antibody and Streptavidin HRP was added for 30 minutes at RT. This was followed by a stringent cycle of 5 washes, with wells being soaked in wash buffer for 30 seconds to 1 minute to minimise background readings. Freshly prepared 3,3′,5,5′-Tetramethylbenzidine (TMB) was then added to the wells and incubated in the dark until colour change occurred. The reaction was stopped using
Sulphuric acid (H₂SO₄) (2M), turning wells from blue to yellow. Absorbance was read at 450nm using a FLUOstar microplate reader (Omega BMG Labtech Ltd, Buckinghamshire, UK). Sample concentration was calculated via reference to the standard curve, and multiplied by the dilution factor of the sample.

Table 2.4. ELISA assays and working antibody concentrations.

<table>
<thead>
<tr>
<th>Target</th>
<th>Capture antibody conc.</th>
<th>Detection antibody conc.</th>
<th>Top standard</th>
<th>Supplier</th>
<th>Product Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>TL1A</td>
<td>1µg/ml</td>
<td>500ng/ml</td>
<td>4000 pg/ml</td>
<td>Peprotech</td>
<td>900-K290</td>
</tr>
<tr>
<td>IgE</td>
<td>-</td>
<td>-</td>
<td>10,000 pg/ml</td>
<td>Biolegend</td>
<td>432405</td>
</tr>
<tr>
<td>CXCL1 (KC)</td>
<td>2.0 µg/mL</td>
<td>200 ng/mL</td>
<td>1000 pg/mL</td>
<td>RnD Systems</td>
<td>DY453</td>
</tr>
<tr>
<td>CXCL2 (MIP-2)</td>
<td>2.0 µg/mL</td>
<td>75 ng/mL</td>
<td>1000 pg/mL</td>
<td>RnD Systems</td>
<td>DY452</td>
</tr>
<tr>
<td>CXCL10 (IP-10)</td>
<td>2.0 µg/mL</td>
<td>600 ng/mL</td>
<td>4000 pg/mL</td>
<td>RnD Systems</td>
<td>DY446</td>
</tr>
<tr>
<td>CXCL13 (BCA-1)</td>
<td>1.0 µg/mL</td>
<td>200 ng/mL</td>
<td>1000 pg/mL</td>
<td>RnD Systems</td>
<td>DY470</td>
</tr>
<tr>
<td>CCL3 (MIP-1α)</td>
<td>0.4 µg/mL</td>
<td>100 ng/mL</td>
<td>500 pg/mL</td>
<td>RnD Systems</td>
<td>DY450</td>
</tr>
<tr>
<td>CCL4 (MIP-1β)</td>
<td>4.0 µg/mL</td>
<td>800 ng/mL</td>
<td>1000 pg/mL</td>
<td>RnD Systems</td>
<td>DY451</td>
</tr>
<tr>
<td>CCL5 (RANTES)</td>
<td>2.0 µg/mL</td>
<td>400 ng/mL</td>
<td>2000 pg/mL</td>
<td>RnD Systems</td>
<td>DY478</td>
</tr>
</tbody>
</table>
2.2.9.2 OVA specific IgG ELISA

Levels of OVA specific IgG were measured using an indirect ELISA method. Briefly, 100μl of 100μg/ml OVA (w/v) in PBS was plated per well of a 96 well plate. This was left at 4°C overnight. Wells were then washed with Wash Buffer 4 times to wash away any unbound protein. Plates were blocked with 200μl of 5% non fat milk (w/v) in PBS for 2 hours at RT. Following washes (x4), 100μl of PBS diluted serum was added to the wells at a concentration of 1/1000 and left at room temperature for 2 hours. Unbound sample was removed following a wash cycle, after which goat anti-mouse IgG detection antibody (Biolegend Inc, Cambridge, UK) was added at a concentration of 20ng/ml in 100μl of PBS. This was left for 2 hours at RT, before a further 4 washes and the addition of Streptavidin HRP (R&D Systems Ltd, Abingdon, UK), at a dilution of 1/200 in PBS. After 30 minutes at RT, wells were washed thoroughly 5 times with Wash buffer, after which TMB (Becton Dickenson Ltd, Oxford, UK) was added, and the ELISA was allowed to develop. When judged to have developed, Sulphuric acid (2M) (Fisher Scientific UK Ltd, Loughborough, UK) was added to stop the reaction and the absorbance determined at 450nm. Without a standard curve, the ELISA was deemed to have worked by varying the dilution of serum by half, so reducing the absorbance by approximately half. If a dose response relationship was seen between optical density levels and the dilution titer of the OVA IgG positive standard serum, the ELISA was classed to have worked. This positive reaction was not seen in the absence of the capture antibody (OVA), serum, or the detection antibody (anti mouse IgG), with these readings used as blanks to indicate background sensitivity. Once achieved, the serum dilution (1/1000) was decided upon for experimental use.
2.2.10 Murine splenocyte tissue culture

2.2.10.1 In vitro proliferation assay

Splenocytes (1x10^5/well) were plated in a flat bottomed 96 well plate in RPMI-10 containing 50mM β mercaptoethanol. Cells were then either left untreated or stimulated with 10μg/ml Ovalbumin, 50μg/ml Ovalbumin or 10μg/ml Concanavalin A. Stimulations were performed in triplicate for each sample. Plates were kept at 37°C 5% CO₂ for 48 hours. Following this, 100μl of supernatant was removed and ³H-thymidine (Perkins Elmer Inc, Cambridge, UK) was added at a concentration of 0.37M bq/well. Cells were pulsed for 18 hours at 37°C 5% CO₂, at which point plates were frozen for later analysis or harvested immediately onto filtermats (Perkins Elmer Inc, Cambridge, UK) using a Tomtec Cell Harvester (Tomtec Inc, Connecticut, USA). Scintillant (Perkins Elmer Inc, Cambridge, UK) was melted onto the radioactive filter mats and ³H-thymidine incorporation was measured using a Micro β Counter (Perkins Elmer Inc, Cambridge, UK). Counts were automatically corrected to account for quenching. Filtermats were then disposed of in line with radiation regulations.

2.2.10.2 Spleen lysate

Spleens were removed from the mouse, placed into an eppendorf and snap frozen using liquid nitrogen. They were then homogenised in 1ml of ice cold protein lysis buffer containing phenylmethylsulfonyl fluoride (PMSF) and a cocktail of protease inhibitors (Roche). Homogenisation was performed using an T10 basic homogeniser (IKA Werke
GMBH & Co, Staufen, Germany). Spleens were then spun at 483g for 5 minutes at 4°C and the supernatant removed. Supernatant was stored at -20°C for analysis by ELISA.

2.2.11 Organ fixing for histological analysis

2.2.11.1 Lung fixing for paraffin embedding

Once lungs were harvested, they were placed into individually labelled tissue histology cassettes, which were subsequently placed into a bottle of 10% neutral buffered formalin (Sigma Aldrich Ltd, Dorset, UK). Tissue was left at 4°C for at least 24 hours to allow the formalin to penetrate and thereby ‘fix’ the whole lung.

2.2.11.2 Paraffin embedding

Lungs were embedded in paraffin wax via an automated Shandon Tissue Processor (Fisher Scientific UK Ltd, Loughborough, UK) (Table 2.5). Lungs underwent increasing cycles of alcohol concentrations and xylene, in order to hydrate the samples, followed by permeation with molton wax (60°C). Samples remained in wax until the machine was manually drained, and immediately transferred to the Shandon Histocentre (Fisher Scientific UK Ltd, Loughborough, UK), used for embedding samples in wax blocks. Lung samples were placed into metal histology cassettes, where molton wax was poured into the moulds and left to set on a cold plate.
Table 2.5. Shandon Tissue Processor cycle to assist in the fixing and subsequently the paraffin embedding of murine lung tissue.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Time</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>70% Alcohol empty tank</td>
<td>30 minutes</td>
<td>Room temperature</td>
</tr>
<tr>
<td>90% Alcohol empty tank</td>
<td>60 minutes</td>
<td>Room temperature</td>
</tr>
<tr>
<td>100% Alcohol empty tank</td>
<td>60 minutes</td>
<td>Room temperature</td>
</tr>
<tr>
<td>100% Alcohol empty tank</td>
<td>60 minutes</td>
<td>Room temperature</td>
</tr>
<tr>
<td>100% Alcohol empty tank</td>
<td>60 minutes</td>
<td>Room temperature</td>
</tr>
<tr>
<td>100% Alcohol empty tank</td>
<td>60 minutes</td>
<td>Room temperature</td>
</tr>
<tr>
<td>Xylene empty tank</td>
<td>60 minutes</td>
<td>37°C</td>
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<td>Xylene empty tank</td>
<td>60 minutes</td>
<td>37°C</td>
</tr>
<tr>
<td>Xylene empty tank</td>
<td>60 minutes</td>
<td>45°C</td>
</tr>
<tr>
<td>Wax empty tank</td>
<td>90 minutes</td>
<td>60°C</td>
</tr>
<tr>
<td>Wax empty tank</td>
<td>60 minutes</td>
<td>60°C</td>
</tr>
<tr>
<td>Wax empty tank</td>
<td>60 minutes</td>
<td>60°C</td>
</tr>
<tr>
<td>Wax empty tank</td>
<td>60 minutes</td>
<td>60°C</td>
</tr>
</tbody>
</table>
2.2.11.3 Microtome sectioning

Embedded lungs were stored at -20°C for at least 24 hours prior to sectioning to allow for easier cutting. All tools used during the procedure were also kept on ice. Lungs were sectioned at 5-7μm using a microtome. Cut sections were placed into distilled water heated to 60°C and then transferred onto neutrally charged superfrost slides. Slides were incubated overnight at 60°C to dry and later stored at room temperature until required.

2.2.12 Histological analysis of murine lung

Sections were stained with Haematoxylin and Eosin (Sigma Aldrich Ltd, Dorset, UK), Periodic Acid Schiff (Leica Biosystems Ltd, Peterborough, UK) and Van Gieson Solution (Sigma Aldrich Ltd, Dorset, UK) to help evaluate the severity of lung inflammation. Following each of the stains, DPX mountant media (Sigma Aldrich Ltd, Dorset, UK) was added to the slides, and a coverslip placed on top ensuring no air bubbles were trapped underneath. Photographs of slides were taken using an Olympus Camedia C-3030 Digital Camera (Olympus UK) attached to a Olympus BX41 microscope (Olympus, UK), at various magnifications.
2.2.12.1 Haematoxylin and Eosin staining protocol

A Haematoxylin and Eosin stain was used to look at general lung morphology, so allowing differentiation between treatment groups (Table 2.6) (Figure 2.7).

Table 2.6. Protocol used for the Haematoxylin and Eosin staining of murine lung.

<table>
<thead>
<tr>
<th>Process</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xylene</td>
<td>5 minutes</td>
</tr>
<tr>
<td>Xylene</td>
<td>5 minutes</td>
</tr>
<tr>
<td>Xylene</td>
<td>5 minutes</td>
</tr>
<tr>
<td>IMS 100%</td>
<td>3 minutes</td>
</tr>
<tr>
<td>IMS 100%</td>
<td>3 minutes</td>
</tr>
<tr>
<td>IMS 90%</td>
<td>3 minutes</td>
</tr>
<tr>
<td>IMS 70%</td>
<td>3 minutes</td>
</tr>
<tr>
<td>Running tap water</td>
<td>5 minutes</td>
</tr>
<tr>
<td>Rinse in distilled water</td>
<td>-</td>
</tr>
<tr>
<td>Harris Haematoxylin</td>
<td>90 seconds</td>
</tr>
<tr>
<td>Running tap water</td>
<td>5 minutes</td>
</tr>
<tr>
<td>Dip in 1X Scotts tap water</td>
<td>10 seconds</td>
</tr>
<tr>
<td>Running tap water</td>
<td>5 minutes</td>
</tr>
<tr>
<td>Rinse in distilled water</td>
<td>-</td>
</tr>
<tr>
<td>1% Eosin</td>
<td>90 seconds</td>
</tr>
<tr>
<td>Running tap water</td>
<td>5 minutes</td>
</tr>
<tr>
<td>Rinse in distilled water</td>
<td>-</td>
</tr>
<tr>
<td>IMS 90%</td>
<td>3 minutes</td>
</tr>
<tr>
<td>IMS 100%</td>
<td>3 minutes</td>
</tr>
<tr>
<td>IMS 100%</td>
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</tr>
<tr>
<td>Xylene</td>
<td>5 minutes</td>
</tr>
<tr>
<td>Xylene</td>
<td>5 minutes</td>
</tr>
</tbody>
</table>
Figure 2.7. A DR3<sup>wt</sup> treated chronic ovalbumin challenged mouse lung stained with Haematoxylin and Eosin (Section 2.2.7.4) (Section 2.2.12.1). The stain was used for general lung morphology analysis. Scale bar=100μm.
2.2.12.2 Haematoxylin and Eosin analysis

Haematoxylin and Eosin stained sections were used for qualitative and semi-quantitative analysis, with a score being applied to each treated mouse depending on the number of inflammatory cells present in the peribronchiolar area (the periphery of the airways). Slides from each treatment protocol were assessed by eye by 2 blind scorers according to the following scoring regime below (Figure 2.8).

0= normal lung

1= minor perivascular inflammation and inflammatory cell infiltration.

2= slight perivascular inflammation and peribronchiolar inflammation.

3= moderate peribronchiolar inflammation and perivascular and airway cuffing.

4= marked peribronchial and perivascular inflammation and cuffing.

5= severe peribronchial and perivascular inflammation and cuffing (almost solid lungs).

Scores were then averaged for each mouse and graphed appropriately.
Figure 2.8. Representative examples of the scoring regime for Haematoxylin and Eosin stained lungs. Lungs were scored from 0 (normal lung) to 5 (severely inflamed lung) and scores averaged for each lung. Scale bar=250μm.
2.2.12.3 Periodic Acid Schiff staining protocol

A Periodic Acid Schiff (PAS) stain was used to look at the presence of mucus and goblet cells in the lung following challenge (Table 2.7) (Figure 2.9).

Table 2.7. Protocol used for the Periodic Acid Schiff staining of murine lung.

<table>
<thead>
<tr>
<th>Process</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xylene</td>
<td>5 minutes</td>
</tr>
<tr>
<td>Xylene</td>
<td>5 minutes</td>
</tr>
<tr>
<td>Xylene</td>
<td>5 minutes</td>
</tr>
<tr>
<td>IMS  100%</td>
<td>3 minutes</td>
</tr>
<tr>
<td>IMS  100%</td>
<td>3 minutes</td>
</tr>
<tr>
<td>IMS  90%</td>
<td>3 minutes</td>
</tr>
<tr>
<td>IMS  70%</td>
<td>3 minutes</td>
</tr>
<tr>
<td>Running tap water</td>
<td>5 minutes</td>
</tr>
<tr>
<td>Rinse in distilled water</td>
<td>-</td>
</tr>
<tr>
<td>Periodic Acid</td>
<td>5 minutes</td>
</tr>
<tr>
<td>Running tap water</td>
<td>5 minutes</td>
</tr>
<tr>
<td>Schiff’s Reagent</td>
<td>10 minutes</td>
</tr>
<tr>
<td>Running tap water</td>
<td>8 minutes</td>
</tr>
<tr>
<td>Rinse in distilled water</td>
<td>-</td>
</tr>
<tr>
<td>Harris Haematoxylin</td>
<td>30 seconds</td>
</tr>
<tr>
<td>Running tap water</td>
<td>5 minutes</td>
</tr>
<tr>
<td>Rinse in distilled water</td>
<td>-</td>
</tr>
<tr>
<td>IMS  90%</td>
<td>3 minutes</td>
</tr>
<tr>
<td>IMS  100%</td>
<td>3 minutes</td>
</tr>
<tr>
<td>IMS  100%</td>
<td>3 minutes</td>
</tr>
<tr>
<td>Xylene</td>
<td>5 minutes</td>
</tr>
<tr>
<td>Xylene</td>
<td>5 minutes</td>
</tr>
<tr>
<td>Xylene</td>
<td>5 minutes</td>
</tr>
</tbody>
</table>
Figure 2.9. A DR3\textsuperscript{wt} treated chronic ovalbumin challenged mouse lung stained with Periodic Acid Schiff Stain (Section 2.2.7.4) (Section 2.2.12.3). The stain was used to highlight mucin associated goblet cells. Goblet cells stain bright pink as shown above. Scale bar=100μm.

2.2.12.4 Periodic Acid Schiff analysis

The number of mucin associated goblet cells was quantified using Leica Qwin V3 software (Leica Biosystems Ltd, Peterborough, UK). The airway area was drawn manually and the goblet cells within the area identified based upon their bright pink staining. The airway spaces were then identified and the % of goblet cells calculated as a % of the airway. The average goblet cell % was then calculated for each mouse.
2.2.12.5 Van Gieson solution staining protocol

A Van Gieson stain was used to look at collagen presence, as well as smooth muscle in challenged murine lungs (Table 2.8) (Figure 2.10).

Table 2.8. Protocol used for a Van Gieson stain on murine lung.

<table>
<thead>
<tr>
<th>Process</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xylene</td>
<td>5 minutes</td>
</tr>
<tr>
<td>Xylene</td>
<td>5 minutes</td>
</tr>
<tr>
<td>Xylene</td>
<td>5 minutes</td>
</tr>
<tr>
<td>IMS 100%</td>
<td>3 minutes</td>
</tr>
<tr>
<td>IMS 100%</td>
<td>3 minutes</td>
</tr>
<tr>
<td>IMS 90%</td>
<td>3 minutes</td>
</tr>
<tr>
<td>IMS 70%</td>
<td>3 minutes</td>
</tr>
<tr>
<td>Running tap water</td>
<td>5 minutes</td>
</tr>
<tr>
<td>Rinse in distilled water</td>
<td></td>
</tr>
<tr>
<td>Celestine Blue</td>
<td>5 minutes</td>
</tr>
<tr>
<td>Running tap water</td>
<td>5 minutes</td>
</tr>
<tr>
<td>Rinse in distilled water</td>
<td></td>
</tr>
<tr>
<td>Harris Haematoxylin</td>
<td>5 minutes</td>
</tr>
<tr>
<td>Running tap water</td>
<td>5 minutes</td>
</tr>
<tr>
<td>Rinse in distilled water</td>
<td></td>
</tr>
<tr>
<td>Van Gieson Solution</td>
<td>5 minutes</td>
</tr>
<tr>
<td>Rinse in distilled water</td>
<td></td>
</tr>
<tr>
<td>IMS 90%</td>
<td>3 minutes</td>
</tr>
<tr>
<td>IMS 100%</td>
<td>3 minutes</td>
</tr>
<tr>
<td>IMS 100%</td>
<td>3 minutes</td>
</tr>
<tr>
<td>Xylene</td>
<td>5 minutes</td>
</tr>
<tr>
<td>Xylene</td>
<td>5 minutes</td>
</tr>
<tr>
<td>Xylene</td>
<td>5 minutes</td>
</tr>
</tbody>
</table>
Figure 2.10. A DR3<sup>wt</sup> treated chronic ovalbumin challenged mouse lung stained with Van Gieson Solution (Section 2.2.7.4) (Section 2.2.12.5). The stain was used to display collagen, which stains bright red as shown above. Scale bar=100μm.
2.2.12.6 Van Gieson stain analysis

Levels of collagen, as identified by the Van Gieson stain, change during allergic lung inflammation as airway remodelling occurs, and consequently pulmonary fibrosis. The severity of this pulmonary fibrosis was assessed using a numerical scale known as the ‘Ashcroft Score’ (Ashcroft, Simpson et al. 1988). Photos were taken of each slide, with each photo being scored. The average score of these photos was then calculated to give an overall measure of fibrosis. Slides from each treatment protocol were assessed by eye by 2 blind scorers according to the following scoring regime (Figure 2.11).

0= normal lung

1= minimal fibrosis thickening of alveolar/bronchial walls

3= moderate thickening of walls without obvious damage to lung architecture

5= increased fibrosis with definite damage to lung architecture and formation of fibrosis bonds/ fibrosis masses

7= severe distortion of architecture and large fibrosis area

8= total fibrosis obliteration of field
Figure 2.11. Representative examples of the Ashcroft scoring regime for Van Gieson stained lungs. Scores run from 0 (normal lung) to maximum of 5 (fibrosis with loss of lung architecture) for this project, therefore there were no examples of score 6-8 in my dataset (Ashcroft, Simpson et al. 1988). Scores were averaged for each lung. Scale bar=250μm.
2.2.12.7 DR3 immunohistochemistry upon paraffin fixed lungs

Once stained, sections were mounted with DPX mounting media and photographs taken using an Olympus Camedia C-3030 Digital Camera (Table 2.9) (Figure 2.12).
Table 2.9. Protocol used for DR3 immunohistochemistry using murine tissue.

<table>
<thead>
<tr>
<th>Location</th>
<th>Process</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fume Hood</td>
<td>Xylene</td>
<td>5 minutes</td>
</tr>
<tr>
<td></td>
<td>Xylene</td>
<td>5 minutes</td>
</tr>
<tr>
<td></td>
<td>IMS 100%</td>
<td>3 minutes</td>
</tr>
<tr>
<td></td>
<td>IMS 100%</td>
<td>3 minutes</td>
</tr>
<tr>
<td></td>
<td>IMS 90%</td>
<td>3 minutes</td>
</tr>
<tr>
<td></td>
<td>IMS 70%</td>
<td>3 minutes</td>
</tr>
<tr>
<td></td>
<td>Running tap water</td>
<td>5 minutes</td>
</tr>
<tr>
<td></td>
<td>Blot excess water</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Peroxidase Block</td>
<td>5 minutes</td>
</tr>
<tr>
<td>Humidity chamber</td>
<td>TBS wash</td>
<td>5 minutes</td>
</tr>
<tr>
<td></td>
<td>Serum Blocking reagent D</td>
<td>15 minutes</td>
</tr>
<tr>
<td></td>
<td>Avidin Block</td>
<td>15 minutes</td>
</tr>
<tr>
<td></td>
<td>Biotin Block</td>
<td>15 minutes</td>
</tr>
<tr>
<td></td>
<td>TBS wash</td>
<td>5 minutes</td>
</tr>
<tr>
<td></td>
<td>Goat anti mDR3- biotin/goat IgG control</td>
<td>2 hours</td>
</tr>
<tr>
<td></td>
<td>(20ug/ml)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3x TBS wash</td>
<td>5 minutes</td>
</tr>
<tr>
<td></td>
<td>HSS-HRP</td>
<td>30 minutes</td>
</tr>
<tr>
<td></td>
<td>3 x TBS wash</td>
<td>2 minutes</td>
</tr>
<tr>
<td></td>
<td>DAB</td>
<td>5 minutes</td>
</tr>
<tr>
<td></td>
<td>Running tap water</td>
<td>5 minutes</td>
</tr>
<tr>
<td></td>
<td>Harris Haematoxylin</td>
<td>90 seconds</td>
</tr>
<tr>
<td>Fume Hood</td>
<td>Running tap water</td>
<td>5 minutes</td>
</tr>
<tr>
<td></td>
<td>90% IMS</td>
<td>3 minutes</td>
</tr>
<tr>
<td></td>
<td>100% IMS</td>
<td>3 minutes</td>
</tr>
<tr>
<td></td>
<td>100% IMS</td>
<td>3 minutes</td>
</tr>
<tr>
<td></td>
<td>Xylene</td>
<td>5 minutes</td>
</tr>
<tr>
<td></td>
<td>Xylene</td>
<td>5 minutes</td>
</tr>
<tr>
<td></td>
<td>Xylene</td>
<td>5 minutes</td>
</tr>
</tbody>
</table>
2.2.12.8 DR3 immunohistochemistry analysis

The % of DR3 was quantified using Leica Qwin V3 software (Leica Biosystems Ltd, Peterborough, UK). DR3 was identified within the perimeter of the photo based upon the brown DAB staining. The % of DR3 was then calculated as a % of the lung area shown. The average DR3% was then calculated for each mouse.

Figure 2.12. A DR3wt treated chronic ovalbumin challenged mouse lung stained with DR3 (Section 2.2.7.4) (Section 2.2.12.7). DR3 identified as the brown staining. Scale bar=100μm.
2.3 Statistical analysis

All results were graphed as the mean ± the standard error of the mean (SEM) and were analysed using GraphPad Prism v5. P values of <0.05 were considered significant. Various statistical tests have been used within the study, including an Unpaired T Test for data that could be assumed to be normally distributed and 1 way and 2 way ANOVA’s, to compare more than 2 groups or variables, followed by a Bonferroni post test.
Chapter 3:

Characterisation of leukocyte subsets within the lung, spleen and peripheral blood of naive DR3 mice
3.1 Introduction

Mice deficient in death receptor 3 (DR3) were generated by Wang et al. (Wang, Thern et al. 2001), so allowing the in vivo functions of DR3 to be studied. Complete lack of DR3 was guaranteed by replacing the whole gene with a cassette, so ensuring that none of the 3 murine DR3 splice variants were expressed. It was found that DR3 had a non-redundant role in TCR induced apoptosis as an impairment in the negative selection of thymocytes was noted (Wang, Thern et al. 2001). The generation of DR3$^{ko}$ mice has provided important advances in discovering the role of the receptor in various inflammatory models, such as regulating the expansion of effector T cells in the gut (Meylan, Song et al. 2011), enhancing joint disease in models of arthritis (Bull, Williams et al. 2008), and mediating acute allergic lung inflammation (Meylan, Davidson et al. 2008). The lung is known to predominantly possess alveolar macrophages as well as CD3$^+$ lymphocytes (van Rijt, Kuipers et al. 2004). Following the induction of acute allergic lung inflammation, numbers of CD4$^+$ lymphocytes, eosinophils and NKT cells also increase (Fang, Adkins et al. 2008; Meylan, Davidson et al. 2008) in DR3$^{wt}$ mice in comparison to mice lacking DR3 expression and function.

Despite the receptor already being implicated in immune regulation (Migone, Zhang et al. 2002), and more specifically, acute allergic lung inflammation (Fang, Adkins et al. 2008; Meylan, Davidson et al. 2008), the influence of DR3 upon resident lung cells in the naive state was unknown. With this in mind, initial baseline experiments were conducted to ensure that DR3$^{ko}$ mice suffered no defect in the homeostatic trafficking of leukocytes within the lung or general lung make-up, prior to the induction of allergic lung inflammation.
In addition, as the majority of cells are recruited from the peripheral blood following an inflammatory stimulus, (Anderson and Anderson 1976; Lukacs, Strieter et al. 1995), detailed baseline measurements were undertaken to ensure that DR3\textsuperscript{ko} mice suffered no impairment in leukocyte number. Furthermore, the cellular compositions of DR3\textsuperscript{wt} and DR3\textsuperscript{ko} spleens provided an added insight into the workings of the immune response in these mice.

Upon commencement of the project, expression patterns of DR3 within an un-sensitised murine lung were unknown, although a recently marketed antibody had proved successful in detecting murine DR3 on CD8\textsuperscript{+} splenocytes (Twohig, Marsden et al. 2012). The degree of receptor expression in steady state conditions was required to enable any differences to be identified when inflammation was induced, thereby providing insight to the way in which DR3 is regulated. DR3 levels were also investigated on numerous leukocyte cell types from the lung and spleen, to aid in the identification of cells that were potentially responsive to TL1A.

Airway inflammation is one of many defining hallmarks of asthma, as is airway hyper-responsiveness, although these have never been measured in DR3 mice. Therefore, an attempt was made to quantify these features in DR3\textsuperscript{ko} mice at a baseline level to establish whether any inherent differences existed between DR3\textsuperscript{wt} and DR3\textsuperscript{ko} mice in basic lung function and airway hyper-responsiveness.
3.2 Aims

The aim of this Chapter was to phenotypically determine the composition of both myeloid and lymphocytic cells in the naive lung cavities, airway passages, peripheral blood and spleens of DR3^{ko} mice and so determine whether leukocyte subset numbers varied compared to their WT counterpart prior to immune challenge. DR3 expression was also quantified within the lung and upon various cell types within the lung and spleen using a recently marketed antibody. Furthermore, lung function measurements were assessed in DR3^{ko} mice to determine whether they suffered any deficiencies or difficulties in response to broncho-constricting agents as well as at baseline level.

3.2.1 Objectives

i) Characterisation of the lungs of DR3^{wt} and DR3^{ko} mice.

ii) Phenotypic characterisation of the spleens of DR3^{wt} and DR3^{ko} mice.

iii) Quantifying DR3 expression within the lungs and spleen of DR3^{wt} and DR3^{ko} mice

iv) Phenotypic characterisation of the peripheral blood of DR3^{wt} and DR3^{ko} mice.
3.3 Results

3.3.1 Characterisation of the naive lungs of DR3\textsuperscript{wt} and DR3\textsuperscript{ko} mice

3.3.1.1 Phenotypic analysis of cells within the lungs of DR3\textsuperscript{wt} and DR3\textsuperscript{ko} mice

3.3.1.1.1 Total cell number from the lungs of unchallenged DR3\textsuperscript{wt} and DR3\textsuperscript{ko} mice did not differ

Lungs from DR3\textsuperscript{wt} and DR3\textsuperscript{ko} mice were perfused and the cells extracted using collagenase lung digestion (Section 2.2.5.1). To investigate whether DR3 affected total leukocyte number within the naive lung, cells were counted using a haemocytometer (Section 2.2.6). Results showed that DR3\textsuperscript{ko} mice (9.7±0.5 x10\textsuperscript{5}) did not significantly differ in total leukocyte number compared to DR3\textsuperscript{wt} mice (9.4±1 x10\textsuperscript{5}) (\textit{p}=0.570) (Figure 3.1).

3.3.1.1.2 There were no significant baseline differences between DR3\textsuperscript{ko} and DR3\textsuperscript{wt} lung derived myeloid or lymphocytic cells

Although the loss of DR3 had no effect on lung total cell number, individual subsets were analysed to examine whether DR3 had any role in maintaining these cell populations. Cells were analysed using flow cytometry (Section 2.2.8) and an extensive panel of antibodies to identify a range of myeloid cells (CD11b, CD11c, Ly6G, 7/4, F4/80 and FSC/SSC properties) and lymphocytic cells (CD3, CD4, CD8, TCRβ, B220, NK1.1 and FSC/SSC properties) via well characterised extracellular markers (Table 2.3).
(Figure 2.4) (Figure 2.5) (Figure 2.6). No significant differences were found between naive DR3^ko and DR3^wt mice in any leukocyte cell subset considered (Table 3.1).
Figure 3.1. Total lung leukocyte cell number in naive DR3\textsuperscript{wt} and DR3\textsuperscript{ko} mice. Lung cells were extracted via collagenase digestion and counted using a haemocytometer with trypan blue. Data is representative of 2 individual experiments. Each symbol represents data from a single mouse. Results showed no significant differences using an Unpaired T test.
3.3.1.2 Phenotypic analysis of cells within the alveolar passage of DR3<sup>wt</sup> and DR3<sup>ko</sup> mice

3.3.1.2.1 Total cell numbers from the alveolar passage of unchallenged DR3<sup>wt</sup> and DR3<sup>ko</sup> mice did not significantly differ

Cells of the bronchial passage were isolated from unchallenged DR3<sup>wt</sup> and DR3<sup>ko</sup> mice via a lavage. Cells were counted using a haemocytometer to determine total leukocyte number (Section 2.2.6.2). Statistical analysis showed that there were no significant differences in alveolar leukocytes isolated from DR3<sup>wt</sup> (2.2±0.7 x10<sup>5</sup>) and DR3<sup>ko</sup> (2.2±0.6 x10<sup>5</sup>) (p=0.972) mice (Figure 3.2).

3.3.1.2.2 There were no significant baseline differences between DR3<sup>ko</sup> and DR3<sup>wt</sup> airway derived myeloid or lymphocyte derived cells

To investigate the effects of DR3 loss upon cells of the alveolar passage, cells were stained with a broad panel of antibodies and analysed using flow cytometry. Results identified alveolar macrophages (defined as CD11b<sup>low</sup> CD11c<sup>+</sup>) as making up the majority of myeloid cells within the BAL fluid, although numbers were comparable between DR3<sup>wt</sup> (11.8±4.9 x10<sup>4</sup>) and DR3<sup>ko</sup> (14.8±5.2 x10<sup>4</sup>) (p=0.653) mice (Table 3.1). Following suit, all other cell types examined of both myeloid and lymphocyte lineage presented similar numbers between DR3<sup>wt</sup> and DR3<sup>ko</sup> mice (Table 3.1).
Figure 3.2. Total BAL leukocyte number isolated from naive DR3<sup>wt</sup> and DR3<sup>ko</sup> mice. Cells were isolated via a bronchoalveolar lavage and counted using a haemocytometer with trypan blue. Data is representative of 2 individual experiments. Each symbol represents data from a single mouse. Results showed no significant differences using an Unpaired T test.
### Table 3.1. Comparison of cell numbers isolated from the lung and BAL of DR3<sup>wt</sup> and DR3<sup>ko</sup> mice

<table>
<thead>
<tr>
<th>Cell Subset</th>
<th>DR3&lt;sup&gt;wt&lt;/sup&gt;</th>
<th>DR3&lt;sup&gt;ko&lt;/sup&gt;</th>
<th>Significance&lt;sup&gt;$&lt;/sup&gt;</th>
<th>DR3&lt;sup&gt;wt&lt;/sup&gt;</th>
<th>DR3&lt;sup&gt;ko&lt;/sup&gt;</th>
<th>Significance&lt;sup&gt;$&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukocytes</td>
<td>9.7 ± 0.5 x10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>9.4 ± 1 x10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>N.S.D</td>
<td>2.2 ± 0.7 x10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>2.2 ± 0.6 x10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>N.S.D</td>
</tr>
<tr>
<td>Alveolar macrophages</td>
<td>5.4 ± 0.5 x10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>6.1 ± 0.6 x10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>N.S.D</td>
<td>11.8 ± 4.9 x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>14.8 ± 5.2 x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>N.S.D</td>
</tr>
<tr>
<td>Myeloid DCs</td>
<td>2.6 ± 0.4 x10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>2.3 ± 0.4 x10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>N.S.D</td>
<td>0.6 ± 0.1 x10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>0.6 ± 0.1 x10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>N.S.D</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>5.6 ± 0.8 x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>5.9 ± 0.9 x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>N.S.D</td>
<td>1.5 ± 0.5 x10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>1.5 ± 0.4 x10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>N.S.D</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>7.6 ± 1.1 x10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>7.0 ± 2.6 x10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>N.S.D</td>
<td>3.2 ± 1.9 x10&lt;sup&gt;2&lt;/sup&gt;</td>
<td>1.5 ± 0.8 x10&lt;sup&gt;2&lt;/sup&gt;</td>
<td>N.S.D</td>
</tr>
<tr>
<td>7/4&lt;sup&gt;+&lt;/sup&gt; Monocytes</td>
<td>1.6± 0.2 x10&lt;sup&gt;2&lt;/sup&gt;</td>
<td>1.3 ±0.2 x10&lt;sup&gt;2&lt;/sup&gt;</td>
<td>N.S.D</td>
<td>29.2 ± 8.0</td>
<td>47.7 ± 17.8</td>
<td>N.S.D</td>
</tr>
<tr>
<td>7/4&lt;sup&gt;−&lt;/sup&gt; Monocytes</td>
<td>3.4 ±0.4 x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>3.0 ± 0.4 x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>N.S.D</td>
<td>9.1 ± 2.2 x10&lt;sup&gt;2&lt;/sup&gt;</td>
<td>8.4 ± 1.5 x10&lt;sup&gt;2&lt;/sup&gt;</td>
<td>N.S.D</td>
</tr>
<tr>
<td>T cells</td>
<td>8.6 ± 1.1 x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>7.1 ± 1.3 x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>N.S.D</td>
<td>3.6 ± 0.8 x10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>2.7 ± 0.4 x10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>N.S.D</td>
</tr>
<tr>
<td>CD4&lt;sup&gt;+&lt;/sup&gt; T cells</td>
<td>3.8 ± 0.5 x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>2.9 ± 0.5 x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>N.S.D</td>
<td>2.3 ± 0.5 x10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>1.6 ± 0.3 x10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>N.S.D</td>
</tr>
<tr>
<td>CD8&lt;sup&gt;+&lt;/sup&gt; T cells</td>
<td>2.5 ± 0.4 x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>2.5 ± 0.4 x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>N.S.D</td>
<td>1.1 ± 0.3 x10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>1.0 ± 0.2 x10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>N.S.D</td>
</tr>
<tr>
<td>B cells</td>
<td>3.5 ± 0.3 x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>3.5 ± 0.6 x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>N.S.D</td>
<td>0.8 ± 0.3 x10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>0.6 ± 0.2 x10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>N.S.D</td>
</tr>
<tr>
<td>NK cells</td>
<td>2.7 ± 0.6 x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>2.8 ± 0.5 x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>N.S.D</td>
<td>1.4 ± 0.5 x10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>1.1 ± 0.3 x10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>N.S.D</td>
</tr>
<tr>
<td>NKT cells</td>
<td>5.8 ± 0.9 x10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>4.0 ± 0.6 x10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>N.S.D</td>
<td>N/A†</td>
<td>N/A†</td>
<td>N/A†</td>
</tr>
</tbody>
</table>

*Values correspond to number of leukocytes within the lung ± SEM (n=10)

^ Values correspond to number of leukocytes in the alveolar passage ± SEM (n= 10)

† N/A corresponds to subsets either not being present or numbers too low to be tested

$ Significance between the two genotypes using an Unpaired T test, N.S.D = no significant difference
3.3.1.3 Measuring lung function in DR3\textsuperscript{wt} and DR3\textsuperscript{ko} mice

3.3.1.3.1 Lung function measurements in DR3\textsuperscript{wt} and DR3\textsuperscript{ko} mice were inconsistent

Lung function was measured in unrestrained mice as Penh using a Buxco whole body plethysmography system. Results suggested that naive DR3\textsuperscript{ko} mice possessed no intrinsic defect in lung function, as there were no significant differences in Penh values when mice were challenged with PBS (DR3\textsuperscript{wt} 0.6±0.2, DR3\textsuperscript{ko} 0.9±0.3) or Methacholine (30mg/ml) (DR3\textsuperscript{wt} 0.7±0.3, DR3\textsuperscript{ko} 1.4±0.7) (2 way ANOVA: Interaction=0.623, Treatment=0.460, Genotype=0.211) (Figure 3.3), a drug typically used to induce bronchoconstriction and therefore study potential changes in respiratory mechanics. However, there was variation in the responses of individual mice to both PBS and Methacholine, independent of genotype, shown by the Residual Error value (Residual Error=13.87). As well as this, DR3\textsuperscript{ko} mice failed to remain still within the plethysmography chamber, despite habituation alongside DR3\textsuperscript{wt} mice. A basic analysis of mouse activity was performed by scoring mice according to their level of movement, with 1 representing activity, and 0 allocated for mice which had settled. Over a 5 day period, DR3\textsuperscript{ko} mice moved significantly more than DR3\textsuperscript{wt} mice, which became accustomed to the chamber and had settled by day 3 (Figure 3.3) (2 way ANOVA: Interaction=0.821, Genotype=0.0006, Mouse=0.498). The possible reasons for this will be discussed further (Section 3.4). Overall, results to study lung function differences between DR3\textsuperscript{wt} and DR3\textsuperscript{ko} mice proved inconclusive.
Figure 3.3. Airway hyper-responsiveness, determined using Penh and activity in naive DR3\textsuperscript{wt} and DR3\textsuperscript{ko} mice. (A) Average Penh readings from naive mice receiving no treatment and Methacholine (30mg/ml) measured using plethysmography. Bars represent mean ± SEM (n=5). Not significant by 2 way ANOVA. (B) Activity of DR3\textsuperscript{wt} and DR3\textsuperscript{ko} mice over a 5 day period. 0 represents still mice, 1 represents active. Bars represent mean ± SEM (n=5). Results significant by 2 way ANOVA ***p<0.001 (Interaction=0.8209, Genotype= ***0.0006, Mouse=0.4979).
3.3.1.4 DR3 expression patterns within the lung of DR3 mice

3.3.1.4.1 DR3 expression can be detected within the lungs of naive DR3\textsuperscript{wt} mice

Prior to this study, the expression profile of DR3 within unchallenged lungs had not been researched meaning the receptor’s location and degree of expression were unknown. Using immunohistochemistry (IHC) and a polyclonal DR3 antibody, DR3 levels were analysed in the airways of DR3\textsuperscript{wt} and DR3\textsuperscript{ko} mice. A control IgG antibody was also used to ensure antibody specificity. Using this method, positive DR3 signals were noted throughout the DR3\textsuperscript{wt} lung, particularly within the smooth muscle, peribronchial areas and occasionally within the alveolar network (Figure 3.4). DR3 concentration was quantified using Leica Qwin V3 software (Section 2.2.12.8) and five different fields of view from each lung. Results were then averaged and DR3 expression quantified for each mouse. DR3 baseline expression patterns in naive DR3\textsuperscript{wt} lung were established at 10.4±1.2\%, significantly higher than DR3\textsuperscript{ko} lung (1.6±0.14\%) (\textit{p}<0.0001) and antibody control stained sections (0.02±0.007\%) (\textit{p}<0.0001) (Table 3.2). Figure 3.4 shows representative photos of DR3 expression within the naive lungs.
Figure 3.4 DR3 expression in naive lungs. (A) Representative pictures of DR3\textsuperscript{wt} and DR3\textsuperscript{ko} lungs stained with DR3 or IgG isotype control (Scale bar=100\textmu m). Arrows show DR3 staining in the peribronchiolar and alveolar network. (B) DR3 expression in the lung was analysed using 5 fields of view of each lung and measuring the % of positive (brown) in each field of view. This was then averaged for each mouse. Analysis was performed using Leica Qwin V3 Software. Bars represent mean ± SEM (n=5). Results are significant using 1 way ANOVA with Bonferroni post test (***p<0.0001).
3.3.2 Phenotypic characterisation of the naive spleens of DR3\textsuperscript{wt} and DR3\textsuperscript{ko} mice

3.3.2.1 Phenotypic analysis of cells within the spleens of DR3\textsuperscript{wt} and DR3\textsuperscript{ko} mice

3.3.2.1.1 Total splenocyte number was not significantly different between naive DR3\textsuperscript{wt} and DR3\textsuperscript{ko} mice

Splenocytes were isolated from DR3\textsuperscript{wt} and DR3\textsuperscript{ko} mice before undergoing a RBC lysis, with the remaining leukocytes being counted using a haemocytometer to determine total spleen cell number (Section 2.2.6). Results showed that there were no significant differences in splenocyte numbers isolated from DR3\textsuperscript{wt} (6.5\pm0.5 \times10^7) compared to DR3\textsuperscript{ko} (6.8\pm0.8 \times10^7) (p=0.687) (Figure 3.5) mice, suggesting DR3 is not required to sustain leukocyte number within the spleen.

3.3.2.1.2 There were no significant baseline differences between splenic DR3\textsuperscript{ko} and DR3\textsuperscript{wt} myeloid and lymphocyte populations

To test whether a lack of DR3 affected individual cell subset numbers within the spleen, splenocytes from DR3 transgenic mice were analysed using flow cytometry and a range of antibodies. Findings confirmed that DR3\textsuperscript{ko} mice suffered no impairment in myeloid or lymphocyte populations within the spleen, as all cell types examined presented similar numbers to their DR3\textsuperscript{wt} equivalent (Table 3.3).
Figure 3.5. Total leukocyte number from the spleens of naive DR3\textsuperscript{wt} and DR3\textsuperscript{ko} mice. Splenocytes were extracted via mashing of the spleen and counted using a haemocytometer with trypan blue. Each symbol represents data from a single mouse. Results showed no significant differences using an Unpaired T test.
3.3.3 Quantifying DR3 expression in the naive lungs and spleens of DR3<sup>wt</sup> mice

3.3.3.1 DR3 was constitutively expressed on T cells within the naive spleen and lung of DR3<sup>wt</sup> mice

Whole splenocyte and lung populations from naive DR3<sup>wt</sup> and DR3<sup>ko</sup> mice were assayed for DR3 expression, using flow cytometry and a polyclonal DR3 antibody. Appropriate isotype controls were also used to ensure that staining was specific and confirm that DR3 binding was due to a specific antibody/cell surface antigen interaction. Median Fluorescent Intensity (MFI) was used to evaluate the DR3 signal as total population shifts were noted. Despite numerous blocking steps (Section 2.2.8.2), a degree of non-specific binding was observed from the polyclonal DR3 antibody, as although T cells from DR3<sup>wt</sup> mice presented a significant shift in fluorescence compared to control IgG treated samples, DR3<sup>ko</sup> cells also increased. To increase the reliability and accuracy of DR3 quantification upon cell types, relative fluorescence (RF) of DR3<sup>wt</sup> and DR3<sup>ko</sup> signal over control samples was calculated, by dividing the test MFI by that of the IgG isotype. Using this method, CD4<sup>+</sup> T cells from the spleens of naive DR3<sup>wt</sup> mice were found to exhibit statistically more signal than DR3<sup>ko</sup> CD4<sup>+</sup> T cells (DR3<sup>wt</sup> 13.5±2.4, DR3<sup>ko</sup> 3.3±0.4) (p=0.003) (Figure 3.6) (Figure 3.7) (Table 3.2). The same was also true of the CD8<sup>+</sup> T cell subset (DR3<sup>wt</sup> 4.1±0.7, DR3<sup>ko</sup> 2.2±0.3) (p=0.003) (Figure 3.6) (Figure 3.7), although DR3 was expressed to a lesser extent (Table 3.2). In contrast, NKT cells, although fewer in number, expressed DR3 to a high level, with DR3<sup>wt</sup> mice displaying a RF of 21.5±2.3 compared to 2.3±0.2 in DR3<sup>ko</sup> mice (p<0.0001) (Figure 3.6) (Figure 3.7) (Table 3.2). These statistically significant results within the naive spleen were mimicked in the lung as both the resident CD4<sup>+</sup> T cell
subset (DR3\textsuperscript{wt} 18.8±0.2, DR3\textsuperscript{ko} 4.0±0.3) (p<0.0001) and CD8\(^{+}\) subset (DR3\textsuperscript{wt} 5.4±0.4, DR3\textsuperscript{ko} 0.9±0.2) (p<0.0001) were observed to express DR3 (Figure 3.8) (Figure 3.9) (Table 3.2), although CD4\(^{+}\) T cells expressed roughly 3.5 times more. Conversely, DR3 could not be detected on NKT cells within the naive lung due to low cell numbers, despite constitutive DR3 expression upon the same cell type within the spleen. However, no significant difference in DR3 signal relative to control was seen on NK cells within the spleen in either DR3\textsuperscript{wt} or DR3\textsuperscript{ko} mice (Figure 3.10). Moreover, myeloid DCs, displayed non-specific staining for the polyclonal antibody, as although DR3\textsuperscript{wt} levels increased in comparison to control antibody, DR3\textsuperscript{ko} cells followed the same pattern (Figure 3.10).
Figure 3.6. Flow cytometry dot plots representing DR3 expression on resident T cell populations from the spleens of DR3\textsuperscript{wt} and DR3\textsuperscript{ko} mice. Representative flow cytometry dot plots following staining with DR3 or control IgG antibody and the % shift of the population. CD4\textsuperscript{+} T cells identified using CD3\textsuperscript{+}, TCRβ\textsuperscript{+} and CD4\textsuperscript{+}. CD8\textsuperscript{+} T cells identified using CD3\textsuperscript{+}, TCRβ\textsuperscript{+} and CD8\textsuperscript{+}. NKT cells identified using CD3\textsuperscript{+}, TCRβ\textsuperscript{+} and NK1.1\textsuperscript{+}. 
Figure 3.7. Ratio of DR3 MFI’s on resident T cell populations in the naive spleens of DR3wt and DR3ko mice. Ratio DR3 expression was calculated by the MFI fold increase compared to the control IgG antibody. (A) CD4+ T cells identified using CD3+, TCRβ+ and CD4+ (**p=0.0029). (B) CD8+ T cells identified using CD3+, TCRβ+ and CD8+ (**p=0.0027). (C) NKT cells identified using CD3+, TCRβ+ and NK1.1+ (**p<0.001). Each symbol represents data from a mouse. Values represent mean ± SEM. Results analysed using an Unpaired T test whereby **p<0.01, ***p<0.001.
Figure 3.8. Flow cytometry dot plots representing DR3 expression on resident T cell populations from the lungs of DR3^wt and DR3^ko mice. Representative flow cytometry dot plots following staining with DR3 or control IgG antibody and the % shift of the population. CD4^+ T cells identified using CD3^+, TCR^+ and CD4^+. CD8^+ T cells identified using CD3^+, TCRβ^+ and CD8^+.
Figure 3.9. Ratio of DR3 MFI’s on resident T cell populations in the naive lungs of DR3\textsuperscript{wt} and DR3\textsuperscript{ko} mice. Ratio DR3 expression was calculated by the MFI fold increase compared to the control IgG antibody. (A) CD4\textsuperscript{+} T cells identified using CD3\textsuperscript{+}, TCR\textbeta\textsuperscript{+} and CD4\textsuperscript{+} (***p<0.0001). (B) CD8\textsuperscript{+} T cells identified using CD3\textsuperscript{+}, TCR\textbeta\textsuperscript{+} and CD8\textsuperscript{+} (***p<0.0001). Each symbol represents data from a mouse. Values represent mean ± SEM. Results analysed using an Unpaired T test whereby ***p<0.001.
Figure 3.10. **DR3 expression on DR3^{wt} NK cells or Myeloid DC’s within the naive spleen.** Splenocytes were isolated and identified (NK cells; CD3^{-} and NK1.1^{+}. mDC’s; CD11c^{+} and CD11b^{+}). (A) Representative flow cytometry dot plots following staining with DR3 or control IgG antibody and the % shift of the population. (B) Representative histograms following staining with DR3 (green=DR3^{ko}, blue=DR3^{wt}) or control IgG antibody (red) on NK cells and (C) mDCs.
### Table 3.2. Summary of DR3 expression

<table>
<thead>
<tr>
<th>Cell Subset</th>
<th>DR3$^{\text{wt}}$</th>
<th>Surface Expression Score$^\wedge$</th>
<th>DR3$^{\text{ko}}$</th>
<th>Significance$^$</th>
</tr>
</thead>
<tbody>
<tr>
<td>% DR3 in naive Lung*</td>
<td>10.4 ± 1.2</td>
<td>N/A†</td>
<td>1.6 ± 0.3</td>
<td>***p&lt;0.0001</td>
</tr>
<tr>
<td>DR3 MFI ratio on Lung CD4$^+$ T cells</td>
<td>18.8 ± 0.8</td>
<td>++</td>
<td>4.0 ± 0.3</td>
<td>***p&lt;0.0001</td>
</tr>
<tr>
<td>DR3 MFI ratio on Lung CD8$^+$ T cells</td>
<td>5.4 ± 0.4</td>
<td>+</td>
<td>0.9 ± 0.2</td>
<td>***p&lt;0.0001</td>
</tr>
<tr>
<td>DR3 MFI ratio on Spleen CD4$^+$ T cells</td>
<td>13.5 ± 2.4</td>
<td>++</td>
<td>3.3 ± 0.1</td>
<td>**p=0.003</td>
</tr>
<tr>
<td>DR3 MFI ratio on Spleen CD8$^+$ T cells</td>
<td>4.1 ± 0.7</td>
<td>+</td>
<td>2.2 ± 0.3</td>
<td>**p=0.003</td>
</tr>
<tr>
<td>DR3 MFI ratio on Spleen NKT cells</td>
<td>21.5 ± 2.3</td>
<td>+++</td>
<td>2.3 ± 0.2</td>
<td>***p&lt;0.0001</td>
</tr>
</tbody>
</table>

* % DR3 expression within the naive lungs of DR3$^{\text{wt}}$ and DR3$^{\text{ko}}$ mice via IHC (n=5)

$^\wedge$ Surface expression scores of DR3 are estimates for comparison purposes + low expression to +++ high expression

† N/A corresponds to subsets either not being present or numbers too low to be tested

$^\$ Significance between two genotypes using Unpaired T Test (**p<0.01, ***p<0.001).

Median fluorescence intensity (MFI) ratio calculated by dividing the test median by the isotype following gating on the specified population ± SEM following flow cytometry
3.3.4 Phenotypic characterisation of the peripheral blood in naive DR3\textsuperscript{wt} and DR3\textsuperscript{ko} mice

3.3.4.1 Phenotypic analysis of cells within the peripheral blood of DR3\textsuperscript{wt} and DR3\textsuperscript{ko} mice

3.3.4.1.1 Total leukocyte number in the peripheral blood of unchallenged DR3\textsuperscript{wt} and DR3\textsuperscript{ko} mice did not significantly differ

To examine whether DR3 had any part to play in maintaining the total number of cells within the peripheral blood in a naive mouse, blood was acquired via a cardiac puncture (Section 2.2.5.4). Red blood cells were lysed and leukocytes counted using a haemocytometer (Section 2.2.6). Results confirmed that the absence of DR3 had no effect on the total number of leukocytes within the peripheral blood as no significant differences were seen between DR3\textsuperscript{ko} (1.6±0.3 x10\textsuperscript{6}) and DR3\textsuperscript{wt} (1.8±0.3 x10\textsuperscript{6}) (\(p=0.818\)) mice (Figure 3.11).

3.3.4.1.2 There were no significant baseline differences between DR3\textsuperscript{wt} and DR3\textsuperscript{ko} myeloid and lymphocyte derived peripheral blood cells

Individual cell subsets were isolated from total leukocytes using flow cytometry to investigate whether DR3 had any effect upon specific populations in the naive state. Findings showed that DR3\textsuperscript{ko} mice had no defect in any myeloid or lymphocyte cell types examined, as the 2 genotypes displayed analogous cell subset numbers (Table 3.3).
Figure 3.11. Total leukocyte number isolated from the peripheral blood of naive DR3<sup>wt</sup> and DR3<sup>ko</sup> mice. Cells were isolated via cardiac puncture and counted using a haemocytometer with trypan blue. Each symbol represents data from a single mouse. Results showed no significant differences using an Unpaired T test.
Table 3.3. Comparison of cell numbers isolated from the spleen and peripheral blood of DR3<sup>wt</sup> and DR3<sup>ko</sup> mice

<table>
<thead>
<tr>
<th>Cell Subset</th>
<th>DR3&lt;sup&gt;wt&lt;/sup&gt;</th>
<th>DR3&lt;sup&gt;ko&lt;/sup&gt;</th>
<th>Significance&lt;sup&gt;$&lt;/sup&gt;</th>
<th>DR3&lt;sup&gt;wt&lt;/sup&gt;</th>
<th>DR3&lt;sup&gt;ko&lt;/sup&gt;</th>
<th>Significance&lt;sup&gt;$&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukocytes</td>
<td>6.5 ± 0.5 x10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>6.8 ± 0.8 x10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>N.S.D</td>
<td>1.6 ± 0.3 x10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>1.8 ± 0.3 x10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>N.S.D</td>
</tr>
<tr>
<td>Macrophages</td>
<td>16.1 ± 4.0 x10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>15.0 ± 3. x10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>N.S.D</td>
<td>N/A†</td>
<td>N/A†</td>
<td>N/A†</td>
</tr>
<tr>
<td>Myeloid DCs</td>
<td>6.2 ± 2.2 x10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>5.4 ± 1.4 x10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>N.S.D</td>
<td>N/A†</td>
<td>N/A†</td>
<td>N/A†</td>
</tr>
<tr>
<td>Plas. DCs</td>
<td>1.2 ± 0.3 x10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>0.9 ± 0.2 x10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>N.S.D</td>
<td>N/A†</td>
<td>N/A†</td>
<td>N/A†</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>6.4 ± 1.8 x10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>8.8 ± 3.7 x10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>N.S.D</td>
<td>1.3 ± 0.3 x10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>1.6 ± 0.2 x10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>N.S.D</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>1.5 ± 0.4 x10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>1.4 ± 0.4 x10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>N.S.D</td>
<td>4.5 ± 1.0 x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>3.6 ± 0.8 x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>N.S.D</td>
</tr>
<tr>
<td>7/4+ Monocytes</td>
<td>6.6± 1.2 x10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>6.7 ±1.3 x10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>N.S.D</td>
<td>8.0 ± 0.2 x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>5.9 ± 0.9 x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>N.S.D</td>
</tr>
<tr>
<td>7/4− Monocytes</td>
<td>13.6 ± 3.4 x10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>14.2± 5.1 x10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>N.S.D</td>
<td>2.8 ± 0.5 x10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>3.3 ± 0.6 x10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>N.S.D</td>
</tr>
<tr>
<td>T cells</td>
<td>1.4 ± 0.2 x10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>1.5 ± 0.2 x10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>N.S.D</td>
<td>3.2 ± 0.7 x10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>3.6 ± 0.7 x10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>N.S.D</td>
</tr>
<tr>
<td>CD4&lt;sup&gt;+&lt;/sup&gt; T cells</td>
<td>0.9 ± 0.1 x10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>0.9 ± 0.1 x10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>N.S.D</td>
<td>1.5 ± 0.4 x10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>1.9 ± 0.4 x10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>N.S.D</td>
</tr>
<tr>
<td>CD8&lt;sup&gt;+&lt;/sup&gt; T cells</td>
<td>0.5 ± 0.1 x10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>0.5 ± 0.1 x10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>N.S.D</td>
<td>1.4 ± 0.3 x10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>1.6 ± 0.3 x10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>N.S.D</td>
</tr>
<tr>
<td>B cells</td>
<td>1.7 ± 0.4 x10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>1.5 ± 0.3 x10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>N.S.D</td>
<td>5.3 ± 1.1 x10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>5.8 ± 1.2 x10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>N.S.D</td>
</tr>
<tr>
<td>NK cells</td>
<td>2.0 ± 0.5 x10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>1.9 ± 0.4 x10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>N.S.D</td>
<td>1.5 ± 0.3 x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>1.7 ± 0.2 x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>N.S.D</td>
</tr>
<tr>
<td>NKT cells</td>
<td>4.7 ± 0.7 x10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>4.9 ± 0.7 x10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>N.S.D</td>
<td>6.6± 1.5 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>1.7± 0.2 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>N.S.D</td>
</tr>
</tbody>
</table>

* Values correspond to number of leukocytes within the spleen ± SEM (n=6)

^ Values correspond to number of leukocytes in the peripheral blood ± SEM per ml of blood (n=6)

† N/A corresponds to subsets either not being present or numbers too low to be tested

$ Significance between the two genotypes using an Unpaired T test, N.S.D = no significant difference
3.4 Discussion

3.4.1 Cellular phenotypes of the lung, alveolar passage, spleen and blood of DR3\textsuperscript{wt} and DR3\textsuperscript{ko} mice

Previous to this study, no data had been published on the cellular components of the lung, alveolar passage or peripheral blood of naive DR3\textsuperscript{ko} mice, knowledge essential for the interpretation of effects following immune challenge. The spleen on the other hand had been studied before (Wang, Thern et al. 2001; Meylan, Davidson et al. 2008), although not to the level of detail described within this Chapter. Overall, no significant differences were found in the lungs or airways of DR3\textsuperscript{wt} and DR3\textsuperscript{ko} mice in the cell types examined, suggesting DR3 has no role in the maintenance of leukocytes in the naive state. This result not only confirms that no intrinsic differences exist in the lungs of DR3\textsuperscript{ko} mice at baseline levels, but also allows a comparison to be drawn when studying mice which have been allergen challenged. Similarly, no significant differences were seen in the peripheral blood or spleen of DR3\textsuperscript{wt} and DR3\textsuperscript{ko} mice at baseline level, ensuring that upon inflammation, both DR3\textsuperscript{wt} and DR3\textsuperscript{ko} mice are able to recruit from sources with equal numbers of leukocytes. The data of this Chapter implies that DR3 has a non-redundant role in upholding the cellular composition of the organs examined in un-challenged mice.

3.4.2 DR3 expression patterns in the lung

The detection of DR3 within the lung via IHC proved successful, despite a small degree of non specific binding exhibited by the polyclonal antibody. DR3 expression has previously been found on healthy and psoriatic skin lesions (Bamias, Evangelou et al.
2011) and the naive peritoneal membrane (Perks, 2013 PhD). However, Receptor expression patterns had never been determined in the lung, although studies have been published which link DR3 to lung inflammation (Fang, Adkins et al. 2008; Meylan, Davidson et al. 2008; Schreiber, Wolf et al. 2010). Being able to not only quantify DR3, but also observe the pattern of expression provides a novel advantage to other methods previously used to look at DR3 expression, such as Northern Blot analysis (Wang, Kitson et al. 2001) and RT-PCR (Bamias, Mishina et al. 2006; Bull, Williams et al. 2008; Pappu, Borodovsky et al. 2008; Taraban, Slebioda et al. 2011). Nevertheless, IHC does present a major disadvantage in comparison to the said techniques. Using RT-PCR and Southern blotting, it was found that DR3 had three murine splice variants; full length, membrane bound truncated and soluble isoforms (Wang, Kitson et al. 2001). Whilst, RT-PCR is able to detect all 3 murine splice variants, the antibody used for DR3 IHC cannot. Consequently, although a general DR3 signal can be detected within the lung, the exact splice variant and therefore functionality of the Receptor cannot.

3.4.3 Leukocyte DR3 expression within the lung and spleen

Lack of suitable commercial murine DR3 reagents has hindered progress within this field of research, as murine models prove to play an ever increasing role in studying the effects of the Receptor. Previous attempts within the lab to generate a monoclonal antibody to mouse DR3 failed (Bull, 2008), although other labs have had more success, notably Fang et al. Although DR3 expression was detected on T lymphocytes in the naive lung and spleen, binding of the polyclonal reagent showed a degree of non-specificity despite several additional blocking steps (Section 2.2.8.2). One reason for this may be the DR3 antibody itself, which as a polyclonal may bind not only to DR3
this may be the DR3 antibody itself, which as a polyclonal may bind not only to DR3 but also proteins which exhibit homology to the receptor, such as TNFR1 which demonstrates 28% amino acid similarity to DR3 (Chinnaiyan, Orourke et al. 1996; Kitson, Raven et al. 1996; Marsters, Sheridan et al. 1996; Screaton, Xu et al. 1997). This may explain the small signal detected by the DR3 antibody upon DR3\textsuperscript{ko} T cells.

Notwithstanding this small degree of non-specificity, DR3 was found to be expressed on naive CD4\textsuperscript{+} T cells, CD8\textsuperscript{+} T cells and NKT cells in the spleen, results which accord with published data (Fang, Adkins et al. 2008; Twohig, Marsden et al. 2012), as well as the novel finding of DR3 on CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells in the lung. Adding to this, DR3 has been found to be expressed on CD4\textsuperscript{+} T cells of the Th1 (Bamias, Martin et al. 2003), Th2 (Fang, Adkins et al. 2008) and Th17 (Pappu, Borodovsky et al. 2008) response, as well as regulatory T cells (Schreiber, Wolf et al. 2010). Although not expressed on naive murine B cells (Fang, Adkins et al. 2008), Cavallini \textit{et al} have reported the presence of DR3 on IgM stimulated human B cells isolated from blood. Furthermore, it appears that antigen-stimulated B cells within the murine spleen white pulp region express DR3, although DR3’s influence on antibody production were not examined (Cavallini, Lovato et al. 2013). The above subsets would be interesting to study using DR3\textsuperscript{ko} mice, in both a naive and allergen challenge situation, allowing a clear comparison between cells which express DR3 and those which do not as well as their differential regulation.

Despite the presence of DR3 on T cells, the antibody does not allow distinction between the 3 murine splice variants, which are known to be differentially expressed following
activation (Bamias, Mishina et al. 2006; Twohig, Marsden et al. 2012). Using RT-PCR, it has been reported that naive CD8+ cells from OT-1 transgenic DR3wt mice express both truncated and full length DR3 mRNA transcripts at low levels. Following activation however, full length DR3 mRNA transcripts dominated with an increase also seen in the variant thought to encode a soluble form of the receptor (Twohig, Marsden et al. 2012). High levels of truncated DR3 have been reported on TregS, whilst Th17 cells are thought to express full length DR3 (Pappu, Borodovsky et al. 2008), resulting in TL1A driven proliferation of Th17 cells as opposed to TregS. This data only highlights the need to understand the DR3 isoform/s being expressed in the basal state as they determine the functional response.

Myeloid DCs on the other hand, displayed similar DR3 signals between DR3wt and DR3ko cells, although both shift in comparison to control IgG, suggesting DR3 is present on these cells but cannot be distinguished from the DR3ko sample. The lack of antibody specificity and general ‘stickiness’ of myeloid cells means that DR3 expression cannot be quantified on myeloid cells using flow cytometry and this particular reagent. However, published data states that DR3 is expressed on naive CD11c+ cells within the spleen and lymph nodes, a typical myeloid marker found upon myeloid DCs (Fang, Adkins et al. 2008), suggesting this monoclonal antibody and the polyclonal used within the study differ in their ability to detect DR3 and its splice variants. DR3 expression has been recorded on un-stimulated cells of the human macrophage cell line, THP-1, using both flow cytometry (Kang, Kim et al. 2005; Su, Chang et al. 2006) and RT-PCR (McLaren, Calder et al. 2010), perhaps suggesting that DR3 is to be found upon murine myeloid cells. However, with 13 known human DR3 splice variants and the inability to distinguish between them using the above techniques,
it is difficult to draw comparisons to the murine system. Fang et al also reported DR3 expression on a subpopulation of NK cells using their monoclonal antibody, whilst the commercial polyclonal antibody used in this study was unable to detect any DR3 on NK cells within the DR3\textsuperscript{wt} spleen, as no shift was noted from either DR3\textsuperscript{wt} or DR3\textsuperscript{ko} cells in comparison to control IgG samples. These inconsistencies may be due to a number of reasons, such as low receptor expression on NK cells making signal detection difficult, or the preferential binding of the different antibodies to specific DR3 isoforms, a difficulty previously suggested when measuring DR3 expression (Taraban, Ferdinand et al. 2011). Without access to the monoclonal antibody and testing it alongside the commercial polyclonal, the differences between the detection abilities of the 2 antibodies are difficult to pinpoint.

DR3 expression was not examined in bronchoalveolar lavage samples due to poor cell recovery in naive mice. Few immunological cells reside in the alveolar passage of naive mice, making the detection of DR3 very difficult without the pooling of multiple samples from different mice.

### 3.4.4 Lung function and airway reactivity of DR3\textsuperscript{wt} and DR3\textsuperscript{ko} mice

Results from the lung function tests were inconsistent between individual mice, irrespective of genotype. The Residual Error, used to define variation amongst sampling units, showed that the variation was too great within the results. Using a Power Equation, it was calculated that 70 mice of each genotype would be required to overcome this variation when measuring airway hyper-responsiveness in DR3\textsuperscript{ko} mice,
which is neither feasible nor ethical. Additionally, despite habituation to the Buxco chamber, selective DR3\(^{\text{ko}}\) mice still continued to move when experimental readings were recorded, which most likely contributed to the variation observed. Considering the results already obtained, this method of measuring airway hyper-responsiveness in DR3\(^{\text{ko}}\) mice is neither dependable nor consistent.

Published studies have already demonstrated that DR3\(^{\text{ko}}\) mice suffer with a form of hyper-activity and behavioural defects (Twohig, Roberts et al. 2010), which although thought to be age dependent, could explain their inability to remain still within the Buxco chamber (Figure 3.12) in comparison to DR3\(^{\text{wt}}\) mice. DR3 is expressed constitutively within both the murine and human brain (Harrison, Roberts et al. 2000; Wang, Kitson et al. 2001; Twohig, Roberts et al. 2010) and its genetic loss is thought to be responsible for murine neurological disease in the form of disorientation, abnormal gait and general decline in motor control function (Twohig, Cuff et al. 2011). However, these features were only noted in mice 24 weeks and above. Figure 3.12 (video) suggests similar characteristics surface in DR3\(^{\text{ko}}\) mice as young as 8 weeks, as despite habituation to the plethysmography chamber, DR3\(^{\text{ko}}\) mice appeared to exhibit behavioural abnormalities compared to DR3\(^{\text{wt}}\) mice. Over the acclimatisation period, DR3\(^{\text{wt}}\) mice seemed to display memory in that they gradually became accustomed to the Buxco chamber, remaining relatively calm by the end of the 2 week time allowance (Figure 3.12 video). In contrast, DR3\(^{\text{ko}}\) mice looked to display no habituation to the chamber procedure despite daily introductions over the same time course, suggesting early penetrance of a behavioural phenotype. This ultimately resulted in inconclusive lung function tests. With this in mind, the use of plethysmography in this project was discontinued.
Whole body unrestrained plethysmography itself has been questioned as a suitable means by which to test for airway hyper-reactivity and bronchoconstriction. Plethysmography is generally used due to its ease, as mice do not need to be restrained and are conscious during the procedure, thereby encouraging normal breathing patterns. Whole body plethysmography works by measuring two changes in box pressure; the reduction in air caused by mouse inspiration and an increase due to lungs expanding (Fernandez-Rodriguez, Ford et al. 2008). In normal circumstances these flows cancel each other out. Following airway responsiveness to a bronchoconstrictor, the change in box pressure during expiration is more pronounced than that during inspiration (Archer, Cramton et al. 2004; Fernandez-Rodriguez, Ford et al. 2008). However, debate continues as to the reliability of the method. Zhang et al suggested Penh alone was not enough to confirm airway resistance in BALB/c mice and that a more invasive technique was required to evaluate airway responsiveness, particularly within the lower airway, where the majority of allergic responses occur (Zhang, Lai et al. 2009). Data suggested that plethysmography presented false positives, as mice with mild inflammation demonstrated airway resistance using Penh as an index, but not with the more invasive technique. However, the invasive technique involved anaesthetised mice and intubation, followed by the monitoring of flow using thoracic movements and ultimately trans-pulmonary pressure and lung resistance. As well as this, mice with severe inflammation required less Methacholine to initiate airway hyper-responsiveness according to plethysmography than those measured using the invasive endotracheal intubation method (Zhang, Lai et al. 2009). Adler et al suggested that Penh was strain specific, in that it responded differently for mice of BALB/c and C57BL/6 lineage (Adler, Cieslewicz et al. 2004), whilst Albertine et al found that Penh readings were inconsistent with measurements taken from anaesthetised cannulated mice during the
latter stages of an OVA challenge protocol (days 18-22) when using C57BL/6 mice (Albertine, Wang et al. 2002). Nevertheless, Hamelmann et al argue that whole body plethysmography is an effective indicator of airway hyper-responsiveness following allergic sensitisation, as Penh readings correlated with other hallmarks of bronchial asthma such as eosinophil lung infiltration and increased IgE production (Hamelmann, Schwarze et al. 1997; Schwarze, Hamelmann et al. 2005). In addition, BALB/c mice responded to Methacholine in the same way using both the invasive and non-invasive techniques, leading Hamelmann et al to conclude that Penh is a suitable index of airway kinetics. It appears that the controversy regarding plethysmography is ongoing, as both the advantages and limitations of the technique continue to be published and debated. Despite the ease of Penh as a measure of airway obstruction, DR3<sup>ko</sup> mice were unable to remain still within the chamber resulting in skewed readings, agitated mice and an inability to study lung mechanics.
3.5 Summary

Attempts to examine lung function and airway hyper-reactivity in unchallenged DR3\textsuperscript{ko} mice were inconclusive. Results implied that there was no difference in the lung mechanics of DR3\textsuperscript{wt} and DR3\textsuperscript{ko} mice but the method used to examine this, plethysmography, was neither reliable nor suitable for studying DR3\textsuperscript{ko} mice. Collectively, the data contained in this Chapter confirms DR3 expression on murine T cells in the spleen and presents novel findings of DR3 expression in the naive lungs of DR3\textsuperscript{wt} mice. However, despite constitutive Receptor expression, it is not involved in the maintenance of cell populations in either the spleen, lung, alveolar passage or blood, suggesting function only arises during inflammatory episodes.
Chapter 4:

The role of DR3 in an acute murine model of allergic lung inflammation
4.1 Introduction

Asthma is defined as a chronic disease of the airways, characterised by airway inflammation, airway hyper-responsiveness and airway remodelling. Although no in vivo model is able to replicate all of these morphological and functional features, animal models have been used to study specific aspects of the disease.

Models of acute allergic lung inflammation have long been employed to identify the mechanisms underlying the immunological and inflammatory responses of asthma, replicating the initiating events leading to disease. This short-term exposure is sufficient to imitate airway inflammatory events, allowing the relationship between cells and inflammatory mediators to be investigated and so potential novel targets for drug therapy to be identified.

DR3 has previously been implicated in acute models of inflammatory disease, including acute allergic lung inflammation (Fang, Adkins et al. 2008; Meylan, Davidson et al. 2008). Using both DR3\(^{ko}\) and dominant negative (DN) DR3 transgenic mice, it was found that DR3 was responsible for cellular accumulation into the alveolar passage, Th2 cytokine production and lung immune pathology. However, it is known the nature of the inflammatory response is influenced by the challenge and sensitisation protocols employed (Kumar and Foster 2002; Zosky and Sly 2007), as well as the readouts used to assess disease. As these differ between the existing published data, it is difficult to compare these results in the context of this project. Therefore, the role of DR3 in acute allergic lung inflammation was assessed using the same sensitisation protocol to be
utilised for chronic allergic lung inflammation (Chapter 5), adapted from Fernandez-Rodriguez et al (2008), to allow comparisons to be drawn between the 2 models (Fernandez-Rodriguez, Ford et al. 2008).
4.2 Aims

The aim of this Chapter was to investigate the role of DR3 in acute allergic lung inflammation. DR3\textsuperscript{wt} and DR3\textsuperscript{ko} mice were sensitised using i.p. injection of OVA and Alum before short-term exposure to OVA or PBS. Mice were harvested at 24 and 72 hours post inhalation challenge, which occurred on day 15 (Section 2.2.7.3). To dissect the impact of DR3 on the allergic responses, leukocyte influx into the lung and BAL were measured, as well as allergic antibody levels in the serum. Histological examination of the lung determined the degree of airway remodelling and analysis of spleen cell subsets the systemic effect of DR3.

4.2.1 Objectives

i) Determine the local inflammatory response following acute allergic lung inflammation

ii) Analyse and quantify airway remodelling

iii) Determine the systemic response following acute allergic lung inflammation
4.3 Results

4.3.1 Airway inflammation following acute allergic lung inflammation

4.3.1.1 Analysis of cellular accumulation into the lung

4.3.1.1.1 DR3 did not regulate leukocyte accumulation into the lung

Lung cells were extracted following collagenase treatment and counted using a haemocytometer. Results showed there were no significant differences in lung total cell numbers between DR3\(^{wt}\) OVA or DR3\(^{ko}\) OVA treated mice at the 24 or 72hr harvest points. However, OVA treated mice of both genotypes did have significantly higher number of leukocytes compared to PBS challenged animals at 24hrs (DR3\(^{wt}\) OVA 3.3±0.4 x10\(^6\), DR3\(^{ko}\) OVA 2.9±0.2 x10\(^6\), DR3\(^{wt}\) PBS 1.9±0.1 x10\(^6\), and DR3\(^{ko}\) PBS 1.8±0.1 x10\(^6\)) (\(p=0.0002\)) (Figure 4.1), thus suggesting that DR3 does not control the accumulation of leukocytes in the lung following allergen challenge.
Figure 4.1. Total leukocyte number from the lungs of DR3<sup>wt</sup> and DR3<sup>ko</sup> mice following acute allergic lung inflammation. Mice were sacrificed (A) 24hrs and (B) 72hr post final inhalation challenge with either OVA or PBS. Cells were isolated via a lung digest and counted using a haemocytometer. (A) **p=0.006 using 1 way ANOVA and Bonferroni post test (B) n.s.d using 1 way ANOVA. Values represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001. Each symbol represents data from a single mouse.
4.3.1.1.2 DR3<sup>wt</sup> OVA treated lungs had significantly more 7/4<sup>+</sup> monocytes than DR3<sup>ko</sup> OVA lungs at 24 but not 72hrs post inhalation

At 24hrs post inhalation, significant differences were seen between DR3<sup>wt</sup> OVA and DR3<sup>ko</sup> OVA mice in 7/4<sup>+</sup> monocytes within the lung (DR3<sup>wt</sup> 8.2 ± 1.2 x 10<sup>4</sup>, DR3<sup>ko</sup> 4.9 ± 0.5 x 10<sup>4</sup>) (p<0.05) (Figure 4.2), but no other myeloid cell subset. However, mice that had undergone OVA inhalation consistently displayed higher cell subset numbers in comparison to PBS treated animals. This was significant between DR3<sup>wt</sup> OVA and DR3<sup>ko</sup> PBS mice when comparing alveolar macrophages, as DR3<sup>wt</sup> OVA mice had 8.2±1.3 x 10<sup>5</sup> macrophages, with DR3<sup>ko</sup> PBS mice at 4.3±0.3 x 10<sup>5</sup> (p=0.008) (Figure 4.2). Lungs of DR3<sup>wt</sup> and DR3<sup>ko</sup> OVA mice contained significantly higher numbers of myeloid DCs compared with PBS treated animals (DR3<sup>wt</sup> OVA 1.8±0.4 x 10<sup>4</sup>, DR3<sup>ko</sup> OVA 1.9±0.2 x 10<sup>4</sup>, DR3<sup>wt</sup> PBS 0.5±0.3 x 10<sup>4</sup>, DR3<sup>ko</sup> PBS 0.7±0.1 x 10<sup>4</sup>) (p=0.001) (Figure 4.2). No significant differences in eosinophils were seen between DR3<sup>wt</sup> OVA (15.4±2.4 x 10<sup>4</sup>) and DR3<sup>ko</sup> OVA mice (14.1±2.6 x 10<sup>4</sup>), but the numbers in these treatment groups were significantly higher than control PBS challenged animals (DR3<sup>wt</sup> PBS 5.1±1.2 x 10<sup>4</sup>, DR3<sup>ko</sup> PBS 14.1±2.6 x 10<sup>4</sup>) (p=0.0003) (Figure 4.2).

At 72hrs post-inhalation, DR3<sup>wt</sup> OVA lungs contained 4.9±1.0 x 10<sup>5</sup> eosinophils, more than all other treatment groups and significantly more than DR3<sup>wt</sup> PBS mice (1.9±0.3 x 10<sup>5</sup>) (p=0.026) (Figure 4.3). This trend was also observed when studying myeloid DCs, alveolar macrophages and 7/4<sup>+</sup> monocytes, although no statistical significance was reached. In contrast to 24hr post inhalation, mice showed equivalent numbers of 7/4<sup>+</sup> monocytes and neutrophils irrespective of treatment and genotype (Figure 4.3).
Figure 4.2. Myeloid cell subsets from the digested lungs of DR3<sup>wt</sup> and DR3<sup>ko</sup> mice 24hrs post acute allergic lung inflammation. Mice were sacrificed 24hr post final inhalation challenge with either OVA or PBS. Lung cells were isolated via collagenase lung digestion and subsets determined using antibody cell markers. Alveolar Macrophages (***p=0.008), Neutrophils (*p=0.035), Eosinophils (***p=0.0003), 7/4<sup>+</sup> Monocytes (*p=0.010), 7/4<sup>-</sup> Monocytes (***p=0.001), Myeloid DCs (***p=0.001). Values represent mean ± SEM. Significance determined using 1 way ANOVA and Bonferroni post test; *p<0.05, **p<0.01, ***p<0.001. Each symbol represents data from a single mouse.
Figure 4.3. Myeloid cell subsets from the digested lungs of DR3^{wt} and DR3^{ko} mice 72hrs post acute allergic lung inflammation. Mice were sacrificed 72hr post final inhalation challenge with either OVA or PBS. Lung cells were isolated via collagenase lung digestion and subsets determined using antibody cell markers. Alveolar Macrophages (*p=0.045), Neutrophils n.s.d, Eosinophils (*p=0.026), 7/4^{+} Monocytes n.s.d, 7/4^{-} Monocytes n.s.d, Myeloid DCs n.s.d. Values represent mean ± SEM. Significance determined using 1 way ANOVA and Bonferroni post test; *p<0.05. Each symbol represents data from a single mouse.
4.3.1.1.3 DR3\textsuperscript{wt} OVA lungs had significantly more NK and NKT cells compared to DR3\textsuperscript{ko} OVA lungs at 24 but not 72hrs post inhalation

Significant differences were seen at 24hrs post inhalation in multiple lymphocytic cell subsets. Most significant was the increase in NKT cells and NK cells in DR3\textsuperscript{wt} OVA lungs (NKT 12.7±1.5 x10\textsuperscript{4}, NK 3.1±0.5 x10\textsuperscript{5}) compared to DR3\textsuperscript{ko} OVA (NKT 7.1±1.0 x10\textsuperscript{4}, NK 1.9±0.2 x10\textsuperscript{5}) (\(p<0.0001\) and \(p=0.0005\) respectively) (Figure 4.4). The lungs of OVA treated groups were also observed to have more CD3\textsuperscript{+} T cells than PBS challenged animals (DR3\textsuperscript{wt} OVA 13.2±2.1 x10\textsuperscript{5}, DR3\textsuperscript{ko} OVA 10.7±1.2 x10\textsuperscript{5}, DR3\textsuperscript{wt} PBS 5.1±1.0 x10\textsuperscript{5}, DR3\textsuperscript{ko} PBS 5.5±0.3 x10\textsuperscript{5}) (\(p=0.0004\)), corresponding to a significant increase in CD4\textsuperscript{+} T cells (DR3\textsuperscript{wt} OVA 7.6±1.3 x10\textsuperscript{5}, DR3\textsuperscript{ko} OVA 5.7±0.7 x10\textsuperscript{5}, DR3\textsuperscript{wt} PBS 2.5±0.5 x10\textsuperscript{5}, DR3\textsuperscript{ko} PBS 2.6±0.2 x10\textsuperscript{5}) (\(p=0.0002\)) and activated (CD44\textsuperscript{+}) CD4\textsuperscript{+} T cells (DR3\textsuperscript{wt} OVA 3.9±0.6 x10\textsuperscript{5}, DR3\textsuperscript{ko} OVA 3.3±0.5 x10\textsuperscript{5}, DR3\textsuperscript{wt} PBS 1.5±0.2 x10\textsuperscript{5}, DR3\textsuperscript{ko} PBS 1.4±0.1 x10\textsuperscript{5}) (\(p=0.0005\)) (Figure 4.4). When analysing CD8\textsuperscript{+} T cells, only DR3\textsuperscript{wt} OVA lungs displayed significance over PBS mice (DR3\textsuperscript{wt} OVA 4.3±0.8 x10\textsuperscript{5}, DR3\textsuperscript{wt} PBS 2.1±0.4 x10\textsuperscript{5}, DR3\textsuperscript{ko} PBS 2.3±0.2 x10\textsuperscript{5}) (\(p=0.005\)) (Figure 4.4). B cell numbers were also significantly greater in DR3\textsuperscript{wt} OVA lungs compared with PBS treated mice (DR3\textsuperscript{wt} OVA 1.5±0.3 x10\textsuperscript{5}, DR3\textsuperscript{wt} PBS 0.7±0.1 x10\textsuperscript{5}, DR3\textsuperscript{ko} PBS 0.6±0.1 x10\textsuperscript{5}) (\(p=0.010\)) (Figure 4.4), although levels were higher overall compared with all other treatment groups.

No such trends were seen at 72hrs post inhalation, as DR3\textsuperscript{wt} and DR3\textsuperscript{ko} mice displayed comparable numbers of T and B cells (Figure 4.5). However, DR3\textsuperscript{wt} OVA mice did have significantly more NKT cells than DR3\textsuperscript{ko} PBS mice, with 12.8±2.8 x10\textsuperscript{4} compared to 3.3±0.8 x10\textsuperscript{4} (\(p=0.018\)) (Figure 4.5).
Figure 4.4. Lymphocyte subsets from the digested lungs of DR3<sup>wt</sup> and DR3<sup>ko</sup> mice 24hrs post acute allergic lung inflammation. Mice were sacrificed 24hr post final inhalation challenge with either OVA or PBS. Lung cells were isolated via collagenase lung digestion and subsets determined using antibody cell markers. CD3<sup>+</sup> T cells (**p=0.0004), CD4<sup>+</sup> T cells (**p=0.0002), CD8<sup>+</sup> T cells (**p=0.005), Activated CD4<sup>+</sup> T cells (**p=0.0005), Activated CD8<sup>+</sup> T cells (*p=0.018), NKT cells (**p<0.0001), NK cells (**p=0.0005), B cells (*p=0.010). Values represent mean ± SEM. Significance determined using 1 way ANOVA and Bonferroni post test; *p<0.05, **p<0.01, ***p<0.001. Each symbol represents data from a single mouse.
Figure 4.5. Lymphocyte subsets from the digested lungs of DR3<sup>wt</sup> and DR3<sup>ko</sup> mice 72hrs post acute allergic lung inflammation. Mice were sacrificed 72hr post final inhalation challenge with either OVA or PBS. Lung cells were isolated via collagenase lung digestion and subsets determined using antibody cell markers. CD3<sup>+</sup> T cells n.s.d, CD4<sup>+</sup> T cells n.s.d, CD8<sup>+</sup> T cells n.s.d, Activated CD4<sup>+</sup> T cells n.s.d, Activated CD8<sup>+</sup> T cells n.s.d, NKT cells (*p=0.018), NK cells n.s.d, B cells n.s.d. Values represent mean ± SEM. Significance determined using 1 way ANOVA and Bonferroni post test; *p<0.05. Each symbol represents data from a single mouse.
4.3.1.2 Analysis of cellular infiltration into the alveolar passage

4.3.1.2.1 DR3\textsuperscript{wt} OVA mice had significantly more leukocytes in the alveolar passage than DR3\textsuperscript{ko} OVA mice

At 24hrs post inhalation, DR3\textsuperscript{wt} OVA mice had $4.8\pm 1.4 \times 10^5$ cells within the alveolar passage, significantly more than DR3\textsuperscript{ko} OVA mice at $1.5\pm 0.3 \times 10^5$ and PBS treated animals (DR3\textsuperscript{wt} PBS $1.2\pm 0.2 \times 10^5$, DR3\textsuperscript{ko} PBS $1.5\pm 0.3 \times 10^5$) ($p=0.0096$) (Figure 4.6). Similarly, at 72hrs post inhalation, BAL fluid from DR3\textsuperscript{wt} OVA treated mice was found to contain $5.2\pm 1.3 \times 10^5$ cells, significantly more than DR3\textsuperscript{ko} OVA mice ($1.0\pm 0.3 \times 10^5$) ($p=0.013$) (Figure 4.6), suggesting DR3 regulates cellular accumulation into the bronchoalveolar passage.
Figure 4.6. Total leukocyte number from the alveolar passage of DR3<sup>wt</sup> and DR3<sup>ko</sup> mice following acute allergic lung inflammation. Mice were sacrificed (A) 24hrs and (B) 72hrs post final inhalation challenge with either OVA or PBS. Cells were isolated via a bronchoalveolar lavage and counted using a haemocytometer. (A) **p=0.0096 using 1 way ANOVA and Bonferroni post test. (B) *p=0.013 using 1 way ANOVA and Bonferroni post test; *p<0.05. Values represent mean ± SEM. Each symbol represents data from a single mouse.
4.3.1.2.2 DR3<sup>wt</sup> OVA mice had significantly more 7/4<sup>+</sup> monocytes and myeloid DCs in the alveolar passage than DR3<sup>ko</sup> OVA mice

BAL fluid isolated from DR3<sup>wt</sup> OVA mice displayed increased cell numbers in numerous myeloid cell subsets in comparison to DR3<sup>ko</sup> OVA mice at both 24 and 72hrs post inhalation. At 24hrs post inhalation, DR3<sup>wt</sup> OVA mice had significantly more 7/4<sup>+</sup> monocytes (14.4±7.8 x10<sup>4</sup>) and myeloid DCs (6.5±3.5 x10<sup>3</sup>) than DR3<sup>ko</sup> OVA (7/4<sup>+</sup> monocytes 0.1±0.1 x10<sup>4</sup>, mDCs 0.2±0.1 x10<sup>3</sup>) and DR3<sup>ko</sup> PBS mice (7/4<sup>+</sup> monocytes 0.2±0.1 x10<sup>4</sup>, mDCs 0.3±0.1 x10<sup>3</sup>) (p=0.017) (p=0.018) (Figure 4.7). Similarly, there were 3.0±1.7 x10<sup>4</sup> eosinophils within the DR3<sup>wt</sup> OVA alveolar passage, significantly more than the 0.1±0.02 x10<sup>4</sup> in DR3<sup>ko</sup> PBS mice (p=0.031) (Figure 4.7). In contrast, DR3<sup>wt</sup> OVA and DR3<sup>ko</sup> OVA groups displayed comparable numbers of alveolar macrophages.

Myeloid cell subset numbers at 72hrs post inhalation were akin to those seen at 24hrs. DR3<sup>wt</sup> OVA BAL was observed to contain significantly more 7/4<sup>+</sup> monocytes (4.7±1.3 x10<sup>3</sup>) and myeloid DCs (2.3±0.6 x10<sup>3</sup>) than DR3<sup>ko</sup> OVA BAL fluid (7/4<sup>+</sup> monocytes 0.2±0.04 x10<sup>3</sup>, mDCs 0.4±0.1 x10<sup>3</sup>), with the difference being significant for both the monocyte (p=0.015) and dendritic cell subset (p=0.038) (Figure 4.8).
Figure 4.7. Myeloid cell subsets from the BAL of DR3<sup>wt</sup> and DR3<sup>ko</sup> mice 24hrs post acute allergic lung inflammation. Mice were sacrificed 24hr post final inhalation challenge with either OVA or PBS. Alveolar cells were isolated via lavage and subsets determined using antibody cell markers. Alveolar Macrophages n.s.d, Neutrophils n.s.d, Eosinophils (*p=0.031), 7/4<sup>+</sup> Monocytes n.s.d, 7/4<sup>-</sup> Monocytes (*p=0.017), Myeloid DCs (*p=0.018). Values represent mean ± SEM. Significance determined using 1 way ANOVA and Bonferroni post test; *p<0.05. Each symbol represents data from a single mouse.
Figure 4.8. Myeloid cell subsets from the BAL of DR3<sup>wt</sup> and DR3<sup>ko</sup> mice 72hrs post acute allergic lung inflammation. Mice were sacrificed 72hr post final inhalation challenge with either OVA or PBS. Alveolar cells were isolated via lavage and subsets determined using antibody cell markers. Alveolar Macrophages (*p=0.0396), Neutrophils n.s.d, Eosinophils (*p=0.035), 7/4<sup>+</sup> Monocytes n.s.d, 7/4<sup>-</sup> Monocytes (*p=0.015), Myeloid DCs (*p=0.0378). Values represent mean ± SEM. Significance determined using 1 way ANOVA and Bonferroni post test; *p<0.05. Each symbol represents data from a single mouse.
4.3.1.2.3 DR3\textsuperscript{wt} OVA mice had significantly more CD4\textsuperscript{T} T cells in the alveolar passage than DR3\textsuperscript{ko} OVA treated mice

At 24hrs post inhalation, DR3\textsuperscript{wt} OVA treated mice displayed increased numbers of multiple lymphocytic subset examined in comparison to DR3\textsuperscript{ko} OVA mice, including NKT cells, which displayed an overall p value of 0.034, and B cells (\(p=0.041\)). When examining NK cells, significance was only detected between DR3\textsuperscript{wt} OVA (15.0±5.8 x10\textsuperscript{3}) and DR3\textsuperscript{ko} PBS mice (1.9±0.9 x10\textsuperscript{3}) (\(p=0.024\)) (Figure 4.9). Analysis of CD3\textsuperscript{+} T cells showed significantly higher numbers in DR3\textsuperscript{wt} OVA lavage fluid at 37.0±8.2 x10\textsuperscript{4}, compared to 2.2±0.1 x10\textsuperscript{4} in DR3\textsuperscript{ko} OVA, 5.2±1.9 x10\textsuperscript{4} in DR3\textsuperscript{wt} PBS and 8.4±3.1 x10\textsuperscript{4} in DR3\textsuperscript{ko} PBS mice (Figure 4.9) (\(p<0.0001\)). CD4\textsuperscript{+} T cells and CD8\textsuperscript{+} T cells were higher in DR3\textsuperscript{wt} OVA treated animals (CD4\textsuperscript{+} T cells 24.4±6.4 x10\textsuperscript{3}, CD8\textsuperscript{+} T cells 11.0±1.8 x10\textsuperscript{3}) than DR3\textsuperscript{ko} OVA (CD4\textsuperscript{+} T cells 1.3±0.4 x10\textsuperscript{3}, CD8\textsuperscript{+} T cells 0.7±0.4 x10\textsuperscript{3}) (\(p=0.0002\) and \(p<0.0001\), respectively) (Figure 4.9). Likewise, DR3\textsuperscript{wt} OVA mice had considerably more activated T cells than DR3\textsuperscript{ko} OVA mice, for both activated CD4\textsuperscript{+} (DR3\textsuperscript{wt} OVA 13.1±3.7 x10\textsuperscript{3}, DR3\textsuperscript{ko} OVA 1.0±0.4 x10\textsuperscript{3}) (\(p=0.0003\) and \(p<0.0001\), respectively) (Figure 4.9). Similarly to the 24hr timepoint, more lymphocytes were isolated from the BAL fluid of DR3\textsuperscript{wt} OVA mice than DR3\textsuperscript{ko} OVA 72hr post inhalation. DR3\textsuperscript{wt} OVA mice displayed increased numbers of CD3\textsuperscript{+} T cells, at 14.7±3.7 x10\textsuperscript{3} compared to 1.0±0.5 x10\textsuperscript{3} and 3.3±0.4 x10\textsuperscript{3} in DR3\textsuperscript{ko} OVA and DR3\textsuperscript{wt} PBS mice, respectively (\(p=0.008\)) (Figure 4.10). Equally, DR3\textsuperscript{wt} OVA lavage fluid contained significantly more CD4\textsuperscript{+} T cells (12.2±2.9 x10\textsuperscript{3}) than DR3\textsuperscript{ko} OVA (2.1±0.7 x10\textsuperscript{3}) and DR3\textsuperscript{wt} PBS (2.3±0.3 x10\textsuperscript{3}) groups.
Interestingly however, no significant differences were seen when studying CD8⁺ T cells or B cells. One-way ANOVA analyses indicated significant differences in NKT cell number between DR3⁺ OVA mice (15.6±6.7 x10³) and other treatment groups (DR3⁻ OVA 1.3±0.6 x10³, DR3⁺ PBS 2.1±0.3 x10³, DR3⁻ PBS 2.7 ± 2.2 x10³) ($p=0.018$), as well as NK cells, whereby significance was noted between DR3⁺ OVA (15.0±5.8 x10³) and DR3⁻ OVA (3.4±2.6 x10³) mice ($p=0.015$) (Figure 4.10). Overall, DR3⁺ OVA treated mice presented with consistently higher numbers of lymphocyte cell subsets, particularly those of CD4⁺ origin.

In summary, DR3⁻ mice display reduced numbers of multiple cell subsets in both the lung and BAL following the induction of acute allergic lung inflammation (Table 4.1).
Figure 4.9. Lymphocyte subsets from the BAL of DR3wt and DR3ko mice 24hrs post acute allergic lung inflammation. Mice were sacrificed 24hr post final inhalation challenge with either OVA or PBS. Alveolar cells were isolated via lavage and subsets determined using antibody cell markers. CD3+ T cells (***p<0.0001), CD4+ T cells (***p=0.0002), CD8+ T cells (***p<0.0001), Activated CD4+ T cells (***p=0.0003), Activated CD8+ T cells (**p=0.005), NKT cells (*p=0.034), NK cells (*p=0.024), B cells (*p=0.041) Values represent mean ± SEM. Significance determined using 1 way ANOVA and Bonferroni post test; *p<0.05, **p<0.01, ***p<0.001. Each symbol represents data from a single mouse.
Figure 4.10. Lymphocyte subsets from the BAL of DR3<sup>wt</sup> and DR3<sup>ko</sup> mice 72hrs post acute allergic lung inflammation. Mice were sacrificed 72hr post final inhalation challenge with either OVA or PBS. Alveolar cells were isolated via lavage and subsets determined using antibody cell markers. CD3<sup>+</sup> T cells (**p=0.008), CD4<sup>+</sup> T cells (*p=0.010), CD8<sup>+</sup> T cells n.s.d, NKT cells (*p=0.018), NK cells (*p=0.015), B cells n.s.d Values represent mean ± SEM. Significance determined using 1 way ANOVA and Bonferroni post test; *p<0.05, **p<0.01. Each symbol represents data from a single mouse.
Table 4.1 Summary of significant cell differences between DR3<sup>wt</sup> and DR3<sup>ko</sup> mice in the lung and BAL at 24hrs and 72hrs post inhalation

<table>
<thead>
<tr>
<th>Cell subset</th>
<th>Lung* Significance&lt;sup&gt;$&lt;/sup&gt;</th>
<th>Bronchoalveolar Lavage&lt;sup&gt;^&lt;/sup&gt; Significance&lt;sup&gt;$&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24hr&lt;sup&gt;Δ&lt;/sup&gt;</td>
<td>72hrs&lt;sup&gt;¥&lt;/sup&gt;</td>
</tr>
<tr>
<td>Myeloid DCs</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7/4&lt;sup&gt;-&lt;/sup&gt; Monocytes</td>
<td>*</td>
<td>-</td>
</tr>
<tr>
<td>CD3&lt;sup&gt;+&lt;/sup&gt; T cells</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CD4&lt;sup&gt;+&lt;/sup&gt; T cells</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CD8&lt;sup&gt;+&lt;/sup&gt; T cells</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Act. CD4&lt;sup&gt;+&lt;/sup&gt; T cells</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Act. CD8&lt;sup&gt;+&lt;/sup&gt; T cells</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NKT cells</td>
<td>**</td>
<td>-</td>
</tr>
<tr>
<td>NK cells</td>
<td>*</td>
<td>-</td>
</tr>
<tr>
<td>B cells</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Corresponds to cells isolated from the lung

^ Corresponds to cells isolated from the alveolar passage

Δ Corresponds to cells harvested 24hrs post inhalation

¥ Corresponds to cells harvested 72hrs post inhalation

† N/A corresponds to subsets not being tested

<sup>$</sup> Significance between DR3<sup>wt</sup> and DR3<sup>ko</sup> cell numbers measured by 1 way ANOVA. Values represent the degree of significance (* p<0.05, ** p<0.01, *** p<0.001) as measured by 1 way ANOVA between DR3<sup>wt</sup> and DR3<sup>ko</sup> mice in cell subsets from both the lung and BAL 24hrs and 72hrs post inhalation.
4.3.1.3 Analysis of DR3 expression in the lung

4.3.1.3.1 DR3wt OVA CD4+ T cells expressed significantly more DR3 than DR3wt PBS challenged lymphocytes

DR3 expression was determined on particular lymphocyte populations 24hrs post inhalation by dividing the test MFI by that of the isotype control, thus giving a relative DR3 fluorescence signal (RF). Results showed that CD4+ T cells from DR3wt OVA treated mice had an RF of 9.8±0.4, significantly more than the 8.2±0.4 exhibited by DR3wt PBS mice \((p<0.001)\) (Figure 4.11) (Table 4.2). Moreover, this up-regulation of DR3 corresponded to increased expression on activated CD4+ T cells from DR3wt OVA cells (RF of 12.1±0.5) (Figure 4.11) (Table 4.2). Overall, activated CD4+ T cells exhibited more DR3 than naive CD44- CD4+ T cells \((p<0.01)\). Both naive and effector CD4+ T cell subsets of DR3wt genotype, expressed significantly higher signal than control DR3ko mice (CD4+ T cell \(p<0.0001\)) (activated CD4+ T cell \(p<0.0001\)) (Figure 4.11) (Table 4.2).

No significant differences were found between the RF signals of DR3wt OVA and PBS challenged animals when examining CD8+ T cells and activated CD8+ T cells, although again, both were significantly higher than DR3ko populations (CD8+ T cell \(p<0.0001\)) (activated CD8+ T cell \(p<0.0001\)) (Figure 4.11) (Table 4.2). DR3 expression was found to be similar between naive and activated CD8+ T cells, although both expressed less DR3 than CD4+ T cell subsets.
Figure 4.11. Ratio of DR3 MFI’s on T cell populations in the lung 24hrs post acute allergic lung inflammation. Mice were sacrificed 24hr post final inhalation challenge with either OVA or PBS. Cells were stained with DR3 antibody. Ratio DR3 was calculated by the MFI fold increase compared to control IgG antibody (n=5). Bars represent mean ± SEM. Results are significant using 2 way ANOVA (Interaction=***<0.0001, Genotype=***<0.0001, Cell Type= ***<0.0001).
4.3.1.3.2 DR3\textsuperscript{wt} OVA lungs expressed more DR3 than DR3\textsuperscript{wt} PBS lungs at 24 but not 72hrs post inhalation

DR3 expression was detected in naive DR3\textsuperscript{wt} lungs (Section 3.3.1.4.1), therefore expression levels following the induction of inflammation were investigated. Results showed that at 24hrs post inhalation, lungs from DR3\textsuperscript{wt} OVA treated mice expressed 6.5±3.0\% DR3, significantly more than DR3\textsuperscript{wt} PBS treated lungs (1.2±0.1\% ) (\(p<0.05\)) and isotype control samples (0.03±0.01\% ) (\(p<0.05\)) (Figure 4.12) (Table 4.2). DR3 expression was focused within the peribronchiolar areas of the lung, with occasional signal noted in the alveolar spaces. Importantly, DR3\textsuperscript{ko} OVA (0.6±0.1\% ) and DR3\textsuperscript{ko} PBS (0.3±0.1\% ) mice expressed significantly less signal than DR3\textsuperscript{wt} mice (\(p=0.042\)) (Figure 4.12), indicating staining was specific.

At 72hrs post inhalation, no significant differences were observed in DR3 expression between DR3\textsuperscript{wt} OVA (4.3±2.3\% ) and DR3\textsuperscript{wt} PBS lungs (1.9±1.0\% ) (\(p>0.05\)) (Figure 4.13) (Table 4.2), although DR3\textsuperscript{wt} mice did exhibit higher signal than control DR3\textsuperscript{ko} mice (\(p=0.042\)) (Figure 4.13). Therefore, whilst inhalation treatment is significant at 24hrs (\(p=0.032\)), affecting the expression levels of DR3 between DR3\textsuperscript{wt} OVA and PBS treated mice, this trend did not continue to 72hrs post inhalation.
Figure 4.12. **DR3 expression 24hrs post acute allergic lung inflammation.** Mice were sacrificed 24hr post final inhalation challenge with either OVA or PBS. Lungs were stained with DR3 antibody. Shown are representative photos of DR3\textsuperscript{wt} OVA, DR3\textsuperscript{ko} OVA DR3\textsuperscript{wt} PBS, DR3\textsuperscript{ko} PBS, DR3\textsuperscript{wt} isotype and DR3\textsuperscript{ko} isotype (Scale bar=100\textmu m). DR3 expression in the lung was analysed using 5 fields of view of each lung and measuring the \% of positive (brown) in each field of view. This was then averaged for each mouse (n=5). Analysis was performed using Leica Qwin V3 Software.. Bars represent mean ± SEM. Results are significant using 2 way ANOVA whereby *p<0.05 (Interaction=0.064, Genotype=*0.042, Inhalation Treatment= * 0.032).
Figure 4.13. DR3 expression 72hrs post acute allergic lung inflammation. Mice were sacrificed 72hr post final inhalation challenge with either OVA or PBS. Lungs were stained with DR3 antibody. Shown are representative photos of DR3\textsuperscript{wt} OVA, DR3\textsuperscript{ko} OVA, DR3\textsuperscript{wt} PBS, DR3\textsuperscript{ko} PBS, DR3\textsuperscript{wt} isotype and DR3\textsuperscript{ko} isotype (Scale bar=100\,\mu m). DR3 expression in the lung was analysed using 5 fields of view of each lung and measuring the % of positive (brown) in each field of view. This was then averaged for each mouse (n=5). Analysis was performed using Leica Qwin V3 Software. Bars represent mean ± SEM. Results are significant using 2 way ANOVA whereby *p<0.05 (Interaction=0.220, Genotype=*0.042, Inhalation treatment=0.196).
### Table 4.2 Summary of DR3 expression

<table>
<thead>
<tr>
<th>Cell Subset</th>
<th>DR3&lt;sup&gt;wt&lt;/sup&gt;</th>
<th>DR3&lt;sup&gt;ko&lt;/sup&gt;</th>
<th>Significance&lt;sup&gt;$&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>% DR3 in Lung (72hrs)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1.9 ± 1.0</td>
<td>4.4 ± 2.3</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>% DR3 in Lung&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1.1 ± 0.2</td>
<td>6.5 ± 3.0</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>DR3 MFI ratio on CD4&lt;sup&gt;+&lt;/sup&gt; T cells</td>
<td>8.2 ± 0.4</td>
<td>9.8 ± 0.4</td>
<td>2.0 ± 0.1</td>
</tr>
<tr>
<td>DR3 MFI ratio on CD8&lt;sup&gt;+&lt;/sup&gt; T cells</td>
<td>3.6 ± 0.1</td>
<td>3.6 ± 0.1</td>
<td>1.9 ± 0.1</td>
</tr>
<tr>
<td>DR3 MFI ratio on Activated CD4&lt;sup&gt;+&lt;/sup&gt; T cells</td>
<td>10.9 ± 0.8</td>
<td>12.1 ± 0.5</td>
<td>1.9 ± 0.04</td>
</tr>
<tr>
<td>DR3 MFI ratio on Activated CD8&lt;sup&gt;+&lt;/sup&gt; T cells</td>
<td>3.6 ± 0.2</td>
<td>4.4 ± 0.2</td>
<td>2.0 ± 0.3</td>
</tr>
</tbody>
</table>

<sup>e</sup> % DR3 expression within the naive lungs of DR3<sup>wt</sup> and DR3<sup>ko</sup> mice via IHC

*PBS corresponds to mice challenged via inhalation with PBS

^ OVA corresponds to mice challenged via inhalation with OVA

$ Significance between DR3<sup>wt</sup> PBS and DR3<sup>wt</sup> OVA groups using 1 way ANOVA and Bonferroni post test. N.S.D= no significant difference, *p<0.05, **p<0.01.

Median fluorescence intensity (MFI) ratio calculated by dividing the test median by the isotype following gating on the specified population ± SEM following flow cytometry. All analysis shown from 24hr post inhalation unless otherwise stated.
4.3.1.4 Levels of chemoattractants in the BAL fluid of DR3\textsuperscript{wt} and DR3\textsuperscript{ko} mice

4.3.1.4.1 Chemokine levels were analogous between DR3\textsuperscript{wt} and DR3\textsuperscript{ko} mice following acute allergic lung inflammation

To assess whether recruitment was responsible for the differences in cell numbers, chemoattractant levels were tested in the BAL fluid following acute allergic lung inflammation. Multiple chemokines reported to be involved in the recruitment of both myeloid (CXCL1, CCL3, CCL4, CCL5) and lymphocyte populations (CXCL2, CXCL10, CXCL13) were analysed. No significant differences were found between any treatment groups for any of the chemokines tested either 24 or 72hrs after the final inhalation challenge (Figure 4.14) (Figure 4.15) (Table 4.3). CXCL1, CCL3, CCL4 and CCL5 were below the detection level of the ELISA assays used (Table 4.3).
Figure 4.14. Chemokine levels within the BAL of DR3wt and DR3ko mice 24hrs post acute allergic lung inflammation. Mice were sacrificed 24hr post final inhalation challenge with either OVA or PBS. BAL fluid was isolated and the supernatant used to determine chemokine levels using ELISA. CXCL2 n.s.d, CXCL10 n.s.d and CXCL13 n.s.d. No significant differences seen. Significance determined using 1 way ANOVA and Bonferroni post test Bars represent mean ± SEM (n=5).
Figure 4.15. Chemokine levels within the BAL of DR3\textsuperscript{wt} and DR3\textsuperscript{ko} mice 72hrs post acute allergic lung inflammation. Mice were sacrificed 72hr post final inhalation challenge with either OVA or PBS. BAL fluid was isolated and the supernatant used to determine chemokine levels using ELISA. CXCL2 n.s.d, CXCL10 n.s.d and CXCL13 n.s.d. No significant differences seen. Significance determined using 1 way ANOVA and Bonferroni post test Bars represent mean ± SEM (n=5).
Table 4.3 Chemoattractant levels in BAL fluid at 24 and 72hrs post inhalation

<table>
<thead>
<tr>
<th>Chemokine (pg/ml)</th>
<th>DR3\textsuperscript{wt}</th>
<th></th>
<th>DR3\textsuperscript{ko}</th>
<th></th>
<th>Significance\textsuperscript{$}</th>
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<tr>
<td></td>
<td>PBS\textsuperscript{*}</td>
<td>OVA\textsuperscript{^}</td>
<td>PBS\textsuperscript{*}</td>
<td>OVA\textsuperscript{^}</td>
<td></td>
</tr>
<tr>
<td>24hrs\textsuperscript{\varepsilon}</td>
<td>CXCL2</td>
<td>90±21</td>
<td>107±7</td>
<td>144±55</td>
<td>85±18</td>
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<tr>
<td></td>
<td>CXCL10</td>
<td>310±36</td>
<td>342±26</td>
<td>359±72</td>
<td>275±33</td>
</tr>
<tr>
<td></td>
<td>CXCL13</td>
<td>229±34</td>
<td>275±52</td>
<td>456±180</td>
<td>292±39</td>
</tr>
<tr>
<td>72hrs\textsuperscript{\textsection}</td>
<td>CXCL2</td>
<td>191±25</td>
<td>219±28</td>
<td>281±115</td>
<td>223±28</td>
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<tr>
<td></td>
<td>CXCL10</td>
<td>419±61</td>
<td>412±94</td>
<td>641±314</td>
<td>477±49</td>
</tr>
<tr>
<td></td>
<td>CXCL13</td>
<td>296±47</td>
<td>352±62</td>
<td>677±348</td>
<td>358±35</td>
</tr>
</tbody>
</table>

* PBS corresponds to mice challenged via inhalation with PBS

^ OVA corresponds to mice challenged via inhalation with OVA

\varepsilon 24hrs corresponds to mice harvested 24hrs after the final inhalation challenge

\$ 72hrs corresponds to mice harvested 72hrs after the final inhalation challenge

\$ Significance between DR3\textsuperscript{wt} OVA and DR3\textsuperscript{ko} OVA groups using 1 way ANOVA and Bonferroni post test. N.S.D = no significant difference.
4.3.2 Airway remodelling following acute allergic lung inflammation

4.3.2.1 Analysis of airway remodelling and lung pathology

4.3.2.1.1 Lung pathology was comparable between DR3\textsuperscript{wt} and DR3\textsuperscript{ko} OVA treated mice

Lungs were analysed post inflammation for the development of pathology as judged against a scoring regime (Section 2.2.12.2). Results indicated that DR3 had no role in the progression of lung pathology at this early stage, as no significant differences were seen between DR3\textsuperscript{wt} and DR3\textsuperscript{ko} OVA mice at either 24 or 72hr post inhalation (Figure 4.16) (Figure 4.17) (Table 4.4). At 72hrs post inhalation however, DR3\textsuperscript{wt} OVA mice (3.8±0.4) were judged to have significantly worse lung pathology, evident by peribronchial and perivascular cuffing, compared to PBS treated groups (DR3\textsuperscript{wt} 2.0±0.6, DR3\textsuperscript{ko} 1.4±0.3) (p=0.002) (Figure 4.17) (Table 4.4).
Figure 4.16. Lung pathology in DR3<sup>wt</sup> and DR3<sup>ko</sup> mice 24hrs post acute allergic lung inflammation. Mice were sacrificed 24hr post final inhalation challenge with either OVA or PBS. Lungs were stained with Haematoxylin and Eosin. Shown are representative photos of DR3<sup>wt</sup> OVA, DR3<sup>ko</sup> OVA, DR3<sup>wt</sup> PBS and DR3<sup>ko</sup> PBS lungs (Scale bar=250μm). Lungs were blind scored by 2 individuals for pathology and an average taken for each lung. (A). Values represent mean ± SEM. Results are not significant using 1 way ANOVA. Each symbol represents data from a single mouse.
Figure 4.17. Lung pathology in DR3\textsuperscript{wt} and DR3\textsuperscript{ko} mice 72hrs post acute allergic lung inflammation. Mice were sacrificed 72hr post final inhalation challenge with either OVA or PBS. Lungs were stained with Haematoxylin and Eosin. Shown are representative photos of DR3\textsuperscript{wt} OVA, DR3\textsuperscript{ko} OVA, DR3\textsuperscript{wt} PBS and DR3\textsuperscript{ko} PBS lungs (Scale bar=100μm). Lungs were blind scored by 2 individuals for pathology and an average taken for each lung. (A). Results are significant using 1 way ANOVA (**p=0.002) and Bonferroni post test; *p<0.05, **p<0.01. Values represent mean ± SEM. Each symbol represents data from a single mouse.
4.3.2.1.2 Mucin associated goblet cell number following acute allergic lung inflammation

Goblet cell hyperplasia is a marker of airway remodeling and a major feature of the asthmatic response. Analysis of goblet cells following acute allergic lung inflammation indicated no significant differences between any treatment groups at 24hrs post inhalation. However, both DR3\textsuperscript{wt} and DR3\textsuperscript{ko} OVA treated mice averaged higher at 1.3±0.9\% and 2.2±1.9\% compared to DR3\textsuperscript{wt} PBS (0.06±0.06\%) and DR3\textsuperscript{ko} PBS mice, where no mucin positive areas were identified (Figure 4.18) (Table 4.4). At 72hrs post inhalation, DR3\textsuperscript{wt} OVA lungs were calculated as having 10.3±5.4\% mucin positive areas, compared to just 0.8±0.4\% in DR3\textsuperscript{ko} OVA mice, 0.2±0.2\% in DR3\textsuperscript{wt} PBS animals and none in DR3\textsuperscript{ko} PBS mice (Figure 4.19) (Table 4.4). This was deemed to be significant overall by 1 way ANOVA ($p=0.043$).
Figure 4.18. Goblet cell hyperplasia in DR3\textsuperscript{wt} and DR3\textsuperscript{ko} mice 24hrs post acute allergic lung inflammation. Mice were sacrificed 24hr post final inhalation challenge with either OVA or PBS. Lungs were stained with Periodic Acid Schiff. Shown are representative photos of DR3\textsuperscript{wt} OVA, DR3\textsuperscript{ko} OVA, DR3\textsuperscript{wt} PBS and DR3\textsuperscript{ko} PBS lungs (Scale bar=100μm). The area of PAS\textsuperscript{+} cells was taken as a % of the airway surround using Leica Qwin V3 software, and an average calculated for each lung (A). Values represent mean ± SEM. Results are not significant using 1 way ANOVA. Each symbol represents data from a single mouse.
Figure 4.19. Goblet cell hyperplasia in DR3<sup>wt</sup> and DR3<sup>ko</sup> mice 72hrs post acute allergic lung inflammation. Mice were sacrificed 72hr post final inhalation challenge with either OVA or PBS. Lungs were stained with Periodic Acid Schiff. Shown are representative photos of DR3<sup>wt</sup> OVA, DR3<sup>ko</sup> OVA, DR3<sup>wt</sup> PBS and DR3<sup>ko</sup> PBS lungs (Scale bar=100 μm). The area of PAS+ cells was taken as a % of the airway surround using Leica Qwin V3 software, and an average calculated for each lung. (A). Results are significant using 1 way ANOVA (*p=0.043) whereby *p<0.05. Values represent mean ± SEM. Each symbol represents data from a single mouse.
4.3.2.1.3 Lung fibrosis scores were comparable between DR3<sup>wt</sup> and DR3<sup>ko</sup> OVA treated mice

Lung fibrosis was measured by comparing the levels of collagen as an indication of alterations to the lung architecture following acute allergen induced inflammation. These levels were then scaled against the Ashcroft Score of fibrosis (Ashcroft, Simpson et al. 1988). There was no significant difference in the degree of fibrosis at 24hrs post inhalation between OVA challenged mice (DR3<sup>wt</sup> 1.6±0.2, DR3<sup>ko</sup> 1.2±0.5) (Figure 4.20) (Table 4.4). Likewise, at the 72hr timepoint, DR3<sup>wt</sup> and DR3<sup>ko</sup> OVA treated mice displayed equivalent scores of fibrosis, although levels in both were significantly higher than scores in PBS treated mice (DR3<sup>wt</sup> OVA 2.6±0.2, DR3<sup>ko</sup> OVA 2.7±0.2, DR3<sup>wt</sup> PBS 1.2±0.2, DR3<sup>ko</sup> PBS 0.7±0.3) (p<0.0001) (Figure 4.21) (Table 4.4)
Figure 4.20. Fibrosis in DR3<sup>wt</sup> and DR3<sup>ko</sup> mice 24hrs post acute allergic lung inflammation. Mice were sacrificed 24hr post final inhalation challenge with either OVA or PBS. Lungs were stained with Van Gieson solution. Shown are representative photos of DR3<sup>wt</sup> OVA, DR3<sup>ko</sup> OVA, DR3<sup>wt</sup> PBS and DR3<sup>ko</sup> PBS lungs (Scale bar=100μm). Lungs were blind scored by 2 individuals using Ashcroft score of fibrosis and an average taken for each lung. (A). Values represent mean ± SEM. Results are not significant using 1 way ANOVA. Each symbol represents data from a single mouse.
Figure 4.21. Fibrosis in DR3^{wt} and DR3^{ko} mice 72hrs post acute allergic lung inflammation. Mice were sacrificed 72hr post final inhalation challenge with either OVA or PBS. Lungs were stained with Van Gieson solution. Shown are representative photos of DR3^{wt} OVA, DR3^{ko} OVA, DR3^{wt} PBS and DR3^{ko} PBS lungs (Scale bar=100μm). Lungs were blind scored by 2 individuals using Ashcroft score of fibrosis and an average taken for each lung (A). Results are significant using 1 way ANOVA (**p<0.001) and Bonferroni post test; *p<0.05, **p<0.01, ***p<0.001. Values represent mean ± SEM. Each symbol represents data from a single mouse.
Table 4.4 Lung pathology and degree of staining following histological staining 24 and 72hrs post inhalation

<table>
<thead>
<tr>
<th>Stain</th>
<th>DR3\textsuperscript{wt}</th>
<th>DR3\textsuperscript{ko}</th>
<th>Significance\textsuperscript{5}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PBS\textsuperscript{*}</td>
<td>OVA\textsuperscript{*}</td>
<td>PBS\textsuperscript{*}</td>
</tr>
<tr>
<td>24hrs\textsuperscript{ε}</td>
<td>H&amp;E</td>
<td>2.0±0.6</td>
<td>3.2±0.5</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>PAS</td>
<td>0.06±0.06</td>
<td>1.3±0.9</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>VG</td>
<td>1.2±0.2</td>
<td>1.6±0.2</td>
</tr>
<tr>
<td></td>
<td>++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>72hrs\textsuperscript{¥}</td>
<td>H&amp;E</td>
<td>2.0±0.6</td>
<td>3.8±0.4</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>PAS</td>
<td>0.2±0.2</td>
<td>10.3±5.4</td>
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<tr>
<td></td>
<td>+</td>
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<tr>
<td></td>
<td>VG</td>
<td>1.2±0.2</td>
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</tbody>
</table>

* PBS corresponds to mice challenged via inhalation with PBS

\^ OVA corresponds to mice challenged via inhalation with OVA

\ε 24hrs corresponds to mice harvested 24hrs after the final inhalation challenge

\¥ 72hrs corresponds to mice harvested 72hrs after the final inhalation challenge

\textsuperscript{5} Significance between DR3\textsuperscript{wt} OVA and DR3\textsuperscript{ko} OVA groups using 1 way ANOVA and Bonferroni post test. N.S.D = no significant difference, *p<0.05.

An arbitrary method of staining intensity was used with + meaning low and +++ meaning high

H&E scores are representative of a scoring regime found in Chapter 2, PAS scores represent the % of PAS+ areas as a % of the lung, VG scores are representative of the Ashcroft score of fibrosis, found in Chapter 2.
4.3.3 The systemic response in acute allergic lung inflammation

4.3.3.1 Splenic responses in acute allergic lung inflammation

4.3.3.1.1 Splenocyte total cell number was significantly higher in DR3\textsuperscript{wt} OVA mice compared to DR3\textsuperscript{ko} OVA mice

Total cell number within the spleen was analysed following acute allergic lung inflammation. At 24hrs post inhalation, DR3\textsuperscript{wt} OVA treated mice contained 13.2±1.0 $\times 10^7$ splenocytes, significantly greater than the 9.5±0.3 $\times 10^7$ in DR3\textsuperscript{ko} PBS ($p<0.05$) and 9.1±0.9 $\times 10^7$ in DR3\textsuperscript{ko} OVA animals ($p=0.005$) (Figure 4.22).
Figure 4.22. Total leukocyte number from the spleens of DR3\textsuperscript{wt} and DR3\textsuperscript{ko} mice following acute allergic lung inflammation. Mice were sacrificed 24hrs post final inhalation challenge with either OVA or PBS. Splenocytes were extracted via mashing of the spleen and counted using a haemocytometer Results significant using 1 way ANOVA and Bonferroni post test (**p= 0.005) whereby *p<0.05, **p<0.01. Values represent mean ± SEM. Each symbol represents data from a single mouse.
4.3.3.1.2 Myeloid cell subsets in the spleen were significantly higher in DR3\textsuperscript{wt} mice

Multiple myeloid cell subset numbers were higher in DR3\textsuperscript{wt} OVA spleens at 24hrs post inhalation, although in the case of macrophages and eosinophils, this increase was not significant (Figure 4.23). However, significant increases were seen in DR3\textsuperscript{wt} OVA 7/4\textsuperscript{+} monocytes and plasmacytoid DCs in comparison to all other treatment groups, and myeloid DCs in comparison to DR3\textsuperscript{ko} mice. DR3\textsuperscript{wt} OVA mice had 3.8±0.4 x10\textsuperscript{6} 7/4\textsuperscript{+} monocytes, compared to 1.8±0.1 x10\textsuperscript{6} in DR3\textsuperscript{ko} OVA mice, 1.5±0.2 x10\textsuperscript{6} in DR3\textsuperscript{ko} PBS and 2.6±0.2 x10\textsuperscript{6} in DR3\textsuperscript{wt} PBS mice (p<0.0001) (Figure 4.23). Similarly, DR3\textsuperscript{wt} OVA mice had 6.0±0.7 x10\textsuperscript{5} plasmacytoid DCs, significantly higher than the 2.7±0.3 x10\textsuperscript{5} in DR3\textsuperscript{ko} OVA, 2.8±0.3 x10\textsuperscript{5} in DR3\textsuperscript{ko} PBS and 3.9±0.2 x10\textsuperscript{5} in DR3\textsuperscript{wt} PBS mice (p=0.0001) (Figure 4.23). DR3\textsuperscript{wt} OVA myeloid DCs (7.2±1.0 x10\textsuperscript{5}) too were statistically higher in number compared to DR3\textsuperscript{ko} OVA (3.8±0.6 x10\textsuperscript{5}) and DR3\textsuperscript{ko} PBS (3.7±0.6 x10\textsuperscript{5}) groups (p=0.004), but not DR3\textsuperscript{wt} PBS mice (5.2±0.2 x10\textsuperscript{5}) (Figure 4.23).

Interestingly, 7/4\textsuperscript{+} monocytes appeared to be increased in DR3\textsuperscript{ko} animals compared to DR3\textsuperscript{wt}, with significance between DR3\textsuperscript{ko} PBS (1.7±0.1 x10\textsuperscript{6}) and DR3\textsuperscript{wt} PBS mice (1.0±0.1 x10\textsuperscript{6}) (p=0.032). The same trend was also seen when analysing neutrophils, although no significance was noted (Figure 4.23).
**Figure 4.23. Myeloid cell subsets from the spleens of DR3wt and DR3ko mice 24hrs post acute allergic lung inflammation.** Mice were sacrificed 24hr post final inhalation challenge with either OVA or PBS. Splenocytes were isolated and subsets determined using antibody cell markers. Macrophages n.s.d, Neutrophils n.s.d, Eosinophils n.s.d, 7/4+ Monocytes (*p=0.032), 7/4- Monocytes (**p<0.0001), Myeloid DCs (**p=0.004), Plasmacytoid DCs (**p=0.0001). Values represent mean ± SEM. Significance determined using 1 way ANOVA and Bonferroni post test; *p<0.05, **p<0.01, ***p<0.001. Each symbol represents data from a single mouse.
4.3.3.1.3 Lymphocyte cell subsets in the spleen were significantly higher in DR3\textsuperscript{wt} mice

At 24hrs post inhalation, all lymphocyte cell subsets examined were significantly higher in DR3\textsuperscript{wt} OVA mice compared to all other treatment groups. This included CD3\textsuperscript{+} T cells (DR3\textsuperscript{wt} OVA 14.1±1.7 x10\textsuperscript{6}, DR3\textsuperscript{wt} PBS 8.6±0.9 x10\textsuperscript{6}, DR3\textsuperscript{ko} OVA 6.0±0.7 x10\textsuperscript{6}, DR3\textsuperscript{ko} PBS 7.7±0.8 x10\textsuperscript{6}) (\(p=0.0002\)), which were subdivided into CD4\textsuperscript{+} T cells (DR3\textsuperscript{wt} OVA 8.6±1.0 x10\textsuperscript{6}, DR3\textsuperscript{wt} PBS 5.0±0.5 x10\textsuperscript{6}, DR3\textsuperscript{ko} OVA 3.7±0.4 x10\textsuperscript{6}, DR3\textsuperscript{ko} PBS 4.8±0.5 x10\textsuperscript{6}) (\(p=0.0002\)), activated CD4\textsuperscript{+} T cells (DR3\textsuperscript{wt} OVA 2.7±0.5 x10\textsuperscript{6}, DR3\textsuperscript{wt} PBS 1.2±0.2 x10\textsuperscript{6}, DR3\textsuperscript{ko} OVA 1.0±0.2 x10\textsuperscript{6}, DR3\textsuperscript{ko} PBS 1.3±0.2 x10\textsuperscript{6}) (\(p=0.001\)), CD8\textsuperscript{+} T cells (DR3\textsuperscript{wt} OVA 4.3±0.9 x10\textsuperscript{6}, DR3\textsuperscript{wt} PBS 2.1±0.4 x10\textsuperscript{6}, DR3\textsuperscript{ko} OVA 1.3±0.2 x10\textsuperscript{6}, DR3\textsuperscript{ko} PBS 1.8±0.2 x10\textsuperscript{6}) (\(p=0.002\)), activated CD8\textsuperscript{+} T cells (DR3\textsuperscript{wt} OVA 0.5±0.2 x10\textsuperscript{6}, DR3\textsuperscript{wt} PBS 0.2±0.04 x10\textsuperscript{6}, DR3\textsuperscript{ko} OVA 0.2±0.02 x10\textsuperscript{6}, DR3\textsuperscript{ko} PBS 0.2±0.03 x10\textsuperscript{6}) (\(p=0.002\)) and NKT cells (DR3\textsuperscript{wt} OVA 7.7±1.2 x10\textsuperscript{5}, DR3\textsuperscript{wt} PBS 3.4±0.4 x10\textsuperscript{5}, DR3\textsuperscript{ko} OVA 3.1±0.4 x10\textsuperscript{5}, DR3\textsuperscript{ko} PBS 3.2±0.3 x10\textsuperscript{5}) (\(p=0.0001\)) (Figure 4.24). NK cells were also higher in DR3\textsuperscript{wt} OVA mice (1.1±0.1 x10\textsuperscript{6}) compared to all other inhalation groups (DR3\textsuperscript{wt} PBS 0.7±0.04 x10\textsuperscript{6}, DR3\textsuperscript{ko} OVA 0.5±0.1 x10\textsuperscript{6}, DR3\textsuperscript{ko} PBS 0.6±0.1 x10\textsuperscript{6}) (\(p<0.0001\)), as were \(\gamma\delta\) T cells (DR3\textsuperscript{wt} OVA 5.9±0.8 x10\textsuperscript{5}, DR3\textsuperscript{wt} PBS 3.6±0.4 x10\textsuperscript{5}, DR3\textsuperscript{ko} OVA 2.5±0.2 x10\textsuperscript{5}, DR3\textsuperscript{ko} PBS 2.5±0.2 x10\textsuperscript{5}) (\(p=0.0001\)) (Figure 4.24).
Figure 4.24. Lymphocyte subsets from the spleens of DR3wt and DR3ko mice 24hrs post acute allergic lung inflammation. Mice were sacrificed 24hr post final inhalation challenge with either OVA or PBS. Splenocytes were isolated and subsets determined using antibody cell markers. CD3+ T cells (**p=0.0002), CD4+ T cells (**p=0.0002), CD8+ T cells (**p=0.002), Activated CD4+ T cells (**p=0.001), Activated CD8+ T cells (**p=0.002), NKT cells (**p=0.0001), NK cells (**p=0.0001), γδ T cells (**p=0.0001). Values represent mean ± SEM. Significance determined using 1 way ANOVA and Bonferroni post test; *p<0.05, **p<0.01, ***p<0.001. Each symbol represents data from a single mouse.
4.3.3.2 Analysis of DR3 expression in the spleen

4.3.1.3.1 DR3<sup>wt</sup> OVA CD4<sup>+</sup> T cells and NKT cells expressed significantly more DR3 than DR3<sup>wt</sup> PBS challenged lymphocytes

DR3 expression was examined on the surface of splenic T cells, many of which are known to express DR3 in the basal state (Chapter 3). Results showed that at 24hrs post inhalation, DR3<sup>wt</sup> OVA CD4<sup>+</sup> T cells exhibited an RF signal of 5.2±0.2, significantly more than DR3<sup>wt</sup> PBS treated mice (4.1±0.5) (<i>p<0.05</i>) (Figure 4.25). However, this up-regulation of DR3 on DR3<sup>wt</sup> OVA cells did not correspond to an increase in expression on activated CD4<sup>+</sup> T cells. Likewise, no significant differences were found between DR3<sup>wt</sup> OVA and PBS mice in DR3 expression on CD8<sup>+</sup> T cells or activated CD8<sup>+</sup> T cells. DR3<sup>wt</sup> OVA NKT cells were found to exhibit significantly higher levels of DR3, with an RF of 5.7±0.3, compared to 4.4±0.2 on DR3<sup>wt</sup> PBS NKT cells (<i>p<0.01</i>) (Figure 4.25). In all cell subsets examined, DR3<sup>wt</sup> mice showed significantly greater RF signals than DR3<sup>ko</sup> mice, indicating the specificity of the staining.
Figure 4.25. Ratio of DR3 MFI's on T cell populations in the spleen 24hrs post acute allergic lung inflammation. Mice were sacrificed 24hr post final inhalation challenge with either OVA or PBS. Cells were stained with DR3 antibody. Ratio DR3 was calculated by the MFI fold increase compared to control IgG antibody. Bars represent mean ± SEM (n=5). Results are significant using 2 way ANOVA (Interaction=***<0.0001, Genotype=***<0.0001, Cell Type= ***<0.0001).
4.3.3.3 The B cell response in acute allergic lung inflammation

4.3.3.3.1 B cell number and antibody production in acute allergic lung inflammation

Allergic antibody levels were measured in the serum by ELISA to determine whether a normal systemic immune response had been mounted following acute allergic lung inflammation. At the 24hr timepoint, no significant differences were seen between any treatment group in the levels of Total IgE (DR3\textsuperscript{wt} PBS 1701±944, DR3\textsuperscript{wt} OVA 299±100, DR3\textsuperscript{ko} PBS 378±284, DR3\textsuperscript{ko} OVA 1243±676) or OVA specific IgG (DR3\textsuperscript{wt} PBS 0.3±0.1, DR3\textsuperscript{wt} OVA 0.3±0.02, DR3\textsuperscript{ko} PBS 0.4±0.02, DR3\textsuperscript{ko} OVA 0.3±0.01), although both increased in comparison to naïve controls (Figure 4.26). This is despite higher numbers of B cells in the spleens of DR3\textsuperscript{wt} OVA mice (3.3±0.5 x10\textsuperscript{7}) compared to DR3\textsuperscript{wt} PBS (1.9±0.2 x10\textsuperscript{7}), DR3\textsuperscript{ko} OVA (1.3±0.2 x10\textsuperscript{7}) and DR3\textsuperscript{ko} PBS animals (1.7±0.2 x10\textsuperscript{7}) \( (p=0.0006) \) (Figure 4.26).
Figure 4.26. The B cell response of DR3\textsuperscript{wt} and DR3\textsuperscript{ko} mice 24hrs post acute allergic lung inflammation. Mice were sacrificed 24hr post final inhalation challenge with either OVA or PBS. (A) B cells were identified using specific antibody markers (***p=0.0006). Serum was isolated from blood obtained from cardiac puncture. Serum was tested for concentrations of (B) Total IgE (n.s.d) and (C) OVA specific IgG (n.s.d) by ELISA. Each point represents the mean of doublets from different mice. Values represent mean ± SEM. Significance determined using 1 way ANOVA and Bonferroni post test; *p<0.05, **p<0.01, ***p<0.001. Each symbol represents data from a single mouse.
4.4 Discussion

4.4.1 DR3 influence on airway inflammation

Following the induction of acute allergic lung inflammation, lung preparations from DR3\(^{\text{ko}}\) mice presented with reduced numbers of 7/4\(^{-}\) monocytes and NKT cells at 24hrs post inhalation, and NK cells 72hrs post inhalation. This is in contrast to published data from Meylan et al, who in addition to NKT cells, also found reduced percentages of CD3\(^{+}\) T cells, CD4\(^{+}\) T cells and eosinophils within the lung (Meylan, Davidson et al. 2008). There are several possible reasons for this discrepancy, one being the use of different sensitisation and aerosolisation protocols, which have previously been suggested as a reason for conflicting results within the literature of murine asthma models (Kumar and Foster 2002). Meylan et al sensitised mice with an OVA/Alum mix on days 0 and 7, followed by an intra-tracheal challenge on day 14 and intranasal challenge on day 15 (Meylan, Davidson et al. 2008). In this thesis, mice were sensitised with an OVA/Alum mix on days 0 and 5, followed by 2 OVA aerosolisation challenges on day 15. Furthermore, whilst Meylan et al harvested mice at 48 and 72hrs after the final challenge, the protocol employed for this Chapter used 24 and 72hr timepoints. The difference in the timing of assessment may also have contributed to divergent results.

Previous papers have reported DR3 to affect NKT cell function, with Fang et al suggesting DR3 co-stimulates IL-13 production from glycosphingolipid activated NKT cells (Fang, Adkins et al. 2008). NKT cells are considered instrumental to the allergic response, as NKT deficient mice fail to develop airway hyper-reactivity, airway eosinophilia and OVA specific IgE antibodies in comparison to WT littermates (Akbari,
Stock et al. 2003; Lisbonne, Diem et al. 2003). Moreover, Fang et al adoptively transferred splenic Dominant Negative (DN) DR3 transgenic NKT cells into OVA/Aluminum potassium sulphate primed NKT cell deficient mice prior to aerosol challenge. Results showed WT NKT cells could instigate eosinophil exudation and Th2 cytokine production, while DN DR3 NKT cells could not, thus demonstrating the importance of DR3 signals from NKT cells (Fang, Adkins et al. 2008). Korsgren et al however, suggest that it is NK cells alone and not NKT cells which drive allergic lung inflammation. CD1d mutant mice, deficient in NKT cells but normal NK cells, still developed lung tissue eosinophilia and allergen specific IgE similar to WT mice (Korsgren, Persson et al. 1999), implying further research is required on the role of these innate cells in allergic lung inflammation.

The function of DR3 on NK cells has not been broadly studied. Fang et al found DR3 expression on a sub-population of NK cells within the naive spleen (Fang, Adkins et al. 2008), although this observation could not be reproduced by us (Chapter 3) or by others (Twohig, Marsden et al. 2012). Using a model of MCMV in C57BL/6 mice, the kinetics of NK cell expansion were observed to be similar between DR3wt and DR3ko mice (Twohig, Marsden et al. 2012). However, using DBA-1 mice, which cannot mount efficient NK mediated resistance to MCMV, DR3ko mice displayed critical weight loss and increased viral titres in comparison to DR3wt mice, suggesting DR3 is crucial for survival in the absence of protective DR3 independent NK cell responses (Twohig, Marsden et al. 2012). Human peripheral blood derived NK cells have been shown to release IFNγ via TL1A synergisation with the IL-12/IL-18 pathway (Papadakis, Prehn et al. 2004). Heidemann et al went on to show that TL1A augments IL-12/IL-18 cytotoxicity against NK cell resistant tumor cell lines (Heidemann, Chavez et al. 2010).
These differences emphasise the variation that can arise when performing experiments in vitro compared to in vivo, as well as comparing the responses of human cells to those in a mouse model. Therefore, although numbers of NK cells increased in DR3wt mice following acute allergic lung inflammation at 72hrs post inhalation, the consequences of this are unclear.

Similar to published data, total cell numbers were higher in the BALF of DR3wt OVA challenged compared to DR3ko mice (Fang, Adkins et al. 2008; Meylan, Davidson et al. 2008). However, unlike Fang et al, who reported reduced eosinophil numbers using both DN DR3 transgenic mice and TL1A antibody blockade, no significant differences were noted in eosinophils using our model. This may again be due to the different protocols employed to induce allergic lung inflammation or alternative means of identifying eosinophils. Whilst flow cytometry was utilised to identify eosinophils in this thesis, defined as F4/80+ SSChi CD11b+, Fang et al used differential cell counts and therefore were reliant on cell morphology and microscopy (Fang, Adkins et al. 2008). Despite the lack of statistical difference in BALF eosinophil number, significant differences were seen in the numbers of 7/4− monocytes, myeloid DCs, CD3+ T cells and CD4+ T cells at 24 and 72hrs post inhalation along with CD8+ T cells 24hrs after the final challenge.

Acting as a transient reservoir of myeloid precursors, monocytes harbor the potential to differentiate into macrophages and dendritic cells (Gordon and Taylor 2005), including lung macrophages and pulmonary DCs under inflammatory conditions (Geissmann, Jung et al. 2003; Landsman, Varol et al. 2007; Varol, Landsman et al. 2007; Yona and
Jung 2010). When transferred into mice lacking the essential co-stimulatory molecules CD80 and CD86, monocytes were observed to restore the CD4^+ T cell response after inflammatory challenge (Landsman, Varol et al. 2007), suggesting monocyte derived DCs share the same priming properties as classical DCs. In asthmatic patients, allergen challenge led to the accumulation of myeloid DCs in the airways, concomitant with a reduction in circulating CD11c^+ cells, suggesting cells were recruited from the bloodstream (Jahnsen, Moloney et al. 2001). Given the plasticity and mobility of monocytes, the increase in 7/4^+ monocytes may account for the increase observed in myeloid DCs.

DCs act as a link between innate and adaptive immunity, sensitising T cells to inhaled allergen, as well as contributing to the inflammatory response via the up-regulation of co-stimulatory molecules and the re-activation of allergen primed T cells following challenge (Vermaelen and Pauwels 2003). DCs accumulate in the airway submucosa, forming multiple contacts with CD4^+ T cells (Constant, Brogdon et al. 2002; Huh, Strickland et al. 2003). Their depletion from the airways abolished the characteristic features of asthma, including eosinophilic inflammation, goblet cell hyperplasia, bronchial hyper-reactivity and Th2 cytokine release from CD4^+ T cells, all of which were restored upon adoptive transfer of DCs (van Rijt, Jung et al. 2005; Van Rijt and Lambrecht 2005). Using SCID mice engrafted with T cells from house dust mite allergic patients, it was shown that allergen pulsed monocyte derived DCs were sufficient to exacerbate inflammation when delivered to sensitised lungs, inducing Th2 effector function following migration to the mediastinal lymph nodes (Hammad, Lambrecht et al. 2002). Differentiated DCs have also recently been reported to proliferate within the lung after aeroallergen treatment, particularly within the epithelial and sub-epithelial layers of the airway mucosa (Veres, Voedisch et al. 2013).
Both human peripheral blood derived DCs and monocytes have been cited to release TL1A in response to FCγR stimulation as well as immune complexes (Cassatella, da Silva et al. 2007; Prehn, Thomas et al. 2007). Moreover, murine DCs have also been shown to produce TL1A (Bamias, Mishina et al. 2006) in response to the TLR stimulants LPS and Soluble Tachyzoite Antigen (STag), as well as cross linked IgG (Meylan, Davidson et al. 2008). The increased numbers of myeloid DCs within the DR3\textsuperscript{wt} allergic alveolar passage may therefore re-activate allergen primed T cells explaining the increased numbers of CD4\textsuperscript{+} and activated CD4\textsuperscript{+} T cells within the BALF.

DR3 was originally thought to be restricted to T cells (Chinnaiyan, Orourke et al. 1996; Kitson, Raven et al. 1996), thus several studies have concentrated on this aspect of DR3 function. Numerous reports have described reduced CD4\textsuperscript{+} T cell infiltration in DR3\textsuperscript{ko} mice during an immune response, in both systemic situations, whereby fewer CD4\textsuperscript{+} T cells were noted in the spleen in a Salmonella enterica bacterial model (Buchan, Taraban et al. 2012), and localised models such as EAE, where fewer CD4\textsuperscript{+} T cells were recorded in the spinal cord (Meylan, Davidson et al. 2008; Pappu, Borodovsky et al. 2008), implying DR3 is essential for CD4\textsuperscript{+} T cell effector responses. DR3\textsuperscript{ko} mice also exhibited reduced CD4\textsuperscript{+} T cells in the lung and BALF following acute allergic lung inflammation (Meylan, Davidson et al. 2008). Their depletion was found to abolish eosinophilic inflammation and airway hyper-reactivity (Gavett, Chen et al. 1994), emphasising the importance of CD4\textsuperscript{+} T cells for disease development.

DR3 expression was found to be higher on activated lung CD4\textsuperscript{+} T cells than naïve CD4\textsuperscript{+} T cells, although levels of both from DR3\textsuperscript{wt} OVA mice significantly increased in
comparison to DR3$^{wt}$ PBS mice. This up-regulation of DR3 may render OVA challenged CD4$^{+}$ T cells more responsive to TL1A. However, the use of the polyclonal antibody in this study does not distinguish between the different murine splice variants, as discussed in Chapter 3. Published data does suggest that the activation status of T cells determines the DR3 isoform expressed. Bamias et al measured mRNA levels of full length DR3 from the terminal ileum of inflamed SAMP1/YitFC and TNF$^{AARE}$ mice (Bamias, Mishina et al. 2006), concluding that activated cells up-regulated the full length DR3 isoform which correlate with the severity of inflammation. DR3 expression has also been shown to tally with disease phases in a model of experimental autoimmune uveitis (EAU) (Qin 2011). Inflammation was detected at day 7, peaked at day 14 and thereafter rapidly resolved, correlating with DR3 mRNA and protein expression on CD4$^{+}$ T cells from the draining lymph node. CD4$^{+}$ T cells from the peak of disease also produced more IL-17 when co-cultured with TL1A compared to cells isolated from the naïve or recovery phase (Qin 2011), emphasising the Receptor’s capacity to potentiate T cell responses. Therefore, the increase in DR3 expression observed on OVA challenged CD4$^{+}$ T cells could represent alterations in splice variant expression triggered by activation.

DR3 has previously been linked to cell activation via the NF-κB pathway (Migone, Zhang et al. 2002) and has more recently been shown to influence the cellular proliferation of CD4$^{+}$ T cells both in vitro, using suboptimal doses of anti-CD3 (Pappu, Borodovsky et al. 2008; Jones, Stumhofer et al. 2011), and in vivo (Buchan, Taraban et al. 2012), whereby BrdU incorporation was reduced in DR3$^{ko}$ CD4$^{+}$ T cells post Salmonella enterica Typhimurium infection. It has also been suggested that DR3 exerts a far greater proliferative effect over activated CD44$^{+}$ CD4$^{+}$ T cells compared to naïve.
CD44^+ cells (Bamias, Mishina et al. 2006). Furthermore, DR3^ko OT-II OVA specific T cells transferred into naïve congenic host mice failed to proliferate when challenged with OVA, corresponding with reduced numbers of inflammatory cells in the BAL (Meylan, Davidson et al. 2008). These experiments confirm that DR3 is necessary for the proliferation of locally re-stimulated T cells, and may be the mechanism by which CD4^+ T cell numbers increased in DR3^wt OVA mice.

Chemoattractants have been shown to play a significant role in murine models of allergic lung inflammation, with some suggesting they orchestrate the immune response (Gonzalo, Lloyd et al. 1998; D'Ambrosio, Mariani et al. 2001) and maintain disease (Murdoch and Finn 2000; Lloyd 2002). Therefore, their levels were assessed as a means by which DR3 could further contribute to the increase in leukocyte numbers observed in DR3^wt OVA mice, especially as data from a peritonitis system in the laboratory had indicated that DR3/TL1A signaling was upstream of chemokine production (Perks, 2013 PhD). Although no significant differences were observed, this may be due to the timing of chemokine assessment, chemokine promiscuity, or the restriction of the panel tested. DCs for example, are a prominent source of the Th2 attracting chemokines CCL17 and CCL22 during allergic lung inflammation, neither of which were investigated within this study (Beaty, Rose et al. 2007; Medoff, Seung et al. 2009). Adding to this, CCL11 neutralisation led to reduced eosinophil numbers, whilst the blocking of CCL2 caused a decrease in monocytes as well as Th2 cytokines (Gonzalo, Lloyd et al. 1998; Rose, Lannigan et al. 2010). Without measuring chemokine levels at multiple timepoints through the course of the model, along with a broader panel of chemoattractants, the potential contribution of DR3 via leukocyte recruitment cannot be discounted.
DR3 has also been reported to interact with E-selectin, an adhesion receptor found on endothelial cells. E-selectin is typically expressed at sites of inflammation or injury, allowing leukocyte homing via the activated vasculature (Kansas 1996). Mice deficient in E-selectin, or their receptors, have displayed defects in leukocyte migration (Frenette, Mayadas et al. 1996). Furthermore, *in vitro* data showed DR3 silencing to decrease colon cancer cells adhesion to E-selectin, impairing trans-endothelial migration (Gout, Morin et al. 2006; Porquet, Poirier et al. 2011). Therefore, it is a possible that the differences observed in this Chapter were due to an inability of DR3\(^{ko}\) leukocytes to adhere to the endothelium via E-selectin, resulting in an impairment in extravasation. However, whilst both *Gout et al* and *Porquet et al* have reported E-selectin/DR3 interaction, others have failed to replicate this result. *Dagia et al* investigated G-CSF mobilised peripheral blood leukocyte adhesion to TNF\(\alpha\) activated vascular endothelium using the same E-selectin pulldown system to identify ligands. Whilst DR3 was not identified as a binding partner in this study, (Dagia, Gadhoum et al. 2006), *Gout et al* failed to identify known E-selectin ligands such as CD44 in their studies. Furthermore, *Winkler et al* attempted to characterise E-selectin ligands on haematopoietic stem and progenitor cells using lysates from Selplg\(^{-/-}\) CD44\(^{-/-}\) mice. However, by quantitative RT-PCR no DR3 was detected (Winkler, Barbier et al. 2012), suggesting further research is required regarding DR3’s proposed capacity to bind E-selectin.

More unexpected was the increase seen in CD8\(^{+}\) T cells in the alveolar lavage of DR3\(^{wt}\) OVA mice. The exact function of CD8\(^{+}\) T cells in murine allergic lung inflammation is unknown, with some suggesting they regulate the immune response via the inhibition of airway hyper-reactivity and limiting IgE production (Stock, Kallinich et al. 2004), whilst others argue they may contribute to the severity of disease via the release of IFN\(\gamma\)
and IL-13 in concert with CD4⁺ T cells (Koya, Miyahara et al. 2007), both of which DR3 signalling has previously been shown to induce (Bamias, Martin et al. 2003; Papadakis, Prehn et al. 2004; Prehn, Mehdizadeh et al. 2004; Bamias, Mishina et al. 2006; Meylan, Song et al. 2011; Taraban, Slebioda et al. 2011). DR3 has also been shown to influence CD8⁺ T cell proliferation in vivo. Following MCMV infection, DR3wt mice had more splenic CD8⁺ T cells than DR3ko mice, correlating with an increase in Ki67 expression on DR3wt CD8⁺ T cells (Twohig, Marsden et al. 2012). Similarly, adoptive transfer of OT-I T cells treated with OVA in combination with TL1A increased CD8⁺ T cell expansion 81 fold, in comparison to 12 fold with OVA alone (Slebioda, Rowley et al. 2011), suggesting that like CD4⁺ T cells, CD8⁺ T cell proliferation is DR3 dependent. However, the mechanisms behind this are thought to differ between the 2 cell types, as whilst CD4⁺ T cell expansion appears to be IL-2 dependent (Meylan, Davidson et al. 2008), CD8⁺ T cells cell expansion does not (Slebioda, Rowley et al. 2011). The exact function of DR3 on CD8⁺ T cells in allergic lung inflammation would require further study.

DR3 it appears is vital for the accumulation and possibly expansion of multiple cell types during acute allergic lung inflammation. The up-regulation of DR3 on T cells, particularly memory T cells, may coincide with the release of TL1A from monocyte derived myeloid DCs, which themselves expand in response to allergic challenge. This could lead to DR3 dependent T cell proliferation within the lung and the release of multiple cytokines, influencing the development of multiple allergic lung inflammation features, such as airway remodelling.
4.4.2 DR3 and airway remodelling in acute allergic lung inflammation

No significant differences were observed between DR3\textsuperscript{wt} and DR3\textsuperscript{ko} OVA treated mice in lung pathology, mucin production or fibrosis 24hrs post inhalation, despite the significant cell increases seen in the BALF. However, 72hrs after the final challenge, DR3\textsuperscript{ko} OVA lungs demonstrated decreased mucin levels, similar to data already published on the role of DR3 in acute allergic lung inflammation (Fang, Adkins et al. 2008; Meylan, Davidson et al. 2008). Decreased CD4\textsuperscript{+} and NKT cell derived IL-13 was proposed to be responsible for this lack of goblet cell hyperplasia (Fang, Adkins et al. 2008; Meylan, Davidson et al. 2008), much like that seen in the intestine (Meylan, Song et al. 2011; Taraban, Slebioda et al. 2011). However, results were variable, as two out of the five experimental DR3\textsuperscript{wt} OVA mice failed to produce any goblet cell derived mucus at all.

Additionally, no significant differences were found between DR3\textsuperscript{wt} and DR3\textsuperscript{ko} mice lung pathology, contrasting with data from Fang \textit{et al} and Meylan \textit{et al}, who described reduced histopathology scores, including peribronchial inflammation in DR3\textsuperscript{ko} mice (Meylan, Davidson et al. 2008), whilst DN lungs exhibited reduced numbers of infiltrating cells around blood vessels and bronchioli (Fang, Adkins et al. 2008). Lung pathology differences may in part be due to the subjective nature of histo-pathological scoring methods. Both groups combined the assessment of mucus production and lung infiltration, whereas they were scored individually in this Chapter, thereby distinguishing between these 2 readouts.
No data has been published on the role of DR3 in lung fibrosis, with the results of this Chapter suggesting the Receptor is not required for its development following acute lung inflammation. Though fibrotic scores were generally quite low, DR3$^{ko}$ mice displayed comparable collagen levels to DR3$^{wt}$ mice. Airway fibrosis is characterised by increased collagen deposition in the reticular basement membrane layer, a process influenced by matrix metalloproteinase (MMP)-9 amongst others (Hoshino, Nakamura et al. 1998; Atkinson and Senior 2003). DR3 has previously been shown to influence MMP-9 levels, as stimulation of the human macrophage cell line THP-1, with an anti-DR3 antibody and IFN$\gamma$ (Kim, Lee et al. 2001) or recombinant human TL1A (Kang, Kim et al. 2005), induced MMP-9 expression in a dose-dependent manner. Moreover, down-regulation of DR3 on pancreatic cancer cells using a natural flavonoid, fisetin, led to decreased levels of activated MMP-9 (Murtaza, Adhami et al. 2009). These reports imply that DR3 is able to control MMP-9 activation, though no work has been reported to date on MMP-9 in murine models of disease. The absence of differences between DR3$^{wt}$ and DR3$^{ko}$ collagen levels would not support this hypothesis, but more extensive research into the relationship between DR3 expression and MMPs is required before any firm conclusions can be drawn.

Alternatively, the lack of differences may be due to the model employed to examine fibrosis. Models of acute allergic lung inflammation do not generally exhibit the characteristics of airway remodelling, particularly fibrosis, which generally occurs as a result of chronic responses as opposed to short-term exposures. Acute models are believed to induce an inflammatory response as opposed to structural alterations (Jarman and Lamb 2005). Overall, it seems that DR3 has little influence on these
processes within the acute phase of allergic lung inflammation, potentially due to the short-term nature of this acute model of disease.

4.4.3 DR3 expression in the lung

Increased DR3 expression was observed throughout the lung in DR3<sup>wt</sup> OVA treated mice in comparison to DR3<sup>wt</sup> PBS challenged animals, with the difference being significant at 24hrs post inhalation. This may be linked to the increased numbers of leukocytes seen within the alveolar passage at the same timepoint, many of which are known to express DR3. However, DR3 expression was also detected in the airway epithelium. Multiple human studies have reported the up-regulation of DR3 in tissues undergoing inflammatory situations, most recently Bamias et al in psoriatic skin lesions (Bamias, Evangelou et al. 2011), as well as Al-Lamki et al who published DR3 up-regulation on renal tubular epithelial cells during acute transplant rejection (Al-Lamki, Wang et al. 2003). This is the first report of DR3 expression in the murine lung, and more importantly, its increased expression in response to inflammation.

4.4.4 The effect of DR3 on the systemic response following acute allergic lung inflammation

DR3 has been described as dispensable for systemic priming against antigen in acute allergic inflammation (Meylan, Davidson et al. 2008). DR3<sup>ko</sup> splenocytes produced comparable levels of IL-5 and IL-13 to DR3<sup>wt</sup> mice following OVA and Alum challenge (Meylan, Davidson et al. 2008), whilst Kayamuro et al have reported an increase in the levels of IL-4 and IL-5 from OVA/TL1A challenged splenocytes compared to OVA alone following intranasal immunisation (Kayamuro, Yoshioka et al.
while analysis in this Chapter revealed DR3\textsuperscript{wt} OVA challenged mice had significantly higher splenocyte numbers than DR3\textsuperscript{ko} mice at 24hrs post inhalation. Spleens of DR3\textsuperscript{ko} OVA mice displayed reduced accumulation of all T cell subsets examined, as well as 7/4\textsuperscript{+} monocytes, myeloid DCs and plasmacytoid DCs. This is the first report of differences in splenic cell populations from DR3\textsuperscript{ko} mice challenged with non-infectious antigen.

Systemic function was assessed via Total IgE and OVA specific IgG measurement. Despite an increase in splenic DR3\textsuperscript{wt} B cell number at 24hrs post inhalation, no significant differences were detected in antibody levels, similar to previous reports (Meylan, Davidson et al. 2008). However, Fang et al reported reduced OVA specific IgE levels in the serum of DN DR3 OVA challenged animals (Fang, Adkins et al. 2008), suggesting DR3 is required for the humoral response. In support of this, elevated levels of TL1A have also been associated with rheumatoid arthritis specific auto-antibody production in RA patients, including rheumatoid factor (RF) IgG and RF-IgM. Moreover, peripheral blood mononuclear cells from RA patients stimulated with TL1A, also displayed increased antibody production (Sun, Zhao et al. 2013). Similarly, TL1A was found to augment OVA specific IgG and IgA responses in the serum and mucosal surfaces of mice when administered intranasally with OVA (Kayamuro, Yoshioka et al. 2009). Collectively, the results of this Chapter imply that DR3 is required for more than the accumulation of effector T cells at the site of inflammation, describing a distinct phenotype in the spleen, the biological impact of which, remain unknown. This will be further investigated in Chapter 6.
4.5 Summary

The data in this Chapter corroborates published literature stating that loss of DR3 function ameliorates acute allergic lung inflammation, shown by reduced cellular infiltration into the BALF and goblet cell hyperplasia, thus suggesting DR3 as a potential therapeutic target. A novel phenotype was also observed in the spleens of DR3^{wt} OVA mice, warranting further investigation into systemic and more specifically splenic responses in DR3^{wt} mice (Chapter 6).
Chapter 5:

The role of DR3 in a chronic murine model of allergic lung inflammation
5.1 Introduction

Asthma is a chronic disease of the airways characterised by lung inflammation, bronchial hyper-responsiveness, airway obstruction and airway remodelling (Hessel, Van Oosterhout et al. 1995; Kay 1996; Wills-Karp 1999). The chronic nature of the disease is mediated by the infiltration of multiple inflammatory cell types into the airway submucosa, including eosinophils, mast cells and activated Th2 lymphocytes (Hamid and Tulić 2009), which in turn lead to structural changes in the airway wall. Airway remodelling is a term that encompasses a range of changes, such as thickening of the airway walls, abnormal extracellular matrix protein deposition and mucus gland hyperplasia (Hogaboam, Blease et al. 2000; Tattersfield, Knox et al. 2002; Lloyd and Robinson 2007). This complex and dynamic process is thought to contribute to the dysregulation of airway function, therefore prolonging the asthmatic response and typifying human asthma.

Originally thought to be exclusively Th2 mediated, the immune regulation and response to innocuous allergen is now considered to be highly heterogeneous (Moore, Meyers et al. 2010). This in part, is due to the increasing use of in vivo models of this complex disease which reflect the varied nature of asthma. Although substantial differences exist between human asthma and murine models, the latter can still provide clues to the mechanisms behind this condition. Mice can be easily manipulated using the several immunological tools available. As well as this, mouse models have been well-characterised and used throughout the literature (Kips, Anderson et al. 2003). Despite this frequency of use, limitations also exist. Mice do not spontaneously develop asthma; it is experimentally induced using allergen and more often than not, adjuvant. In order
to mimic the chronic nature of asthma, the mice also undergo multiple airway challenges, as occurs with asthmatic patients that are chronically exposed to allergen. No model however is able to replicate the entire asthma phenotype, so extrapolation of results to human asthma should be made with caution.

DR3 has been known to play a prominent role in several chronic inflammatory diseases, including Crohns disease (Bamias, Martin et al. 2003; Takedatsu, Michelsen et al. 2008; Kamada, Hisamatsu et al. 2010) and Rheumatoid Arthritis (Cassatella, da Silva et al. 2007; Bamias, Siakavellas et al. 2008; Zhang, Wang et al. 2009). The use of murine models have established DR3 as a key player in the proliferation (Pappu, Borodovsky et al. 2008), differentiation (Bull, Williams et al. 2008) and maintenance (Jones, Stumhofer et al. 2011) of leukocyte populations during these chronic diseases. DR3 has been cited as a critical trigger of murine acute allergic lung inflammation, promoting CD4+ cell effector function, co-stimulating IL-13 and IL-5 release and enhancing general lung pathology (Fang, Adkins et al. 2008; Meylan, Davidson et al. 2008). However, DR3’s role in chronic lung inflammation is undefined and worth exploring considering immune responses to chronic virus infections are not impaired in DR3<sup>ko</sup> mice (Twohig, Marsden et al. 2012).
5.2 Aims

The aim of this Chapter was to investigate the role of DR3 in chronic allergic lung inflammation. DR3\textsuperscript{wt} and DR3\textsuperscript{ko} mice underwent recurrent inhalation challenges with OVA or PBS over an 8 week period and were harvested at 24 and 72 hours (hrs) post final inhalation challenge, which occurred on day 57 (Section 2.2.7.4). Myeloid and lymphocyte cell influx was measured in the lung and alveolar passages using flow cytometry, serum antibody measurements (Total IgE and OVA specific IgG) determined the allergic antibody response and histological examination of the lung was used to assess airway remodelling. The accumulation of leukocytes in the spleen was also examined to determine whether inflammatory responses were localised or systemic. Despite being a hallmark of the asthma phenotype, airway hyper-responsiveness could not be measured in DR3\textsuperscript{ko} mice, as discussed in Chapter 3.

5.2.1 Objectives

i) Determine the local inflammatory response following chronic allergic lung inflammation

ii) Analyse and quantify airway remodelling

iii) Determine the systemic response following chronic allergic lung inflammation
5.3 Results

5.3.1 Cellular accumulation following chronic allergic lung inflammation

5.3.1.1 Analysis of cellular accumulation into the lung

5.3.1.1.1 DR3 did not regulate leukocyte accumulation into the lung

Following chronic allergic lung inflammation, one lung lobe was harvested specifically to assess leukocyte accumulation. There were no significant differences in the total lung cell numbers of DR3\textsuperscript{wt} and DR3\textsuperscript{ko} OVA treated lungs at either the 24hr (DR3\textsuperscript{wt} 2.2±0.1 \times 10^6, DR3\textsuperscript{ko} 2.8±0.1 \times 10^6) (\(p=0.094\)) or 72hr timepoint (DR3\textsuperscript{wt} 2.0±0.3 \times 10^6, DR3\textsuperscript{ko} 3.1±0.3 \times 10^6) (\(p=0.097\)), suggesting that DR3 does not control the accumulation of leukocytes into the lung following multiple allergen challenges. However, the total lung cell number of mice which underwent OVA inhalation was higher than those treated with PBS of the same genotype. This was significant at 24hrs post inhalation between DR3\textsuperscript{ko} PBS and DR3\textsuperscript{ko} OVA treated mice (1.9±0.2 \times 10^6, 2.8±0.1 \times 10^6) (\(p=0.022\)). Significance was also noted between DR3\textsuperscript{wt} PBS and DR3\textsuperscript{ko} OVA mice at 72hrs (1.8±0.2 \times 10^6, 3.1±0.4 \times 10^6) (\(p=0.022\)) (Figure 5.1).
Figure 5.1. Total leukocyte cell number from the lungs of DR3wt and DR3ko mice following chronic allergic lung inflammation. Mice were sacrificed (A) 24hr and (B) 72hr post final inhalation challenge with either OVA or PBS. Cells were isolated via a lung digest and counted using a haemocytometer. Total cell number from the lung showed significant differences at 24hr (*p=0.022) and 72hr (*p=0.022). Significance determined using 1 way ANOVA and Bonferroni post test; *p<0.05. Values represent mean ± SEM. Each symbol represents data from a single mouse.
5.3.1.1.2 There were significantly more myeloid dendritic cells in DR3\(^{wt}\) OVA treated lungs compared to DR3\(^{ko}\) OVA treated lungs at 24 but not 72hrs post inhalation.

At 24hrs post inhalation, significant differences were seen between DR3\(^{wt}\) OVA and DR3\(^{ko}\) OVA mice in myeloid DC number (DR3\(^{wt}\) 1.4±0.1 \times 10^3, DR3\(^{ko}\) 0.5±0.1 \times 10^3) \((p<0.0001)\) (Table 5.1), but no other myeloid cell subset. However, mice that had undergone OVA inhalation treatment as opposed to PBS, appeared to trend towards increased numbers, with this increase being significant between DR3\(^{wt}\) 7/4 monocytes and PBS subsets \((p=0.0006)\).

At 72hrs post inhalation, no differences were seen in myeloid DCs between DR3\(^{wt}\) and DR3\(^{ko}\) OVA treatment groups, as DR3\(^{wt}\) PBS mice instead showed the highest numbers, this increase being significant over DR3\(^{ko}\) PBS mice \((p=0.023)\). Interestingly, DR3\(^{ko}\) OVA mice appeared to display a statistically greater number of alveolar macrophages compared to all other treatment groups, including DR3\(^{wt}\) OVA mice and PBS controls \((p=0.0003)\) (Table 5.2). All other myeloid cell subsets studied showed equivalent numbers between treatment groups.
5.3.1.1.3 Lymphocyte accumulation into the lung at 24 and 72hr post inhalation

At 24hrs post inhalation, DR3\textsuperscript{ko} OVA mice displayed a significantly higher number of CD3\textsuperscript{+} T cells compared to PBS treatment groups ($p=0.004$), although this difference was not statistically significant between DR3\textsuperscript{wt} OVA and DR3\textsuperscript{ko} OVA mice. This corresponded to a significant increase in CD4\textsuperscript{+} T cells over all other treatment groups ($p=0.002$) as well as CD8\textsuperscript{+} T cells, although like CD3\textsuperscript{+} T cells, significance was only seen between DR3\textsuperscript{ko} OVA and PBS challenged mice ($p=0.015$) (Table 5.1). There were also significantly increased numbers of NK cells in OVA treated lungs compared to PBS challenged mice ($p=0.037$) (Table 5.1).

In comparison, at 72hrs post inhalation, no significant differences were seen between any treatment groups when studying CD3\textsuperscript{+}, CD4\textsuperscript{+} or CD8\textsuperscript{+} T cells (Table 5.2). However, DR3\textsuperscript{ko} OVA mice did have lower numbers of CD8\textsuperscript{+} activated T cells, defined by the marker CD44, in comparison to DR3\textsuperscript{wt} OVA treated mice ($p=0.017$) (Table 5.2). DR3\textsuperscript{ko} PBS mice had significantly more NKT cells than DR3\textsuperscript{ko} OVA mice ($p=0.010$) (Table 5.2), whilst DR3\textsuperscript{wt} PBS mice displayed increased NK cell numbers over all other treatment groups, irrespective of inhalation treatment and genotype ($p=0.002$) (Table 5.2). Overall, results were inconsistent. At the 24hr timepoint OVA treated mice appeared to show increased cell numbers compared to PBS challenged animals, thus suggesting that DR3 does not regulate the accumulation of lymphocytes into the lung following chronic allergic inflammation.
Table 5.1 Comparison of cell numbers isolated from the lungs of DR3<sup>wt</sup> and DR3<sup>ko</sup> mice 24hr post inhalation

<table>
<thead>
<tr>
<th>Cell Subset</th>
<th>DR3&lt;sup&gt;wt&lt;/sup&gt;</th>
<th>DR3&lt;sup&gt;ko&lt;/sup&gt;</th>
<th>Significance&lt;sup&gt;$&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PBS*</td>
<td>OVA^</td>
<td>PBS*</td>
</tr>
<tr>
<td>Alveolar Macrophages</td>
<td>4.4 ± 0.6 x10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>7.0 ± 0.5 x10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>5.1 ± 0.5 x10&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
<tr>
<td>Myeloid DCs</td>
<td>0.5 ± 0.1 x10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>1.4 ± 0.1 x10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>0.3 ± 0.1 x10&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>3.5 ± 0.7 x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>4.1 ± 0.9 x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>4.5 ± 1.6 x10&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>0.2 ± 0.1 x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>1.2 ± 0.6 x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>0.2 ± 0.04 x10&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>7/4&lt;sup&gt;+&lt;/sup&gt; Monocytes</td>
<td>1.5 ± 0.3 x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>2.7 ± 0.2 x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>1.8 ± 0.5 x10&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>7/4&lt;sup&gt;-&lt;/sup&gt; Monocytes</td>
<td>1.7 ± 0.3 x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>3.7 ± 0.3 x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>1.7 ± 0.2 x10&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>T cells</td>
<td>1.1 ± 0.1 x10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>1.5 ± 0.2 x10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>1.1 ± 0.3 x10&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td>CD4&lt;sup&gt;+&lt;/sup&gt; T cells</td>
<td>7.4 ± 0.7 x10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>8.7 ± 1.3 x10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>7.9 ± 0.8 x10&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
<tr>
<td>CD8&lt;sup&gt;+&lt;/sup&gt; T cells</td>
<td>1.7 ± 0.3 x10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>3.0 ± 0.5 x10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>1.7 ± 0.4 x10&lt;sup&gt;5&lt;/sup&gt;</td>
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<tr>
<td>B cells</td>
<td>3.7 ± 0.8 x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>6.6 ± 0.7 x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>3.8 ± 1.0 x10&lt;sup&gt;4&lt;/sup&gt;</td>
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<tr>
<td>NK cells</td>
<td>3.1 ± 0.3 x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>4.7 ± 0.7 x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>2.3 ± 0.3 x10&lt;sup&gt;4&lt;/sup&gt;</td>
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<tr>
<td>NKT cells</td>
<td>1.8 ± 0.3 x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>3.1 ± 0.4 x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>1.8 ± 0.4 x10&lt;sup&gt;4&lt;/sup&gt;</td>
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</table>

*Corresponds to mice challenged with PBS
^ Corresponds to mice challenged with OVA
$ Significance tested between DR3<sup>wt</sup> and DR3<sup>ko</sup> OVA groups using 1 way ANOVA and Bonferroni post test; N.S.D = no significant difference, ***p<0.001
<table>
<thead>
<tr>
<th>Cell Subset</th>
<th>DR3&lt;sup&gt;wt&lt;/sup&gt;</th>
<th>OVA&lt;sup&gt;^&lt;/sup&gt;</th>
<th>DR3&lt;sup&gt;ko&lt;/sup&gt;</th>
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<td>4.4 ± 0.7 x10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>4.4 ± 0.7 x10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>8.1 ± 0.9 x10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>**p=0.01</td>
</tr>
<tr>
<td>Myeloid DCs</td>
<td>0.9 ± 0.1 x10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>0.6 ± 0.2 x10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>0.3 ± 0.1 x10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>0.4 ± 0.1 x10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>N.S.D</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>10.4 ± 1.4 x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>8.9 ± 1.6 x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>13.4 ± 0.7 x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>10.3 ± 2.3 x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>N.S.D</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>2.4 ± 0.5 x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>2.0 ± 0.2 x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>2.1 ± 0.4 x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>2.2 ± 0.3 x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>N.S.D</td>
</tr>
<tr>
<td>7/4&lt;sup&gt;+&lt;/sup&gt; Monocytes</td>
<td>3.5 ± 0.5 x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>4.1 ± 0.4 x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>4.9 ± 1.2 x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>3.4 ± 0.7 x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>N.S.D</td>
</tr>
<tr>
<td>7/4&lt;sup&gt;-&lt;/sup&gt; Monocytes</td>
<td>2.3 ± 0.4 x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>1.7 ± 0.2 x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>2.7 ± 0.4 x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>2.2 ± 0.9 x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>N.S.D</td>
</tr>
<tr>
<td>T cells</td>
<td>1.2 ± 0.2 x10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>1.8 ± 0.3 x10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>1.5 ± 0.2 x10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>1.0 ± 0.1 x10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>N.S.D</td>
</tr>
<tr>
<td>CD4&lt;sup&gt;+&lt;/sup&gt; T cells</td>
<td>6.5 ± 0.9 x10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>9.5 ± 1.6 x10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>7.5 ± 0.9 x10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>6.9 ± 0.8 x10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>N.S.D</td>
</tr>
<tr>
<td>CD8&lt;sup&gt;+&lt;/sup&gt; T cells</td>
<td>5.3 ± 0.7 x10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>7.6 ± 1.2 x10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>6.8 ± 1.0 x10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>4.1 ± 0.6 x10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>N.S.D</td>
</tr>
<tr>
<td>Act. CD4&lt;sup&gt;+&lt;/sup&gt; T cells</td>
<td>1.3 ± 0.3 x10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>1.8 ± 0.5 x10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>0.8 ± 0.1 x10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>1.1 ± 0.2 x10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>N.S.D</td>
</tr>
<tr>
<td>Act. CD8&lt;sup&gt;+&lt;/sup&gt; T cells</td>
<td>0.9 ± 0.1 x10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>1.1 ± 0.2 x10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>0.6 ± 0.1 x10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>0.5 ± 0.04 x10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>*p=0.017</td>
</tr>
<tr>
<td>B cells</td>
<td>8.9 ± 2.0 x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>10.7 ± 2.2 x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>12.1 ± 2.5 x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>8.3 ± 1.3 x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>N.S.D</td>
</tr>
<tr>
<td>NK cells</td>
<td>2.4 ± 0.4 x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>1.4 ± 0.1 x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>1.4 ± 0.2 x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>1.0 ± 0.1 x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>N.S.D</td>
</tr>
<tr>
<td>NKT cells</td>
<td>0.6 ± 0.1 x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>0.8 ± 0.1 x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>0.9 ± 0.1 x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>0.5 ± 0.1 x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>N.S.D</td>
</tr>
</tbody>
</table>

*Corresponds to mice challenged with PBS

^ Corresponds to mice challenged with OVA

$ Significance tested between DR3<sup>wt</sup> and DR3<sup>ko</sup> OVA groups using 1 way ANOVA and Bonferroni post test; N.S.D = no significant difference, **p<0.01
5.3.1.3 Analysis of cellular accumulation into the alveolar passage

5.3.1.2.1 Leukocyte accumulation in the alveolar passage

Following chronic allergic lung inflammation, the alveolar passages of mice were lavaged and total cell number determined. At 24hrs post inhalation, DR3\textsuperscript{wt} mice that had undergone PBS inhalation displayed significantly fewer leukocytes than DR3\textsuperscript{ko} mice that had suffered OVA inhalation challenges (DR3\textsuperscript{wt} PBS 1.1±0.1 x10\textsuperscript{5}, DR3\textsuperscript{ko} OVA 2.2±0.3 x10\textsuperscript{5}) \( (p=0.004) \) (Figure 5.2). The same trend was seen at 72hrs post inhalation, although results were not significant (DR3\textsuperscript{wt} PBS 1.5±0.4 x10\textsuperscript{5}, DR3\textsuperscript{wt} OVA 1.9±0.4 x10\textsuperscript{5}, DR3\textsuperscript{ko} PBS 2.5±0.5 x10\textsuperscript{5}, DR3\textsuperscript{ko} OVA 3.1±0.6 x10\textsuperscript{5}) \( (p=0.204) \) (Figure 5.2).
Figure 5.2. Total leukocyte number from the alveolar passage of DR3wt and DR3ko mice following chronic allergic lung inflammation. Mice were sacrificed (A) 24hr and (B) 72hr post final inhalation challenge with either OVA or PBS. Cells were isolated via a bronchoalveolar lavage and counted using a haemocytometer. Total cell number from the BAL showed significant differences at 24hr (*p=0.004) but not 72hr n.s.d. Significance determined using 1 way ANOVA and Bonferroni post test; *p<0.05, **p<0.01. Values represent mean ± SEM. Each symbol represents data from a single mouse.
5.3.1.2.2 Myeloid cell subsets in the alveolar passage did not significantly differ between DR3\textsuperscript{wt} and DR3\textsuperscript{ko} OVA challenged mice

At both 24 and 72hrs post inhalation no significant differences were noted for any of the myeloid cell subsets studied within the bronchoalveolar lavage (Table 5.3) (Table 5.4).

5.3.1.2.3 Lymphocyte accumulation into the alveolar passage did not significantly differ between DR3\textsuperscript{wt} and DR3\textsuperscript{ko} OVA challenged mice

After the final inhalation challenge (24hrs), OVA challenged mice of both genotypes contained a significantly higher number of CD3\textsuperscript{+} T cells than DR3\textsuperscript{wt} PBS challenged mice \((p=0.005)\) (Table 5.3). This corresponded with higher numbers of CD4\textsuperscript{+} T cells \((p=0.005)\) and CD8\textsuperscript{+} T cells \((p=0.018)\), but not NKT cells. When studying B cells, DR3\textsuperscript{ko} PBS mice displayed increased cell numbers over both DR3\textsuperscript{wt} OVA and DR3\textsuperscript{wt} PBS mice \((p=0.008)\), whilst equivalent numbers of NK cells were observed (Table 5.3).

However, at 72hrs post inhalation no significant differences were observed between treatment groups (Table 5.4).
Table 5.3 Comparison of cell numbers isolated from the alveolar passage of DR3\textsuperscript{wt} and DR3\textsuperscript{ko} mice 24hrs post inhalation

<table>
<thead>
<tr>
<th>Cell Subset</th>
<th>DR3\textsuperscript{wt}</th>
<th></th>
<th>DR3\textsuperscript{ko}</th>
<th></th>
<th>Significance\textsuperscript{S}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PBS\textsuperscript{*}</td>
<td>OVA\textsuperscript{^}</td>
<td>PBS\textsuperscript{*}</td>
<td>OVA\textsuperscript{^}</td>
<td></td>
</tr>
<tr>
<td>Alveolar Macrophages</td>
<td>1.5 ± 0.5 x10\textsuperscript{4}</td>
<td>1.8 ± 0.2 x10\textsuperscript{4}</td>
<td>5.5 ± 1.4 x10\textsuperscript{4}</td>
<td>4.2 ± 1.8 x10\textsuperscript{4}</td>
<td>N.S.D</td>
</tr>
<tr>
<td>Myeloid DC’s</td>
<td>0.2 ± 0.1 x10\textsuperscript{3}</td>
<td>0.2 ± 0.1 x10\textsuperscript{3}</td>
<td>0.5 ± 0.2 x10\textsuperscript{3}</td>
<td>0.7 ± 0.3 x10\textsuperscript{3}</td>
<td>N.S.D</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>1.1 ± 0.3 x10\textsuperscript{4}</td>
<td>0.7 ± 0.4 x10\textsuperscript{4}</td>
<td>0.5 ± 0.1 x10\textsuperscript{4}</td>
<td>2.0 ± 0.5 x10\textsuperscript{4}</td>
<td>N.S.D</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>0.5 ± 0.1 x10\textsuperscript{3}</td>
<td>0.8 ± 0.2 x10\textsuperscript{3}</td>
<td>0.7 ± 0.2 x10\textsuperscript{3}</td>
<td>0.7 ± 0.2 x10\textsuperscript{3}</td>
<td>N.S.D</td>
</tr>
<tr>
<td>7/4\textsuperscript{+} Monocytes</td>
<td>0.2 ± 0.1 x10\textsuperscript{3}</td>
<td>0.4 ± 0.1 x10\textsuperscript{3}</td>
<td>0.4 ± 0.1 x10\textsuperscript{3}</td>
<td>0.3 ± 0.1 x10\textsuperscript{3}</td>
<td>N.S.D</td>
</tr>
<tr>
<td>7/4\textsuperscript{-} Monocytes</td>
<td>0.3 ± 0.1 x10\textsuperscript{3}</td>
<td>0.6 ± 0.2 x10\textsuperscript{3}</td>
<td>0.9 ± 0.3 x10\textsuperscript{3}</td>
<td>1.1 ± 0.3 x10\textsuperscript{3}</td>
<td>N.S.D</td>
</tr>
<tr>
<td>T cells</td>
<td>2.3 ± 0.3 x10\textsuperscript{4}</td>
<td>9.0 ± 2.3 x10\textsuperscript{4}</td>
<td>0.3 ± 0.1 x10\textsuperscript{4}</td>
<td>7.5 ± 1.2 x10\textsuperscript{4}</td>
<td>N.S.D</td>
</tr>
<tr>
<td>CD4\textsuperscript{+} T cells</td>
<td>0.8 ± 0.2 x10\textsuperscript{4}</td>
<td>6.9 ± 2.2 x10\textsuperscript{4}</td>
<td>1.9 ± 0.5 x10\textsuperscript{4}</td>
<td>5.6 ± 1.2 x10\textsuperscript{4}</td>
<td>N.S.D</td>
</tr>
<tr>
<td>CD8\textsuperscript{+} T cells</td>
<td>0.3 ± 0.03 x10\textsuperscript{4}</td>
<td>0.7 ± 0.2 x10\textsuperscript{4}</td>
<td>0.5 ± 0.1 x10\textsuperscript{4}</td>
<td>0.5 ± 0.1 x10\textsuperscript{4}</td>
<td>N.S.D</td>
</tr>
<tr>
<td>B cells</td>
<td>1.1 ± 0.3 x10\textsuperscript{4}</td>
<td>3.4 ± 0.5 x10\textsuperscript{4}</td>
<td>3.4 ± 0.5 x10\textsuperscript{4}</td>
<td>2.4 ± 0.8 x10\textsuperscript{4}</td>
<td>N.S.D</td>
</tr>
<tr>
<td>NK cells</td>
<td>0.5 ± 0.1 x10\textsuperscript{3}</td>
<td>1.2 ± 0.5 x10\textsuperscript{3}</td>
<td>2.7 ± 2.2 x10\textsuperscript{3}</td>
<td>1.0 ± 0.1 x10\textsuperscript{3}</td>
<td>N.S.D</td>
</tr>
<tr>
<td>NKT cells</td>
<td>1.1 ± 0.3 x10\textsuperscript{3}</td>
<td>0.9 ± 0.3 x10\textsuperscript{3}</td>
<td>3.4 ± 0.5 x10\textsuperscript{3}</td>
<td>0.7 ± 0.1 x10\textsuperscript{3}</td>
<td>N.S.D</td>
</tr>
</tbody>
</table>

\textsuperscript{*}Corresponds to mice challenged with PBS
\textsuperscript{^} Corresponds to mice challenged with OVA
\textsuperscript{S} Significance tested between DR3\textsuperscript{wt} and DR3\textsuperscript{ko} OVA groups using 1 way ANOVA and Bonferroni post test; N.S.D = no significant difference,
Table 5.4 Comparison of cell numbers isolated from the alveolar passage of DR3\textsuperscript{wt} and DR3\textsuperscript{ko} mice 72hrs post inhalation

<table>
<thead>
<tr>
<th>Cell Subset</th>
<th>DR3\textsuperscript{wt}</th>
<th></th>
<th>DR3\textsuperscript{ko}</th>
<th></th>
<th>Significance$^5$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PBS(^*)</td>
<td>OVA(^^)</td>
<td>PBS(^*)</td>
<td>OVA(^^)</td>
<td></td>
</tr>
<tr>
<td>Alveolar Macrophages</td>
<td>3.2 ± 1.0 x10\textsuperscript{4}</td>
<td>3.2 ± 0.1 x10\textsuperscript{4}</td>
<td>8.6 ± 2.0 x10\textsuperscript{4}</td>
<td>6.7 ± 2.2 x10\textsuperscript{4}</td>
<td>N.S.D</td>
</tr>
<tr>
<td>Myeloid DC’s</td>
<td>0.5 ± 0.1 x10\textsuperscript{3}</td>
<td>0.7 ± 0.3 x10\textsuperscript{3}</td>
<td>0.6 ± 0.2 x10\textsuperscript{3}</td>
<td>0.6 ± 0.2 x10\textsuperscript{3}</td>
<td>N.S.D</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>2.1 ± 1.1 x10\textsuperscript{4}</td>
<td>3.4 ± 1.8 x10\textsuperscript{4}</td>
<td>4.4 ± 1.5 x10\textsuperscript{4}</td>
<td>12.4 ± 6.3 x10\textsuperscript{4}</td>
<td>N.S.D</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>0.3 ± 0.1 x10\textsuperscript{3}</td>
<td>8.5 ± 3.7 x10\textsuperscript{3}</td>
<td>0.8 ± 0.4 x10\textsuperscript{3}</td>
<td>6.9 ± 3.3 x10\textsuperscript{3}</td>
<td>N.S.D</td>
</tr>
<tr>
<td>7/4(^+) Monocytes</td>
<td>0.6 ± 0.4 x10\textsuperscript{3}</td>
<td>1.1 ± 0.6 x10\textsuperscript{3}</td>
<td>0.8 ± 0.4 x10\textsuperscript{3}</td>
<td>2.7 ± 1.6 x10\textsuperscript{3}</td>
<td>N.S.D</td>
</tr>
<tr>
<td>7/4(^-) Monocytes</td>
<td>0.3 ± 0.2 x10\textsuperscript{3}</td>
<td>1.2 ± 0.5 x10\textsuperscript{3}</td>
<td>0.6 ± 0.3 x10\textsuperscript{3}</td>
<td>1.6 ± 0.7 x10\textsuperscript{3}</td>
<td>N.S.D</td>
</tr>
<tr>
<td>T cells</td>
<td>0.3 ± 0.1 x10\textsuperscript{4}</td>
<td>1.8 ± 0.9 x10\textsuperscript{4}</td>
<td>0.3 ± 0.1 x10\textsuperscript{4}</td>
<td>1.7 ± 0.6 x10\textsuperscript{4}</td>
<td>N.S.D</td>
</tr>
<tr>
<td>CD4(^+) T cells</td>
<td>2.2 ± 1.0 x10\textsuperscript{3}</td>
<td>15.0 ± 7.3 x10\textsuperscript{3}</td>
<td>1.9 ± 0.5 x10\textsuperscript{3}</td>
<td>13.7 ± 4.8 x10\textsuperscript{3}</td>
<td>N.S.D</td>
</tr>
<tr>
<td>CD8(^+) T cells</td>
<td>0.9 ± 0.4 x10\textsuperscript{3}</td>
<td>2.6 ± 1.4 x10\textsuperscript{3}</td>
<td>0.9 ± 0.2 x10\textsuperscript{3}</td>
<td>2.4 ± 0.8 x10\textsuperscript{3}</td>
<td>N.S.D</td>
</tr>
<tr>
<td>B cells</td>
<td>3.3 ± 2.3 x10\textsuperscript{3}</td>
<td>2.6 ± 1.3 x10\textsuperscript{3}</td>
<td>11.1 ± 6.5 x10\textsuperscript{3}</td>
<td>2.9 ± 0.6 x10\textsuperscript{3}</td>
<td>N.S.D</td>
</tr>
<tr>
<td>NK cells</td>
<td>2.0 ± 0.1 x10\textsuperscript{2}</td>
<td>3.4 ± 1.0 x10\textsuperscript{2}</td>
<td>3.7 ± 1.2 x10\textsuperscript{2}</td>
<td>3.6 ± 0.5 x10\textsuperscript{2}</td>
<td>N.S.D</td>
</tr>
<tr>
<td>NKT cells</td>
<td>0.9 ± 0.5 x10\textsuperscript{3}</td>
<td>3.1 ± 1.5 x10\textsuperscript{3}</td>
<td>0.9 ± 0.3 x10\textsuperscript{3}</td>
<td>3.1 ± 1.1 x10\textsuperscript{3}</td>
<td>N.S.D</td>
</tr>
</tbody>
</table>

\(^*\)Corresponds to mice challenged with PBS

\(^^\) Corresponds to mice challenged with OVA

\(^5\) Significance tested between DR3\textsuperscript{wt} and DR3\textsuperscript{ko} OVA groups using 1 way ANOVA and Bonferroni post test; N.S.D = no significant difference,
5.3.2 Airway remodelling following chronic allergic lung inflammation

5.3.2.1 Analysis of airway remodelling and lung pathology

5.3.2.1.1 Lung pathology was more severe in DR3<sup>wt</sup> mice that had been exposed to OVA inhalation than DR3<sup>ko</sup> mice

A H&E stain was used to assess general lung pathology. Lungs were graded according to a severity scale with a maximum score of 5 (Section 2.2.12.2). Staining revealed that at both 24 and 72hrs post inhalation, DR3<sup>wt</sup> OVA mice exhibited worse lung pathology, marked by increased peribronchial and perivascular inflammation and cellular cuffing compared to all other treatment groups. At 24hrs post inhalation, DR3<sup>wt</sup> OVA mice scored an average of 4.5±0.3, compared to DR3<sup>wt</sup> PBS (2.2±0.5), DR3<sup>ko</sup> OVA (2.5±0.3) and DR3<sup>ko</sup> PBS (2.0±0.4) mice (p=0.003) (Figure 5.3) (Table 5.5). This was also true at the 72hr timepoint whereby DR3<sup>wt</sup> OVA samples displayed a mean lung pathology score of 4.0±0.5, significantly higher than DR3<sup>wt</sup> PBS (2.4±0.3), DR3<sup>ko</sup> OVA (2.6±0.5) and DR3<sup>ko</sup> PBS (2.4±0.3) mice (p=0.035) (Figure 5.4) (Table 5.5), thus suggesting the development of lung pathology during chronic allergic lung inflammation was DR3 dependent.
Figure 5.3. Analysis of lung pathology 24hrs post chronic allergic lung inflammation. Mice were sacrificed 24hr post final inhalation challenge with either OVA or PBS. Lungs were stained with Haematoxylin and Eosin. Shown are representative photos of DR3\textsuperscript{wt} OVA, DR3\textsuperscript{ko} OVA, DR3\textsuperscript{wt} PBS and DR3\textsuperscript{ko} PBS lungs (Scale bar=250\(\mu\)m).

(A) Lungs were blind scored by 2 individuals for pathology and an average taken for each lung. Results were significant using 1 way ANOVA (*\(p=0.003\)) whereby *\(p<0.05\), **\(p<0.01\), ***\(p<0.001\). Values represent mean ± SEM. Each symbol represents data from a single mouse.
Figure 5.4. Analysis of lung pathology 72hrs post chronic allergic lung inflammation. Mice were sacrificed 72hr post final inhalation challenge with either OVA or PBS. Lungs were stained with Haematoxylin and Eosin. Shown are representative photos of DR3\textsuperscript{wt} OVA, DR3\textsuperscript{ko} OVA, DR3\textsuperscript{wt} PBS and DR3\textsuperscript{ko} PBS lungs (Scale bar=100\textmu m). (A) Lungs were blind scored by 2 individuals for pathology and an average taken for each lung. Results were significant using 1 way ANOVA (*p=0.035) whereby *p<0.05. Values represent mean ± SEM. Each symbol represents data from a single mouse.
5.3.2.1.2 Mucin associated goblet cell number was significantly different between 

**DR3\(^{wt}\) and DR3\(^{ko}\) mice**

Lungs were stained with Periodic Acid Schiff to quantitate levels of mucin producing goblet cells. Results showed that the lungs of DR3\(^{wt}\) OVA treated mice produced significantly more mucin than all other experimental groups at both 24 and 72hrs post inhalation. This goblet cell hyperplasia was particularly apparent 24hrs after the final aerosolisation challenge, as DR3\(^{wt}\) OVA mice presented with 4.1±0.5% of its airways as positive, compared to 1.7±0.8% in DR3\(^{ko}\) OVA chronically challenged airways, 0.08±0.05% in DR3\(^{wt}\) PBS mice and 0.1±0.06% in DR3\(^{ko}\) PBS mice (\(p<0.0001\)) (Figure 5.5) (Table 5.5). A similar pattern was detected at 72hrs post inhalation, as DR3\(^{wt}\) OVA mice had significantly more goblet cells than other experimental groups (DR3\(^{wt}\) OVA 7.6±3.6%, DR3\(^{wt}\) PBS 0.07±0.05%, DR3\(^{ko}\) OVA 2.0±1.1%, DR3\(^{ko}\) PBS 0.02±0.01%) (\(p=0.030\)) (Figure 5.6) (Table 5.5).
Figure 5.5. Analysis of goblet cell numbers 24hrs post chronic allergic lung inflammation. Mice were sacrificed 24hr post final inhalation challenge with either OVA or PBS. Lungs were stained with Periodic Acid Schiff. Shown are representative photos of DR3<sup>wt</sup> OVA, DR3<sup>ko</sup> OVA, DR3<sup>wt</sup> PBS and DR3<sup>ko</sup> PBS lungs (Scale bar=250μm). (A) The area of PAS<sup>+</sup> cells was taken as a % of the airway surround using Leica Qwin V3 software, and an average calculated for each lung. Results were significant using 1 way ANOVA (**p<0.0001) whereby *p<0.05, **p<0.01, ***p<0.001. Values represent mean ± SEM. Each symbol represents data from a single mouse.
Figure 5.6. Analysis of goblet cell numbers 72hrs post chronic allergic lung inflammation. Mice were sacrificed 72hr post final inhalation challenge with either OVA or PBS. Lungs were stained with Periodic Acid Schiff. Shown are representative photos of DR3<sup>wt</sup> OVA, DR3<sup>ko</sup> OVA, DR3<sup>wt</sup> PBS and DR3<sup>ko</sup> PBS lungs (Scale bar=100μm). (A) The area of PAS<sup>+</sup> cells was taken as a % of the airway surround using Leica Qwin V3 software, and an average calculated for each lung. Results were significant using 1 way ANOVA (*p=0.030) whereby *p<0.05. Values represent mean ± SEM. Each symbol represents data from a single mouse.
5.3.2.1.3 Lung fibrosis scores were comparable between DR3<sup>wt</sup> and DR3<sup>ko</sup> OVA mice

Lungs were stained with Van Gieson solution to assay the level of collagen and thereby acquire an arbitrary measure of fibrosis. Lung fibrosis following multiple allergen challenge was not DR3 dependent at either the 24 or 72hr timepoint, as both DR3<sup>wt</sup> OVA (24hrs 3.4±0.3, 72hrs 3.0±0.3) and DR3<sup>ko</sup> OVA (24hrs 2.8±0.3, 72hrs 1.8±0.5) treated mice scored comparably using the Ashcroft measure of Fibrosis (Ashcroft, Simpson et al. 1988). Both OVA challenged groups displayed higher scores than mice challenged consecutively with PBS, with this difference being significant at the 24hr (DR3<sup>wt</sup> OVA 3.4±0.3, DR3<sup>wt</sup> PBS 1.5±2.6, DR3<sup>ko</sup> OVA 2.8±0.3, DR3<sup>ko</sup> PBS 1.1±0.3) (<i>p<0.0001</i>) (Figure 5.7) and 72hr timepoints (DR3<sup>wt</sup> OVA 3.0±0.3, DR3<sup>wt</sup> PBS 1.4±0.3, DR3<sup>ko</sup> PBS 1.1±0.3) (<i>p=0.009</i>) (Figure 5.8) (Table 5.5).
Figure 5.7. Analysis of fibrosis 24hrs post chronic allergic lung inflammation. Mice were sacrificed 24hr post final inhalation challenge with either OVA or PBS. Lungs were stained with Van Gieson solution. Shown are representative photos of DR3wt OVA, DR3ko OVA, DR3wt PBS and DR3ko PBS lungs (Scale bar=100μm). (A) Lungs were blind scored by 2 individuals using Ashcroft score of fibrosis and an average taken for each lung. Results were significant using 1 way ANOVA (***p<0.0001) whereby *p<0.05, **p<0.01, ***p<0.001. Values represent mean ± SEM. Each symbol represents data from a single mouse.
Figure 5.8. Analysis of fibrosis 72hrs post chronic allergic lung inflammation. Mice were sacrificed 72hr post final inhalation challenge with either OVA or PBS. Lungs were stained with Van Gieson solution. Shown are representative photos of DR3\textsuperscript{wt} OVA, DR3\textsuperscript{ko} OVA, DR3\textsuperscript{wt} PBS and DR3\textsuperscript{ko} PBS lungs (Scale bar=100μm). (A) Lungs were blind scored by 2 individuals using Ashcroft score of fibrosis and an average taken for each lung. Results were significant using 1 way ANOVA (\(*p=0.009\)) whereby *p<0.05, **p<0.01. Values represent mean ± SEM. Each symbol represents data from a single mouse.
Table 5.5 Lung pathology and degree of staining following histological analysis 24 and 72hrs post inhalation

<table>
<thead>
<tr>
<th>Stain</th>
<th>DR3&lt;sup&gt;wt&lt;/sup&gt;</th>
<th>DR3&lt;sup&gt;ko&lt;/sup&gt;</th>
<th>Significance&lt;sup&gt;5&lt;/sup&gt;</th>
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<tr>
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<td>OVA&lt;sup&gt;^&lt;/sup&gt;</td>
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</tr>
<tr>
<td>24hrs&lt;sup&gt;ε&lt;/sup&gt;</td>
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<td><strong>4.5±0.3</strong></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>PAS</td>
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</tr>
<tr>
<td></td>
<td>+</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>VG</td>
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<td><strong>3.4±0.3</strong></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>72hrs&lt;sup&gt;¥&lt;/sup&gt;</td>
<td>H&amp;E</td>
<td>2.4±0.3</td>
<td><strong>4.0±0.5</strong></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>PAS</td>
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<tr>
<td></td>
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</tbody>
</table>

* PBS corresponds to mice challenged via inhalation with PBS

^ OVA corresponds to mice challenged via inhalation with OVA

ε 24hrs corresponds to mice harvested 24hrs after the final inhalation challenge

¥ 72hrs corresponds to mice harvested 72hrs after the final inhalation challenge

<sup>5</sup> Significance between DR3<sup>wt</sup> OVA and DR3<sup>ko</sup> OVA groups using 1 way ANOVA and Bonferroni post test. N.S.D = no significant difference, *p<0.05.

An arbitrary measure of staining intensity was used with + meaning low and +++ meaning high

H&E scores are representative of a scoring regime found in Chapter 2, PAS scores represent the % of PAS+ areas a % of the lung, VG scores are representative of the Ashcroft core of fibrosis, found in Chapter 2
5.3.2.2 DR3 expression following chronic allergic lung inflammation

5.3.2.2.1 DR3 expression in the lungs was similar between DR3\textsuperscript{wt} OVA and DR3\textsuperscript{wt} PBS challenged mice

DR3 signals were analogous between DR3\textsuperscript{wt} OVA (7.0±2.8\%) and DR3\textsuperscript{wt} PBS (6.5±2.7\%) mice at 72hrs post inhalation, although both were significantly greater than their DR3\textsuperscript{ko} counterparts (\textit{p}=0.025) and isotype controls (Figure 5.10). At 24hrs post inhalation however, DR3\textsuperscript{wt} PBS mice showed 11.0±4.1\% DR3 signal, significantly more than DR3\textsuperscript{wt} OVA treated mice at 8.9±1.7\% (\textit{p}=0.006). Both treatments groups of DR3\textsuperscript{wt} genotype gave significantly greater signals than DR3\textsuperscript{ko} mice (\textit{p}=0.004) (Figure 5.9). DR3 expression appeared to be focused in the epithelium of the mouse bronchioles, in a similar location to the goblet cells identified using Periodic Acid Schiff stain (Section 5.3.2.1.2). Occasional signals were also noted in the alveolar spaces and smooth muscle.
Figure 5.9. DR3 expression 24hrs post chronic allergic lung inflammation. Mice were sacrificed 24hr post final inhalation challenge with either OVA or PBS. Lungs were stained with DR3 antibody. Shown are representative photos of DR3wt OVA, DR3ko OVA, DR3wt PBS, DR3ko PBS, DR3wt isotype and DR3ko isotype (Scale bar=100μm). DR3 expression in the lung was analysed using 5 fields of view of each lung and measuring the % of positive (brown) in each field of view. This was then averaged for each mouse (n=5). Analysis was performed using Leica Qwin V3 Software. Results were significant using 2 way ANOVA (Interaction=0.073, Genotype=**0.004, Inhalation treatment=**0.006) whereby *p<0.05, **p<0.01. Bars represent mean ± SEM.
Figure 5.10. DR3 expression 72hrs post chronic allergic lung inflammation. Mice were sacrificed 72hr post final inhalation challenge with either OVA or PBS. Lungs were stained with DR3 antibody. Shown are representative photos of DR3\textsuperscript{wt} OVA, DR3\textsuperscript{ko} OVA DR3\textsuperscript{wt} PBS, DR3\textsuperscript{ko} PBS, DR3\textsuperscript{wt} isotype and DR3\textsuperscript{ko} isotype (Scale bar=100μm). DR3 expression in the lung was analysed using 5 fields of view of each lung and measuring the % of positive (brown) in each field of view. This was then averaged for each mouse (n=6). Analysis was performed using Leica Qwin V3 Software. Results were significant using 2 way ANOVA ($\text{Interaction}=0.266$, $\text{Genotype}=0.025$, $\text{Inhalation treatment}=0.054$) whereby *p<0.05. Bars represent mean ± SEM.
5.3.3 The systemic response in chronic allergic lung inflammation

5.3.3.1 Splenic responses in chronic allergic lung inflammation

5.3.3.1.1 Splenocyte total cell number was significantly higher in DR3<sup>wt</sup> OVA mice

The spleen was harvested following chronic allergic lung inflammation to assess whether a systemic response had occurred. At 24hrs post inhalation, DR3<sup>wt</sup> OVA mice contained $11.1\pm1.0 \times 10^7$ splenocytes, significantly more than DR3<sup>ko</sup> PBS ($6.2\pm0.8 \times 10^7$) and DR3<sup>ko</sup> OVA ($5.3\pm1.0 \times 10^7$) mice ($p=0.002$) (Figure 5.11). At 72hrs post inhalation both DR3<sup>wt</sup> OVA ($13.2\pm1.1 \times 10^7$) and PBS ($11.5\pm1.1 \times 10^7$) groups displayed significantly more leukocytes than DR3<sup>ko</sup> OVA ($5.3\pm0.6 \times 10^7$) and PBS ($7.8\pm0.7 \times 10^7$) groups ($p<0.0001$) (Figure 5.11).
**Figure 5.11.** Total leukocyte number from the spleens of DR3\(^{wt}\) and DR3\(^{ko}\) mice following chronic allergic lung inflammation. Mice were sacrificed at (A) 24hr and (B) 72hr post final inhalation challenge with either OVA or PBS. Splenocytes were extracted via mashing of the spleen and counted using a haemocytometer. Total splenocyte number showed significant differences at 24hr (**\(p=0.002\)) and 72hr (**\(*p<0.0001\)). Significance determined using 1 way ANOVA and Bonferroni post test; *\(p<0.05\), **\(p<0.01\), ***\(p<0.001\). Values represent mean ± SEM. Each symbol represents data from a single mouse.
5.3.3.1.2 Myeloid cell subsets in the spleen were significantly higher in DR3<sup>wt</sup> groups

Multiple myeloid cell subsets were significantly higher in DR3<sup>wt</sup> OVA mice compared to other treatment groups at 24hrs post inhalation. There were significantly more monocytes/macrophages at 1.4±0.1 x10<sup>7</sup> compared to DR3<sup>ko</sup> OVA mice at 0.5±0.1 x10<sup>7</sup> (p=0.001), as well as neutrophils (DR3<sup>wt</sup> OVA 1.1±0.2 x10<sup>6</sup>, DR3<sup>ko</sup> OVA 0.5±0.1 x10<sup>6</sup>) (p=0.012) (Figure 5.12). Dendritic cells, both myeloid and plasmacytoid, also differed between DR3<sup>wt</sup> OVA mice and other treatment groups, at 0.3±0.03 x10<sup>6</sup> myeloid DCs in contrast to 0.2±0.06 x10<sup>6</sup> in DR3<sup>ko</sup> PBS and 0.1±0.06 x10<sup>6</sup> in DR3<sup>ko</sup> OVA groups (p=0.001). Similarly, plasmacytoid DC cell number was significantly higher in DR3<sup>wt</sup> OVA mice (2.6±0.2 x10<sup>5</sup>) than DR3<sup>wt</sup> PBS (1.5±0.2 x10<sup>5</sup>), DR3<sup>ko</sup> OVA (1.3±0.2 x10<sup>5</sup>) and DR3<sup>ko</sup> PBS (1.2±0.3 x10<sup>5</sup>) treated animals (p=0.001) (Figure 5.12). Eosinophils were not significantly different at the 24hr harvest timepoint (p=0.20).

At 72hrs post inhalation, DR3<sup>wt</sup> groups again presented with higher myeloid cell numbers than DR3<sup>ko</sup> mice. There were significantly more splenic macrophages in DR3<sup>wt</sup> OVA (1.4±0.2 x10<sup>7</sup>) and DR3<sup>wt</sup> PBS mice (1.4±0.1 x10<sup>7</sup>) than DR3<sup>ko</sup> OVA (0.3±0.1 x10<sup>7</sup>) and DR3<sup>ko</sup> PBS (0.3±0.03 x10<sup>7</sup>) groups (p<0.0001), as well as 7/4<sup>+</sup> monocytes (DR3<sup>wt</sup> OVA 3.6±0.5 x10<sup>6</sup>, DR3<sup>wt</sup> PBS 3.8±0.3 x10<sup>6</sup>, DR3<sup>ko</sup> OVA 0.8±0.2 x10<sup>6</sup>, DR3<sup>ko</sup> PBS 0.7±0.1 x10<sup>6</sup>) (p<0.0001) and 7/4<sup>-</sup> monocytes (DR3<sup>wt</sup> OVA 10.9±0.8 x10<sup>6</sup>, DR3<sup>wt</sup> PBS 9.9±1.3 x10<sup>6</sup>, DR3<sup>ko</sup> OVA 3.7±1.1 x10<sup>6</sup>, DR3<sup>ko</sup> PBS 2.8±1.2 x10<sup>6</sup>) (p<0.0001) (Figure 5.13). Similarly, DR3<sup>wt</sup> OVA (2.1±0.3 x10<sup>6</sup>) and DR3<sup>wt</sup> PBS (2.4±0.3 x10<sup>6</sup>) mice had significantly higher levels of neutrophils than DR3<sup>ko</sup> OVA (0.4±0.1 x10<sup>6</sup>) and DR3<sup>ko</sup> PBS (0.3±0.04 x10<sup>6</sup>) (p<0.0001) treated animals. In contrast to 24hrs post inhalation, eosinophils were also different at the 72hr timepoint. DR3<sup>wt</sup>
genotypes had increased subset numbers in relation to DR3\textsuperscript{ko} mice (DR3\textsuperscript{wt} OVA 1.0±0.2 x10\textsuperscript{6}, DR3\textsuperscript{wt} PBS 1.4±0.2 x10\textsuperscript{6}, DR3\textsuperscript{ko} OVA 0.2±0.04 x10\textsuperscript{6}, DR3\textsuperscript{ko} PBS 0.2±0.03 x10\textsuperscript{6}) (p<0.0001) (Figure 5.13). When studying DCs, it was found that whilst myeloid DCs followed the trend of reduced cell numbers in DR3\textsuperscript{ko} groups (DR3\textsuperscript{wt} OVA 1.6±0.3 x10\textsuperscript{6}, DR3\textsuperscript{wt} PBS 1.9±0.2 x10\textsuperscript{6}, DR3\textsuperscript{ko} OVA 0.3±0.1 x10\textsuperscript{6}, DR3\textsuperscript{ko} PBS 0.3±0.1 x10\textsuperscript{6}) (p<0.0001), plasmacytoid DCs were comparable between DR3\textsuperscript{wt} OVA, DR3\textsuperscript{wt} PBS and DR3\textsuperscript{ko} PBS. It was only DR3\textsuperscript{ko} OVA mice that showed diminished plasmacytoid DC number, this being significantly different between DR3\textsuperscript{wt} PBS (1.6±0.2 x10\textsuperscript{6}) and DR3\textsuperscript{ko} OVA (0.5±0.2 x10\textsuperscript{6}) groups (p=0.027) (Figure 5.13).
Figure 5.12. Myeloid cell subsets from the spleens of DR3wt and DR3ko mice 24hrs post chronic allergic lung inflammation. Mice were sacrificed 24hr post final inhalation challenge with either OVA or PBS. Splenocytes were isolated and subsets determined using antibody cell markers. Monocytes/Macrophages (**p=0.001), Neutrophils (*p=0.012), Eosinophils n.s.d, Myeloid DCs (**p=0.001), Plasmacytoid DCs (**p=0.001). Significance determined using 1 way ANOVA and Bonferroni post test; *p<0.05, **p<0.01, ***p<0.001. Values represent mean ± SEM. Each symbol represents data from a single mouse.
Figure 5.13. Myeloid cell subsets from the spleens of DR3wt and DR3ko mice 72hrs post chronic allergic lung inflammation. Mice were sacrificed 72hr post final inhalation challenge with either OVA or PBS. Splenocytes were isolated and subsets determined using antibody cell markers. Macrophages (***p<0.0001), Neutrophils (***p<0.0001), Eosinophils (***p<0.0001), 7/4+ Monocytes (***p<0.0001), 7/4- Monocytes (***p<0.0001), Myeloid DCs (***p<0.0001) and Plasmacytoid DCs (*p=0.027). Significance determined using 1 way ANOVA and Bonferroni post test; *p<0.05, **p<0.01, ***p<0.001. Values represent mean ± SEM. Each symbol represents data from a single mouse.
Lymphocyte cell subsets in the spleen were significantly higher in DR3\textsuperscript{wt} groups

To examine T cell accumulation in the spleen, lymphocyte cell subset numbers were analysed following chronic inhalation challenges. DR3\textsuperscript{wt} OVA mice displayed more T cells at 24hrs post inhalation, shown by the increase in CD3\textsuperscript{+} cells compared to other treatment groups. DR3\textsuperscript{wt} OVA mice had 2.5±0.5 x 10\textsuperscript{7} T cells whilst DR3\textsuperscript{ko} PBS and DR3\textsuperscript{ko} OVA had 1.1±0.2 x 10\textsuperscript{7} and 1.0±0.2 x 10\textsuperscript{7} respectively. This difference was significant by 1 way ANOVA (\(p=0.009\)), and translated into significant differences in CD3\textsuperscript{+} T cell subsets, such as CD4\textsuperscript{+} T cells (DR3\textsuperscript{wt} OVA 15.4±2.9 x 10\textsuperscript{6}, DR3\textsuperscript{wt} PBS 7.5±1.3 x 10\textsuperscript{6}, DR3\textsuperscript{ko} OVA 5.5±1.3 x 10\textsuperscript{6}, DR3\textsuperscript{ko} PBS 6.3±0.9 x 10\textsuperscript{6}) (\(p=0.007\)), CD8\textsuperscript{+} T cells (DR3\textsuperscript{wt} OVA 9.3±1.9 x 10\textsuperscript{6}, DR3\textsuperscript{ko} OVA 3.4±0.9 x 10\textsuperscript{6}, DR3\textsuperscript{ko} PBS 4.0±0.6 x 10\textsuperscript{6}) (\(p=0.015\)) and NKT cells (DR3\textsuperscript{wt} OVA 9.0±0.3 x 10\textsuperscript{6}, DR3\textsuperscript{ko} OVA 0.3±0.1 x 10\textsuperscript{6}) (\(p=0.015\)) (Figure 5.14). Moreover, differences were also noted in the numbers of NK cells, as DR3\textsuperscript{wt} OVA mice had 2.9±0.3 x 10\textsuperscript{6} cells, compared to 1.8±0.1 x 10\textsuperscript{6} in DR3\textsuperscript{wt} PBS, 1.5±0.1 x 10\textsuperscript{6} in DR3\textsuperscript{ko} PBS and 1.4±0.2 x 10\textsuperscript{6} in DR3\textsuperscript{ko} OVA groups, this being significant (\(p=0.0002\)) (Figure 5.14).

At 72hrs post inhalation, the increase in splenic T cell numbers was not just restricted to DR3\textsuperscript{wt} OVA treated mice, as DR3\textsuperscript{wt} PBS challenged animals also displayed considerable increases compared to DR3\textsuperscript{ko} mice. At 72hrs, the disparity between the groups appeared to be genotype specific as opposed to treatment specific, as both OVA and PBS aerosolised DR3\textsuperscript{wt} mice consistently showed significantly higher T cell subset numbers in comparison to the DR3\textsuperscript{ko} genotype. CD3\textsuperscript{+} T cells measured at 2.2±0.3 x 10\textsuperscript{7} and 2.1±0.2 x 10\textsuperscript{7} in DR3\textsuperscript{wt} OVA and DR3\textsuperscript{wt} PBS mice, compared to 0.4±0.1 x 10\textsuperscript{7} and
0.3±0.04 x10^7 in DR3^ko OVA and DR3^ko PBS, a significant difference of p<0.0001 (Figure 5.15). This difference converted into the relevant T cell subsets as DR3^wt CD4^+ T cells were significantly higher than that of DR3^ko mice, (DR3^wt OVA 12.2±1.6 x10^6, DR3^wt PBS 10.7±0.7 x10^6, DR3^ko OVA 1.7±0.2 x10^6, DR3^ko PBS 1.6±0.2 x10^6) (p<0.0001), as were CD8^+ T cells (DR3^wt OVA 8.5±0.8 x10^6, DR3^wt PBS 8.0±0.7 x10^6, DR3^ko OVA 1.6±0.2 x10^6, DR3^ko PBS 1.3±0.2 x10^6) (p<0.0001) and NKT cells (DR3^wt OVA 4.2±0.7 x10^5, DR3^wt PBS 4.6±0.5 x10^5, DR3^ko OVA 0.9±0.1 x10^5, DR3^ko PBS 0.6±0.1 x10^5) (p<0.0001) (Figure 5.15). In addition to total T cells being higher in DR3^wt groups, activated T cell numbers, defined by the marker CD44, were also significantly greater in the CD8^+ T cell lineage (DR3^wt OVA 1.8±0.6 x10^6, DR3^wt PBS 1.9±0.2 x10^6, DR3^ko OVA 0.3±0.04 x10^6, DR3^ko PBS 0.2±0.02 x10^6) (p=0.0003) and CD4^+ cells, although significance was only seen between DR3^wt OVA mice (2.7±09 x10^6) and DR3^ko groups (DR3^ko OVA 0.3±0.1 x10^6, DR3^ko PBS 0.3±0.03 x10^6) (p=0.001) in this subset (Figure 5.15). Splenic NK cell numbers also increased in DR3^wt OVA (7.3±1.0 x10^6) and DR3^wt PBS (7.4±0.8 x10^6) mice after chronic allergic lung inflammation in comparison to DR3^ko OVA (1.6±0.1 x10^6) and DR3^ko PBS animals (1.2±0.2 x10^6) (p<0.0001) (Figure 5.15).
Figure 5.14. T cell subsets from the spleens of DR3wt and DR3ko mice 24hrs post chronic allergic lung inflammation. Mice were sacrificed 24hr post final inhalation challenge with either OVA or PBS. Splenocytes were isolated and subsets determined using antibody cell markers. CD3+ T cells (**p=0.009), CD4+ T cells (**p=0.007), CD8+ T cells (*p=0.015), NKT cells (*p=0.015), NK cells (**p=0.0002). Significance determined using 1 way ANOVA and Bonferroni post test; *p<0.05, **p<0.01, ***p<0.001. Values represent mean ± SEM. Each symbol represents data from a single mouse.
**Figure 5.15.** T cell subsets from the spleens of DR3<sup>wt</sup> and DR3<sup>ko</sup> mice 72hrs post chronic allergic lung inflammation. Mice were sacrificed 72hr post final inhalation challenge with either OVA or PBS. Splenocytes were isolated and subsets determined using antibody cell markers. CD3<sup>+</sup> T cells (**p<0.0001), CD4<sup>+</sup> T cells (**p<0.0001), CD8<sup>+</sup> T cells (**p<0.0001), Activated CD4<sup>+</sup> T cells (**p<0.0001), Activated CD8<sup>+</sup> T cells (**p<0.0001), NKT cells (**p<0.0001), NK cells (**p<0.0001). Significance determined using 1 way ANOVA and Bonferroni post test; *p<0.05, **p<0.01, ***p<0.001. Values represent mean ± SEM. Each symbol represents data from a single mouse.
5.3.3.2 The B cell response in chronic allergic lung inflammation

5.3.3.2.1 B cell number and antibody production in chronic allergic lung inflammation

Splenic B cell numbers were also quantified as were the levels of Total IgE and OVA specific IgG. Following OVA priming and the subsequent induction of chronic inflammation, DR3\textsuperscript{ko} mice displayed comparable antibody responses to DR3\textsuperscript{wt} mice. At both 24 and 72hrs, there were no significant differences in either Total IgE or OVA specific IgG antibody levels, although both increased in comparison to naive mice (Figure 5.16) (Figure 5.17) (Table 5.6). This was despite a greater number of B cells within the spleens of DR3\textsuperscript{wt} OVA mice at 24hrs (DR3\textsuperscript{wt} OVA 2.8±0.5 x10\textsuperscript{7}, DR3\textsuperscript{ko} OVA 1.1±0.3 x10\textsuperscript{7}, DR3\textsuperscript{ko} PBS 1.1±0.2 x10\textsuperscript{7}) (\(p=0.006\)) (Figure 5.16) and DR3\textsuperscript{wt} OVA and PBS mice at 72hrs (DR3\textsuperscript{wt} OVA 1.4±0.2 x10\textsuperscript{7}, DR3\textsuperscript{wt} PBS 2.0±0.3 x10\textsuperscript{7}, DR3\textsuperscript{ko} OVA 0.3±0.1 x10\textsuperscript{7}, DR3\textsuperscript{ko} PBS 0.3±0.1 x10\textsuperscript{7}) (\(p<0.0001\)) (Figure 5.17).
Figure 5.16. The B cell response of DR3\textsuperscript{wt} and DR3\textsuperscript{ko} mice 24hrs post chronic allergic lung inflammation. Mice were sacrificed 24hr post final inhalation challenge with either OVA or PBS. (A) B cells were identified using specific antibody markers (*p=0.006). Serum was isolated from blood obtained from cardiac puncture. Serum was tested for concentrations of (B) IgE (n.s.d) and (C) OVA specific IgG (n.s.d) by ELISA. Each point represents the mean of doublets from different mice. Significance tested using 1 way ANOVA and Bonferroni post test; *p<0.05. Values represent mean ± SEM. Each symbol represents data from a single mouse.
Figure 5.17. The B cell response of DR3<sup>wt</sup> and DR3<sup>ko</sup> mice 72hrs post chronic allergic lung inflammation. Mice were sacrificed 72hr post final inhalation challenge with either OVA or PBS. (A) B cells were identified using specific antibody markers (***(p<0.0001). Serum was isolated from blood obtained from cardiac puncture. Serum was tested for concentrations of (B) IgE (n.s.d) and (C) OVA specific IgG (n.s.d) by ELISA. Each point represents the mean of doublets from different mice. Significance tested using 1 way ANOVA and Bonferroni post test; ***(p<0.001. Values represent mean ± SEM. Each symbol represents data from a single mouse.
Table 5.6 Antibody levels following chronic allergic lung inflammation harvested 24 and 72hrs post inhalation challenge

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<td>PBS$^*$</td>
<td>OVA$^\text{^}$</td>
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<tr>
<td>24hrs$^\varepsilon$</td>
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<td>OVA IgG</td>
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<td>0.3±0.01</td>
</tr>
<tr>
<td>72hrs$^\upsilon$</td>
<td>Total IgE</td>
<td>1417±798</td>
<td>1407±141</td>
</tr>
<tr>
<td></td>
<td>OVA IgG</td>
<td>0.3±0.01</td>
<td>0.3±0.01</td>
</tr>
</tbody>
</table>

$^*$ PBS corresponds to mice challenged via inhalation with PBS

$^\text{^}$ OVA corresponds to mice challenged via inhalation with OVA

$^\varepsilon$ 24hrs corresponds to mice harvested 24hrs after the final inhalation challenge

$^\upsilon$ 72hrs corresponds to mice harvested 72hrs after the final inhalation challenge

$^\$ Significance between DR3^{wt} OVA and DR3^{ko} OVA groups using 1 way ANOVA, N.S.D = no significant difference

Values represent the mean±SEM. Total IgE (pg/ml) measured by ELISA. OVA IgG scores are the optical density reading from the OVA specific IgG ELISA using serum from DR3^{wt} and DR3^{ko} mice.
5.4 Discussion

5.4.1 DR3 influence on airway inflammation

To date, no data has been published on the role of DR3 in chronic allergic lung inflammation. Results within this Chapter suggest that leukocyte accumulation into the lungs following chronic challenge is variable. Twenty four hours after the final inhalation challenge, both DR3\(^{wt}\) and DR3\(^{ko}\) OVA challenged mice displayed increased numbers of multiple cell types compared to PBS challenged animals, although this was rarely significant. Similarly, mice harvested 72hrs post inhalation showed similar numbers of numerous cell types, including monocytes, T cells and B cells. It can therefore be said that for the majority of subsets studied and in contrast to the acute model, a lack of DR3 did not significantly affect leukocyte accumulation into the lungs or BALF during chronic allergic lung inflammation.

An exception however, were myeloid DCs, as DR3\(^{wt}\) OVA lungs contained significantly more than all other treatment groups, though this increase did not correlate with an increase in T cells. Myeloid DCs are thought to be key in the generation of an allergen specific T cell response (van Rijt, Jung et al. 2005; Lambrecht and van Rijt 2006) and are found throughout the murine lung, within the intraepithelial layer of the large conducting airways as well as in the lung interstitium (Wikstrom and Stumbles 2007). Upon allergen exposure, DCs migrate to the draining lymph nodes in response to homing chemokines and undergo maturation. It has also been suggested that airway epithelial cells control DC activation and migration. Chimaeric mice with radioresistant stromal cells and radiosensitive haematopoietic cells, but deficient in the LPS receptor TLR4, were challenged via inhalation with LPS. Results demonstrated that DC
migration to lymph nodes was dependent on TLR4 signalling on epithelial cells (Hammad, Chieppa et al. 2009), suggesting that as well as lymph node homing chemokines, epithelial cells also function to control DC activity. Upon maturation, DCs are able to directly prime naive T cells to differentiate into effector T cells. It has been reported that human DCs activated by thymic stromal lymphopoietin (TSLP), a DC modulating cytokine, could also directly prime CD4⁺ T cells to differentiate into pro-inflammatory Th2 cells via the release of the TNFSF ligand, OX40-L (Ito, Wang et al. 2005). Furthermore, TSLP has been shown to play a significant role in murine DC responses to antigen, as mice that conditionally over-expressed the cytokine mounted vigorous Th2 responses (Headley, Zhou et al. 2009), compared to TSLPRKO mice which failed to develop an antigen-specific response unless adoptively transferred with WT CD4⁺ T cells (Al-Shami, Spolski et al. 2005). It would be interesting to investigate whether TL1A release by both stimulated human (Prehn, Thomas et al. 2007) and murine (Bamias, Mishina et al. 2006; Meylan, Davidson et al. 2008) DCs, could be influenced by TSLP similar to OX40-L.

Previous studies have found DR3 to have an active role in cellular infiltration and accumulation in chronic disease models. In the AIA model of Rheumatoid Arthritis, DR3KO mice displayed reduced cellular infiltration at day 21, correlating with reduced structural damage to the joint, assessed by absence of synovial hyperplasia, lack of pannus formation and no bone erosion (Bull, Williams et al. 2008). Moreover, anti-TL1A treatment at the point of arthritic induction significantly reduced clinical signs of disease in a collagen induced model of arthritis (CIA). This was partly characterised by reduced leukocyte infiltration into the synovial tissue (Bull, Williams et al. 2008). Similarly in a CIA model, human TL1A was found to aggravate disease and enhance
disease manifestation, including increased cartilage damage, pannus formation and cell infiltration (Zhang, Wang et al. 2009). In a DSS model of chronic colitis, increased levels of memory T cells and B cells were noticed in the lamina propria, as well as levels of IFNγ and IL-17. However, administration of an anti-TL1A antibody neutralised these effects, as mice demonstrated reduced CD4+ T cells and cytokine production from the gut associated lymphoid tissue as well as mucosal T cells (Takedatsu, Michelsen et al. 2008). In a G protein αi2−/− T cell transfer model, spontaneous development of colitis can be abrogated by injection of anti-TL1A, reducing inflammatory cell infiltration into the mucosa and down-regulating T cell activation (Takedatsu, Michelsen et al. 2008). Furthermore, reduced numbers of CD4+ T cells were detected in the spinal cords of DR3ko and TL1Ako mice compared to DR3wt mice following the induction of EAE (Meylan, Davidson et al. 2008; Pappu, Borodovsky et al. 2008), all suggesting that the DR3/TL1A axis is important in the development of disease which is often concurrent with cellular infiltration. However, whilst allergic lung inflammation is predominately considered a Th2 mediated disease, experimental antigen induced arthritis, DSS induced chronic colitis and EAE, are believed to be Th1/Th17 mediated.

When studying the immune response to *Toxoplasma gondii*, DR3wt and DR3ko mice were immunised via the peritoneal cavity with cysts from the brains of infected animals. Infection and survival rates were comparable between the 2 genotypes, as were levels of infiltrating CD3+ T cells. Stimulated spleen cells also produced equivalent amounts of TNFα, IFNγ and IL-10 in response to STAg, suggesting that this chronic model of infection was not DR3 dependent (Meylan, Davidson et al. 2008). As well as this, in a chronic model of peritoneal fibrosis, whereby mice were injected weekly over a 4 week
period with an inactivated bacterial suspension of *Staphylococcus epidermis* (SES), no differences were seen in the recruitment of leukocytes into the peritoneal cavity at day 49. However, a difference was seen between DR3\(^{wt}\) and DR3\(^{ko}\) mice in fibrosis development (Perks, 2013 PhD), indicating that structural measures of disease pathology do not necessarily correlate with leukocyte accumulation.

Numerous cell subset numbers, including eosinophils, did not significantly differ within the lung or BALF from DR3\(^{wt}\) and DR3\(^{ko}\) mice following multiple aerosolisation challenges. This is in keeping with published literature (Sakai, Yokoyama et al. 2001) and a common occurrence when modelling chronic allergic lung protocols, which continues to be problematic in exhibiting the characteristics of human asthma. Koerner-Rettberg *et al* noted sustained airway lymphocytosis but short lived eosinophilia upon long term challenge (Koerner-Rettberg, Doths et al. 2008). Meanwhile, Swirski *et al* describes diminishing eosinophilia by 3 weeks and complete resolution after 4 weeks of antigen exposure (Swirski, Sajic et al. 2002), suggesting that chronic exposure to antigen does not sustain airway inflammation but rather leads to airway unresponsiveness. This could explain the apparent lack of differences between OVA and PBS challenged mice, with inflammation not being a predominant feature of some chronic asthma mouse models (Locke, Royce et al. 2007). This apparent lack of airway inflammation after multiple aerosol challenges is not just restricted to DR3 amongst the TNFRSF. TNFR\(^{ko}\) mice, deficient in both TNFR1 and 2, have also shown no differences compared to WT mice, in airway hyper-responsiveness, BAL leukocyte numbers and cytokine levels after 7 days of consecutive OVA aerosolisation, suggesting that TNF\(\alpha\) alone is unable to abrogate allergic lung inflammation and other cytokines have the ability to compensate for its loss (Rudmann, Moore et al. 2000).
Another potential explanation for my observations is the development of immune mediated tolerance alongside long-term challenge (Jungsuwadee, Benkovszky et al. 2004; Van Hove, Maes et al. 2007). One suggested regulatory method is an alteration in the interaction between DCs and T cells via the suppression of the co-stimulatory molecules CD28 and ICOS, thereby inhibiting DC maturation (Van Hove, Maes et al. 2007). This would be consistent with the lack of T cell expansion within the lung following chronic challenges, despite increased myeloid DC number. The measurement of these co-stimulatory molecules would therefore make an interesting point of study. Furthermore, regulatory T cells, which hold the ability to ablate inflammation upon transfer into an established chronic allergic lung inflammation mouse model (Kearley, Barker et al. 2005; Kearley, Robinson et al. 2008), could also be measured upon repeat of these experiments.

Regulatory T cells are known to express DR3 (Pappu, Borodovsky et al. 2008; Schreiber, Wolf et al. 2010; Taraban, Ferdinand et al. 2011; Taraban, Slobioda et al. 2011), though controversy exists as to the levels in comparison to conventional CD4$^+$ T cells. Schreiber et al reported preferential binding of a monoclonal DR3 antibody to T$_{reg}$, suggesting DR3 expression is higher on T$_{reg}$ than conventional CD4$^+$ T cells (Schreiber, Wolf et al. 2010), whilst Taraban et al, reported similar DR3 expression between the 2 subsets using a different commercially available polyclonal DR3 antibody. This was supported with data using soluble recombinant TL1A, which bound equally well to both conventional and regulatory CD4$^+$ T cells (Taraban, Ferdinand et al. 2011). These differences may be due to the use of different reagents, which may preferentially bind specific isoforms on T$_{reg}$. It has previously been shown that T$_{reg}$ express less full length DR3 in comparison to Th17 cells (Pappu, Borodovsky et al. 2008).
2008), with Taraban et al stating that the antibody used throughout their studies was capable of detecting both full length and truncated DR3, confirmed through retroviral expression studies with the 2 isoforms in the mouse embryo fibroblast cell line, NIH3T3 (Taraban, Ferdinand et al. 2011). It is therefore possible that the antibody used by Schreiber et al, binds epitopes of DR3, that detect splice variants that are differentially expressed on distinct CD4+ T cells subsets.

Functional effects of DR3 signalling on the T_{reg} subset have also been studied, with Schreiber et al using an agonistic DR3 antibody to expand the pool of T_{regs} within the lung, reversing the ratio of conventional T cells to T_{reg}s. This was reported to lead to the suppression of acute allergic lung inflammation (Schreiber, Wolf et al. 2010). Similarly, Reddy et al observed T_{reg} expansion in mice virally infected with Herpes Simplex Virus-1 (HSV-1), reducing the severity of stromal keratitis lesions, particularly when administered with galectin-9 (J Reddy, Schreiber et al. 2012). However, in a model of small intestinal inflammation, Taraban et al argued that whilst DR3 activation did increase the levels of T_{reg}s, it also conferred co-stimulatory effects on conventional CD4+ T cells, causing a suppressive effect on the ability of T_{reg}s to attenuate inflammation (Taraban, Ferdinand et al. 2011; Taraban, Slebioda et al. 2011). In response to this, Schreiber et al suggested that the balance between DR3 triggering regulatory or effector immunity was likely dependent upon the availability of cognate antigen to the T_{reg} or effector T cell subsets, rather than the difference in receptor expression (Schreiber, Khan et al. 2011). This conflicting data emphasises the need to study T_{reg}s in more detail in this particular chronic model of allergic lung inflammation.
Alternatively, the lack of lung leukocyte differences between OVA and PBS challenged mice, and possibly DR3\textsuperscript{wt} and DR3\textsuperscript{ko} mice, may be the extraction method employed throughout this study. Once lungs were removed, they were sliced into small pieces, treated with collagenase and passed through a cell strainer (Section 2.2.5.1). This process resulted in relatively high levels of cell death. However, the protocol also allowed access to the cells locked within the lung compartments, which would otherwise not have been examined. Intriguingly, despite flow cytometry results suggesting few differences in leukocyte accumulation, the H&E stains showed greater cellular infiltration in the peribronchial and perivascular areas of DR3\textsuperscript{wt} OVA mice. This discrepancy could be explained by inefficient extraction of leukocyte subsets and/or preferential viability dependent on DR3 expression. However, alternative methods also have disadvantages. Mechanical digestion or mincing are more aggressive than enzymatic digestion, whilst multi-colour microscopy on lung sections are more limited in the number of fluorescence markers that can be used to differentiate cell subsets.

Myeloid cells, particularly macrophages, are known to be especially adhesive when activated (Taylor, personal communication), which may be enhanced amid repeated exposure to allergen, making cells more difficult to extract. Having said this, the lack of leukocyte differences between DR3\textsuperscript{wt} and DR3\textsuperscript{ko} mice following chronic allergic lung inflammation may be model rather than DR3 related.

Aside from myeloid DCs, no other cell subset was observed to significantly increase in number in comparison to DR3\textsuperscript{ko} mice. It is therefore unsurprising that no chemokine
differences were observed in the BALF of DR3\textsuperscript{ko} and DR3\textsuperscript{wt} mice (Appendix figure 1.1) (Appendix Table 1.1).

5.4.2 Effects of DR3 on airway remodelling

Whilst chronic models of allergic lung disease have been shown to have modest levels of inflammation, airway remodelling is considered a fundamental characteristic feature of the disease. My results showed that the lungs of DR3\textsuperscript{wt} OVA mice underwent airway remodelling to a significantly higher degree than DR3\textsuperscript{ko} OVA mice, implying DR3 is involved in the regulation of lung pathology. DR3\textsuperscript{wt} OVA mice had higher levels of mucin than DR3\textsuperscript{ko} OVA mice and PBS treated groups. Mucin production is a key contributor to asthma and via the production of mucus plugs, also to fatalities (Locke, Royce et al. 2007). It is unknown however, whether this increase in mucin is due to the differentiation or proliferation of goblet cells. The transcription factor SPDEF is thought to be involved in the conversion of Clara cells, goblet cell precursors, into mucin producing goblet cells (Chen, Korfhagen et al. 2009). Loss of function experiments using SPDEF would confirm if the observed goblet cell increase was due to differentiation or proliferation, and whether DR3 acted upstream or downstream of this transcription factor. Moreover, DR3 has already been shown to have a role in goblet cell hyperplasia, albeit in a model of small intestinal inflammation (Meylan, Song et al. 2011; Shih, Barrett et al. 2011; Taraban, Slebioda et al. 2011). Mice which constitutively expressed TL1A on both T cells (Meylan, Davidson et al. 2008) and DCs (Taraban, Slebioda et al. 2011) spontaneously developed small bowel disease which was characterised by goblet cell hypertrophy and hyperplasia. This was shown to be IL-13 driven.
IL-13 has been shown to be a key contributor to goblet cell hyperplasia in the lung (Wills-Karp, Luyimbazi et al. 1998; Chen, Sivaprasad et al. 2013), along with IL-5 (Lee, McGarry et al. 1997). Compared to DN DR3 or DR3<sup>ko</sup> mice, DR3<sup>wt</sup> animals were found to have increased numbers of goblet cells in acute allergic lung inflammation, which correlated with increases in IL-13 (Fang, Adkins et al. 2008; Meylan, Davidson et al. 2008). While CD4<sup>+</sup> Th2 cells are considered one of the main sources for this cytokine, Doherty et al found that CD4<sup>+</sup> T cell depletion had no effect on remodelling progression (Doherty, Soroosh et al. 2009), indicating the central source of IL-13 may lie elsewhere. Epithelial cells themselves have been implicated in the release of remodelling cytokines (Polito and Proud 1998; Holgate, Lackie et al. 2000; Allahverdian, Harada et al. 2008), as have more recently, a population of innate cells known as nuocytes or type 2 innate lymphoid cells (ILC) (Barlow, Bellosi et al. 2012). ILC2s can be found in the lung (Monticelli, Sonnenberg et al. 2011) and have the capacity to release IL-13 in response to IL-33 and IL-25, which themselves have been implicated in the airway remodelling process. IL-33 release from epithelial cells triggers goblet cell hyperplasia, Th2 cytokine production, as well as Th2 cell differentiation via the programming of DCs through the IL-33 receptor ST2 (Rank, Kobayashi et al. 2009). Alveolar macrophages too have been shown to release IL-33 and in turn, induce remodelling. Macrophage depletion abolished airway remodelling as opposed to CD4<sup>+</sup> T cell depletion, where the process continued to occur (Mizutani, Nabe et al. 2013). IL-25, a member of the IL-17 cytokine family has also been implicated in the airway remodelling process following allergic lung inflammation. Released by differentiated Th2 cells (Fort, Cheung et al. 2001), activated eosinophils, bone marrow derived mast cells, FceRI activated basophils and epithelial cells (Hammad, Chieppa et al. 2009), IL-
25 is thought to act on stromal cells (Swaidani, Bulek et al. 2009) and macrophages (Yang, Grinchuk et al. 2013). IL-25 levels have been shown to increase in response to MMP-7, which is induced in allergen activated epithelial cells, cleaving the cytokine to its more active form to promote Th2 cytokine production (Goswami, Angkasekwinai et al. 2009). Systemic administration of recombinant IL-25 for 10 days in C57BL/6 mice induced peripheral blood, lung and spleen eosinophilia, elevated serum antibody levels and pulmonary goblet cell metaplasia. It would be interesting to investigate the interaction between these cytokines and the DR3/TL1A pathway in regulation of mucin production in the lung.

When studying the fibrotic response, there were no significant differences between DR3\textsuperscript{wt} and DR3\textsuperscript{ko} OVA mice in collagen production. The DR3/TL1A pathway has previously been shown to be involved in intestinal fibrosis (Shih, Barrett et al. 2011; Barrett, Zhang et al. 2012; Zheng, Zhang et al. 2013). Mice which constitutively expressed TL1A exhibited enhanced gut fibrosis compared to WT mice (Barrett, Zhang et al. 2012), which correlated with an increase in TGFβ1 (Shih, Barrett et al. 2011), a known mediator of fibrosis (Le, Cho et al. 2007). Furthermore, TL1A and crosslinking of DR3 with a monoclonal antibody has been shown to induce TGFβ1 \textit{in vitro} from the macrophage cell line, THP-1 (Lee, Kim et al. 2010). Thus, whilst published work suggests a role for the DR3/TL1A pathway in the induction of TGFβ1 and therefore collagen production, the data described in this Chapter does not. Further work would be required to determine how much this is due to organ and animal model-specific DR3 function.
Interestingly, other TNFR members have been found to have a role in the fibrotic response during chronic allergic lung inflammation. Inhibition of LIGHT led to a reduction in the level of fibrosis as well as smooth muscle mass, thought to be mediated via the inhibition of TGFβ and IL-13 release (Doherty, Soroosh et al. 2011). Similarly, TNFR p55/p75 deficient mice demonstrated significantly reduced peribronchial fibrosis, smooth muscle layer and deposition of extracellular matrix proteins in a model of chronic allergic lung inflammation (Cho, Kwon et al. 2006). The lack of differences seen within this Chapter suggests that among the TNFRSF, DR3 is not essential for the initiation and development of lung fibrosis.

5.4.3 DR3 expression in the lungs following chronic allergic lung inflammation

DR3 expression in the lung was comparable between DR3<sup>wt</sup> OVA and PBS treated mice following chronic allergic lung inflammation at 72hrs, but significantly higher in DR3<sup>wt</sup> PBS mice at 24hrs post inhalation. However, as mentioned previously (Section 3.4.2) the DR3 antibody used was unable to distinguish between the 3 murine splice variants; a full-length membrane-bound variant, a soluble form and truncated membrane-bound form (Wang, Kitson et al. 2001). Expression of the different isoforms has already been shown to change depending on cellular activation (Bamias, Mishina et al. 2006; Twohig, Marsden et al. 2012), prompting careful consideration of DR3 splice variant function following T cell activation. Without the identification of the splice variant within the chronic lungs of DR3<sup>wt</sup> OVA and PBS mice, it is difficult to draw conclusions regarding function. Additionally, DR3 has been shown to have a transient nature in certain cell subsets, namely CD<sup>8+</sup> T cells. Purified splenocyte derived T cells were stimulated <em>in vitro</em> with an anti-CD3 antibody, and the pattern of DR3 expression
observed. CD8\(^+\) T cells up-regulated DR3 rapidly, peaking at 24hrs, before declining to baseline levels at 72hrs post stimulation (Twohig, Marsden et al. 2012). DR3 expression may be transient in the lungs of DR3\(^{wt}\) OVA challenged mice, explaining the temporary decrease in DR3 signal at 24hrs in comparison to DR3\(^{wt}\) PBS mice.

DR3 signal was predominately observed in the epithelial areas of the lung bronchioles, correlating with the location of goblet cells. Given the significant and consistent differences noted in goblet cell specific signal, it is tempting to speculate that DR3 is expressed on these mucin producing cells, with signalling through the receptor directly driving mucin production. However, attempts to test this using a dual PAS/DR3 stain proved unsuccessful. Despite the complexity in isolating goblet cells from mice, Wang et al have reported Clara cells can be identified using flow cytometry with a marker known as Clara cell secretory protein (CSSP) (Wang, Keefe et al. 2012). Purified CSSP\(^+\) cells can then be grown in a culture to mimic the lung environment (Wang, Keefe et al. 2012). This could potentially provide a means to analyse DR3 expression on goblet cells, although removal of cells for expansion in vitro may change their physiological and phenotypic properties.

5.4.4 The effects of DR3 on the systemic response

DR3 had no effect on the production of allergic antibodies as comparable levels of Total IgE and OVA specific IgG were seen between DR3\(^{wt}\) and DR3\(^{ko}\) mice, agreeing with previously published acute allergic lung inflammation findings (Meylan, Davidson et al. 2008). This is also similar to Bull et al, who following the induction of AIA, observed normal anti-BSA antibody levels in DR3\(^{ko}\) mice (Bull, Williams et al. 2008). In a model
of CIA however, the addition of TL1A acted to increase the levels of collagen specific 
IgG and IgG2a, suggesting TL1A can enhance the humoral response. This was 
supported with an increase in the size of splenic germinal centres in mice treated with 
TL1A daily for 10 days, required for B cell activation and differentiation into antibody 
producing plasma cells (Zhang, Wang et al. 2009).

DR3 has previously shown to be expressed on human antigen stimulated B cells in the 
germinal centres of tonsils and the spleen (Cavallini, Lovato et al. 2013). Human in 
vitro studies have shown that TL1A is able to reduce the proliferation of B cells which 
have been suboptimally activated with IgM and IL-2 (Cavallini, Lovato et al. 2013), 
contrasting with the results of Zhang et al who suggest TL1A supports the humoral 
response and potentially B cell proliferation. These discrepancies may be due to the use 
of different systems and species to examine the relationship between DR3 and B cells. 
Results within this Chapter also suggests DR3 regulates splenic B cell number 
following antigen challenge, though the immunological impact of this remains obscure 
as quantitative antibody production was unaffected.

The effects on the central immune system were further scrutinised via the analysis of 
leukocyte subsets in the spleen following chronic allergic lung inflammation. Data 
showed that DR3\textsuperscript{wt} OVA mice at 24hrs and DR3\textsuperscript{wt} OVA and PBS mice at 72hrs had 
significantly higher numbers of both myeloid and lymphocytic cells than DR3\textsuperscript{ko} mice, 
suggesting DR3 regulates global cellular accumulation in the spleen, in this case after 
repeated localised exposure to antigen in the lung. The analogous production of OVA 
specific IgG between DR3\textsuperscript{wt} and DR3\textsuperscript{ko} OVA aerosolised animals implies that the 
sensitisation stages in this model were not impaired. Others have also reported that DR3
is not required for central immune responses following acute allergic lung inflammation (Meylan, Davidson et al. 2008). Another possible explanation for the data presented in this Chapter is a defect in leukocyte recirculation from the spleen to the lung upon allergen challenge. Potential reasons for these considerable differences will be discussed further in Chapter 6.
5.5 Summary

The relationship between airway inflammation, impaired lung function and airway remodelling is poorly understood. This is made more difficult by the variety of sensitisation and challenge protocols as well as the array of read-outs used to assess the responses. In this model of chronic allergic lung inflammation, it was found that airway inflammation and allergic antibody production were not DR3 dependent. However, DR3 did regulate mucin production and peribronchiolar cellular infiltration, although the underlying mechanisms behind this are unknown. There was also a clear phenotype within the spleen, as DR3\(^{ko}\) mice displayed decreased leukocyte numbers, the mechanisms for which will be investigated further in Chapter 6.
Chapter 6:

Investigating the role of DR3 in early priming events
6.1 Introduction

The phenotypes observed in Chapter’s 4 and 5 were both striking and unexpected. Despite the use of DR3\textsuperscript{ko} mice to study allergic lung inflammation, no defects in the splenic responses following antigenic challenge had been reported (Meylan, Davidson et al. 2008). Therefore, this clear-cut defect in DR3\textsuperscript{ko} mice was explored further using a single priming event with a range of Alum concentrations in conjunction with OVA.

Alum is a commonly used adjuvant in both murine research and human vaccines, although the mechanisms by which it exerts its stimulatory effects are poorly understood. It has been suggested that the adjuvant forms an antigen depot at the site of injection, thereby prolonging the exposure to antigen and so the immune response (Glenny and Barr 1931). However, this theory has been challenged by several reports, which demonstrate that Alum is in fact absorbed within hours of injection (Weissburg, Berman et al. 1995; Gupta, Chang et al. 1996). More recently, Hutchinson et al also discredited the theory, claiming that removal of the injection site as early as 2hrs post injection had no effect on the development of antigen specific B or T cell responses (Hutchison, Benson et al. 2012).

Immuno-stimulation has also been cited as a mechanism of Alum action. Arguments persist as to whether Alum is able to directly activate antigen presenting cells (Mannhalter, Neychev et al. 1985; Sun, Pollock et al. 2003; Sokolovska, Hem et al. 2007), as the adjuvant was observed to increase antigen uptake by DCs \textit{in vitro}, as well as enhance antigen presentation magnitude and duration (Morefield, Sokolovska et al.
As well as antigen uptake, Alum is also thought to reduce the rate of antigen degradation once internalised, thereby sustaining presentation (Ghimire, Benson et al. 2012). In vivo, the effects of Alum were abolished upon depletion of CD11c+ monocytes or DCs, suggesting a central role for the cell type in Alum induced antigen uptake, as well as maturation (Kool, Soullie et al. 2008). Despite previously being thought to utilise the TLR pathway (Schnare, Barton et al. 2001), the use of MyD88-TRIF double mutant mice confirmed that the adjuvant works independently of TLRs (Gavin, Hoebe et al. 2006).

It has recently been suggested that the NALP3 inflammasome is required for Alum activity via the release of IL-1β (Li, Nookala et al. 2007; Eisenbarth, Colegio et al. 2008; Kool, Petrilli et al. 2008; Li, Willingham et al. 2008), promoting antigen specific IgE responses (Kool, Petrilli et al. 2008). Adding to this, Alum induced proliferation of antigen specific T cells in the draining lymph nodes was found to be dependent on uric acid, a known activator of NALP3. It was therefore hypothesised that Alum caused necrosis of target cells, resulting in the production of uric acid, activation of the NALP3 inflammasome, which in turn activated caspase 1 to process pro-IL-1β, pro-IL-18 and pro-IL-33 into their mature bioactive forms (Kool, Soullie et al. 2008; Martinon, Mayor et al. 2009). Neutralisation of uric acid before Alum injection by the uric acid degrading enzyme, uricase, abolished the recruitment of inflammatory monocytes to the draining lymph nodes (Kool, Willart et al. 2011), suggesting uric acid has a prominent role in Alum mediated activation of the innate immune reaction. Furthermore, phagocytosis of Alum was shown to induce phagosomal destabilisation, resulting in the release of the lysosomal protease Cathepsin B into the cytosol, initiating the activation of the NALP3 inflammasome (Hornung, Bauernfeind et al. 2008). However, the involvement of
NALP3 has been disputed by others (Franchi and Nunez 2008), whilst the use of NALP3 deficient mice only partially reduced IL-6 production and inflammatory monocyte influx after intra-peritoneal administration of Alum (Kool, Petrilli et al. 2008). In addition, the use of caspase 1 deficient mice, which cannot produce IL-1β, had no effect on Alum induced inflammation (McKee, Munks et al. 2009), suggesting Alum may work through NALP3 independent mechanisms. Kuroda et al suggest a crucial role for prostaglandin E₂ (PGE₂) to induce Alum mediated effects, via the induction of cyclooxygenase-2 (COX-2) and membrane associated PGE synthase-1 (mPGES-1). Antigen specific IgE levels were reduced in mPGES-1 deficient mice following immunisation with Alum, whilst immunisation with nitrous oxide, which induces PGE₂ secretion but not IL-1β, enhanced antibody titres, thus suggesting the mechanism was IL-1β independent. COX-2 and mPGES-1 require induction via a pro-inflammatory stimulus, whilst Alum acts to cause lysosomal damage, leading to cytosolic phospholipase A₂ (cPLA₂) activation, resulting in the release of arachidonic acid from membrane lipids, a precursor for PGE₂ (Kuroda, Ishii et al. 2011).

It has also been suggested that Alum stimulates the release of host cell DNA, which like uric acid, acts as a potent immuno-stimulatory danger signal. Host DNA was reported to induce primary B cell responses and IgG1 production, whilst also promoting canonical Th2 responses, leading to IgE isotype switching through interferon response factor 3 (Irf-3) dependent mechanisms. Antibody responses, as well as the migration of inflammatory monocytes, were reduced in the presence of DNase alongside Alum and antigen (Marichal, Ohata et al. 2011). However, DNase treatment was found to have no effect on the appearance of antigen loaded DCs in the draining lymph node, nor the expression of co-stimulatory molecules on these cells, instead prolonging the length of
interaction time between antigen loaded cells and antigen specific T cells and the ability of DCs to present peptide to CD4+ T cells using MHCII molecules. It was thus suggested that Alum acts to allow host DNA access to the cytoplasm of DCs, activating pathways to increase peptide presentation to antigen specific T cells in the draining lymph node (McKee, Burchill et al. 2013).

Despite the debate concerning Alum, it is frequently used to encourage the Th2 phenotype in murine models of allergic lung inflammation, promoting an immune response to otherwise non-immunogenic antigens. Although adjuvant free protocols have been described using OVA, (Blyth, Pedrick et al. 1996; Blyth, Wharton et al. 2000; Janssen, van Oosterhout et al. 2000), multiple exposures are often required to achieve suitable sensitisation. Furthermore, the strain of mouse was also found to be important when utilising adjuvant free protocols, as C57BL/6 mice (the strain used in these studies) were observed to exhibit a lesser inflammatory response to allergen in comparison to BALB/c animals (Conrad, Yildirim et al. 2009).
6.2 Aims

Considering the results obtained previously (Chapters 4 and 5), the aim of this Chapter was to examine the splenic response of DR3\textsuperscript{ko} mice following a single injection via the peritoneum with different concentrations of Alum alongside OVA on day 0 and harvesting on day 7. As DR3 has previously been reported to influence the efficiency of the T cell response to antigen (Meylan, Davidson et al. 2008), various concentrations of adjuvant were considered necessary. Upon harvest, leukocyte accumulation was examined as well as chemokine levels. To determine the T cell response, splenocytes were stimulated with OVA \textit{ex vivo} and proliferation measured by \(^{3}\)H-thymidine uptake, whilst the B cell response was assessed via OVA specific IgG measurements.

6.2.1 Objectives

i) Determine cellular accumulation in the spleens of DR3\textsuperscript{wt} and DR3\textsuperscript{ko} mice in response to varying concentrations of Alum

ii) Examine antigen specific responses

iii) Assess the mechanisms involved in cellular accumulation
6.3 Results

6.3.1 Cellular accumulation in the spleen

6.3.1.1 DR3 regulated leukocyte accumulation into the spleen but had no effect on spleen mass as a % of body mass

Ascending concentrations of Alum (0.1mg-50mg) mixed with OVA, as well as OVA alone were used to look at the response of DR3 to adjuvant and antigen challenge in the spleen. Mice were injected i.p once and spleens harvested after 7 days. Weights of the mice and their spleens were also recorded. Results showed that DR3 had no effect on spleen mass, expressed as a % of total body mass, as no genotype specific differences were seen ($p=0.242$). Increasing concentrations of Alum were significantly associated with increases in spleen mass in both DR3$^{ko}$ and DR3$^{wt}$ mice ($p<0.0001$), which also corresponded with higher total cell numbers in the spleen ($p=0.013$). DR3 was, however, found to regulate cellular accumulation in the spleen ($p=0.004$), as DR3$^{ko}$ mice consistently displayed lower cell numbers compared to DR3$^{wt}$ animals, particularly when suboptimal doses of Alum were used (Alum 50mg DR3$^{wt}$ 11.8±1.2 x10$^7$, DR3$^{ko}$ 8.8±1.2 x10$^7$, Alum 0.4mg DR3$^{wt}$ 8.8±1.1 x10$^7$, DR3$^{ko}$ 5.3±1.1 x10$^7$) (Figure 6.1).
Figure 6.1. Total leukocyte number and spleen mass of DR3<sup>wt</sup> and DR3<sup>ko</sup> mice injected with Alum and OVA. Mice were injected on day 0 via the peritoneal cavity and sacrificed on day 7. Splenocytes were extracted via mashing of the spleen and counted using a haemocytometer. Total cell number ($\text{Interaction}=0.206$, $\text{Genotype}=**0.004$, $\text{Alum}=*0.013$). Spleen mass was calculated as a % of total body mass. Spleen mass ($\text{Interaction}=0.755$, $\text{Genotype}=0.242$, $\text{Alum} = ***p<0.0001$). Significance determined using 2 way ANOVA; *$p<0.05$, **$p<0.01$, ***$p<0.001$. Bars represent mean ± SEM (n=6). Data shown is a combination of 2 individual experiments.
6.3.1.2 DR3\(^{ko}\) mice displayed reduced numbers of dendritic cells at all concentrations of Alum

DR3\(^{ko}\) mice displayed decreased numbers in several myeloid cell subsets examined. The most consistent of these were DCs, including both myeloid and plasmacytoid DCs. DR3\(^{wt}\) myeloid DC number did not change in relation to Alum (\(p=0.718\)), with similar cell numbers being seen across all the concentrations tested. DR3\(^{wt}\) myeloid DC number was reliably increased compared to DR3\(^{ko}\) mice at all Alum concentrations, from 0.1mg (DR3\(^{wt}\) 0.9±0.1 x10\(^6\), DR3\(^{ko}\) 0.7±0.1 x10\(^6\)) to 50mg (DR3\(^{wt}\) 1.2±0.2 x10\(^6\), DR3\(^{ko}\) 0.7±0.1 x10\(^6\)) (\(p<0.0001\)) (Figure 6.2). However, DR3\(^{ko}\) mice injected with OVA alone, displayed analogous cell numbers to DR3\(^{wt}\) mice (DR3\(^{wt}\) 1.0±0.1 x10\(^6\), DR3\(^{ko}\) 1.1±0.2 x10\(^6\)), suggesting Alum, is required to induce the differences seen, which resulted in a fall of numbers in DR3\(^{ko}\) mice.

In contrast, DR3\(^{wt}\) plasmacytoid DCs varied relative to Alum concentration. Cell numbers were similar between doses 0.4mg-1mg, at which point plasmacytoid DCs increased in a dose dependent manner (\(p=0.031\)). DR3\(^{wt}\) numbers were higher than DR3\(^{ko}\) mice at all concentrations, ranging from 1.5±0.3 x10\(^5\) compared to 0.8±0.1 x10\(^5\) at 0.1mg Alum, respectively, to 2.3±0.9 x10\(^5\) compared to a DR3\(^{ko}\) average of 1.2±0.3 x10\(^5\) using 50mg Alum dose (\(p<0.0001\)) (Figure 6.2). Interestingly, unlike myeloid DCs, numbers of plasmacytoid DC in DR3\(^{ko}\) and DR3\(^{wt}\) spleens were not found to be dependent on the presence of Alum, as significant differences were noted after priming with OVA alone (DR3\(^{wt}\) 1.5±0.3 x10\(^5\), DR3\(^{ko}\) 0.8±0.2 x10\(^5\)), suggesting that antigenic stimulation without adjuvant is sufficient to induce this phenotype. However, results from both cell subsets imply DR3 is essential for DC accumulation in the spleen.
As well as DC differences, DR3\textsuperscript{wt} mice were also observed to have higher numbers of macrophages when injected with 2mg of Alum (DR3\textsuperscript{wt} 3.3±0.5 \times10^6, DR3\textsuperscript{ko} 2.2±0.3 \times10^6) upwards to 50mg (DR3\textsuperscript{wt} 5.2±0.9 \times10^6, DR3\textsuperscript{ko} 3.4±0.4 \times10^6) \textit{(p}=0.004\textit{), as well as eosinophils between the same concentrations (Alum 2mg DR3\textsuperscript{wt} 0.8±0.2 \times10^6, DR3\textsuperscript{ko} 0.6±0.1 \times10^6, Alum 50mg DR3\textsuperscript{wt} 1.0±0.2 \times10^6, DR3\textsuperscript{ko} 0.7±0.1 \times10^6) \textit{(p}=0.030\textit{) (Figure 6.2).}

Neutrophil accumulation was significantly affected by the higher concentrations of Alum \textit{(p}<0.0001\textit{), as both genotypes immunised with 50mg of Alum displayed higher cell numbers than all other experimental groups (DR3\textsuperscript{wt} 4.0±0.9 \times10^6, DR3\textsuperscript{ko} 2.6±0.4 \times10^6). No significant differences were noted when analysing the 7/4\textsuperscript{+} or 7/4\textsuperscript{−} monocyte subsets (Figure 6.2).}

Overall, the absence of DR3 affected the content of multiple myeloid cell subsets in the spleen following challenge. At all concentrations of Alum, myeloid DC numbers were lower in DR3\textsuperscript{ko} mice, whilst the same was true for plasmacytoid DCs but included OVA immunisation without Alum. Decreased macrophage and eosinophil numbers in DR3\textsuperscript{ko} mice was dependent on Alum dose, as decreases were seen between the 2mg and 50mg Alum range.
Figure 6.2. Myeloid cell subsets from the spleens of DR3wt and DR3ko mice injected with Alum and OVA. Mice were injected on day 0 via the peritoneal cavity and sacrificed on day 7. Splenocytes were isolated and subsets determined using antibody cell markers. Macrophages (Interaction=0.528, Genotype=**0.004, Alum=0.053), Neutrophils (Interaction=0.224, Genotype=0.382, Alum=***p<0.0001), Eosinophils (Interaction=0.579, Genotype=0.031, Alum=***p<0.0001), 7/4+ Monocytes n.s.d, 7/4- Monocytes n.s.d, Myeloid DCs (Interaction=0.310, Genotype=***p<0.0001, Alum=0.718), Plasmacytoid DCs (Interaction=0.841, Genotype=***p<0.0001, Alum=0.031). Significance determined using 2 way ANOVA; *p<0.05, **p<0.01, ***p<0.001. Values represent mean ± SEM (n=6). Data shown is a combination of 2 individual experiments.
6.3.1.3 DR3 regulated the accumulation of multiple lymphocyte subsets into the spleen following Alum/OVA injection

Multiple lymphocytic subsets were affected by the loss of DR3. DR3<sup>ko</sup> mice displayed reduced numbers of CD3<sup>+</sup> T cells, with evidence of differences noted at 0.4mg (DR3<sup>wt</sup> 2.0±0.2 x10<sup>7</sup>, DR3<sup>ko</sup> 1.0±0.3 x10<sup>7</sup>) and 10mg of Alum (2.7±0.3 x10<sup>7</sup>, DR3<sup>ko</sup> 2.0±0.1 x10<sup>7</sup>) (p=0.001) (Figure 6.3). This correlated with decreased numbers of CD4<sup>+</sup> T cells (Alum 0.4mg DR3<sup>wt</sup> 1.2±0.1 x10<sup>7</sup>, Alum 10mg 1.6±0.2 x10<sup>7</sup>, DR3<sup>ko</sup> 1.2±0.1 x10<sup>7</sup>) (p=0.005) and CD8<sup>+</sup> T cells at the same Alum doses (Alum 0.4mg DR3<sup>wt</sup> 0.7±0.1 x10<sup>7</sup>, Alum 10mg 1.0±0.1 x10<sup>7</sup>, DR3<sup>ko</sup> 0.7±0.04 x10<sup>7</sup>) (p=0.0001) (Figure 6.3). Numbers of activated T cells in DR3<sup>ko</sup> mice, detected via the marker CD44, were also lower at all concentrations of Alum as well as when injected with OVA alone. This was true of both activated CD4<sup>+</sup> (DR3<sup>wt</sup> 2.8±0.2 x10<sup>6</sup>, DR3<sup>ko</sup> 1.9±0.3 x10<sup>6</sup>) and CD8<sup>+</sup> T cell populations (DR3<sup>wt</sup> 1.6±0.2 x10<sup>6</sup>, DR3<sup>ko</sup> 1.2±0.3 x10<sup>6</sup>) (Figure 6.3). DR3<sup>ko</sup> mice were found to have significantly fewer B cells than DR3<sup>wt</sup> mice (p<0.0001), even in the absence of adjuvant (DR3<sup>wt</sup> 2.7±0.2 x10<sup>7</sup>, DR3<sup>ko</sup> 2.1±0.4 x10<sup>7</sup>). This depletion in DR3<sup>ko</sup> B cells continued across the majority of Alum doses, barring Alum 1mg, whereby numbers were found to be equivalent between the 2 genotypes (DR3<sup>wt</sup> 2.1±0.3 x10<sup>7</sup>, DR3<sup>ko</sup> 2.1±0.3 x10<sup>7</sup>) (Figure 6.3)

NKT cell number also differed between DR3<sup>wt</sup> and DR3<sup>ko</sup> mice across all concentrations of Alum, with both factors influencing the results (Genotype p<0.0001, Alum p=0.0008). Like activated T cell subsets, the DR3<sup>wt</sup> spleen contained greater numbers of NKT cells after OVA stimulation alone (DR3<sup>wt</sup> 0.8±0.1 x10<sup>6</sup>, DR3<sup>ko</sup> 0.6±0.1 x10<sup>6</sup>). Genotype was also found to significantly effect γδ T cell number.
(p=0.003), particularly when using 0.1mg Alum (DR3\textsuperscript{wt} 6.5±1.6 x10\textsuperscript{5}, DR3\textsuperscript{ko} 3.9±0.2 x10\textsuperscript{5}), 0.4mg Alum (DR3\textsuperscript{wt} 4.2±1.4 x10\textsuperscript{5}, DR3\textsuperscript{ko} 1.5±0.1 x10\textsuperscript{5}) and 10mg Alum (DR3\textsuperscript{wt} 7.1±1.2 x10\textsuperscript{5}, DR3\textsuperscript{ko} 4.4±0.6 x10\textsuperscript{5}). No significant differences were observed between DR3\textsuperscript{wt} and DR3\textsuperscript{ko} mice in NK cells, although Alum had a significant effect on cell accumulation in both genotypes by 2 way ANOVA (p=0.032) (Figure 6.4).

In summary, DR3 controlled the accrual and/or retention of many lymphocyte subsets into the spleen including CD4\textsuperscript{+} T cells, CD8\textsuperscript{+} T cells, B cells, NKT cells and γδ T cells. Adding to this, the absence of DR3 also decreased the numbers of activated T cells noted within the spleen.
Figure 6.3. Lymphocyte subsets from the spleens of DR3wt and DR3ko mice injected with Alum and OVA. Mice were injected on day 0 via the peritoneal cavity and sacrificed on day 7. Splenocytes were isolated and subsets determined using antibody cell markers. CD3+ T cells (Interaction=0.413, Genotype=**0.001, Alum=***0.0006), CD4+ T cells (Interaction=0.494, Genotype=**0.005, Alum=***0.0005), CD8+ T cells (Interaction=0.538, Genotype=***p<0.0001, Alum=***p<0.0008), Act. CD4+ T cells (Interaction=0.813, Genotype=***p<0.0001, Alum=***p<0.0001), Act. CD8+ T cells (Interaction=0.977, Genotype=***p<0.0001, Alum=0.071), B cells (Interaction=0.524, Genotype=***p<0.0001, Alum=*0.042). Significance determined using 2 way ANOVA; *p<0.05, **p<0.01, ***p<0.001. Values represent mean ± SEM (n=6). Data shown is a combination of 2 individual experiments.
Figure 6.4. NK cells and innate like lymphocyte subsets from the spleens of DR3\textsuperscript{wt} and DR3\textsuperscript{ko} mice injected with Alum and OVA. Mice were injected on day 0 via the peritoneal cavity and sacrificed on day 7. Splenocytes were isolated and subsets determined using antibody cell markers. NK cells (Interaction=0.981, Genotype=0.058, Alum=*0.032), NKT cells (Interaction=0.736, Genotype=***p<0.0001, Alum=***0.0008), γδ T cells (Interaction=0.612, Genotype=**0.003, Alum=*0.041). Significance determined using 2 way ANOVA; *p<0.05, **p<0.01, ***p<0.001. Values represent mean ± SEM (n=6). Data shown is a combination of 2 individual experiments.
6.3.2 DR3 expression in the spleen

6.3.2.1 DR3 expression on splenic lymphocytes

Using Flow cytometry, relative (RF) DR3 cell surface expression was determined on particular lymphocyte populations within the spleen. The highest levels of DR3 cell surface expression were seen on NKT cells. However, the concentration of Alum determined the degree of NKT cell surface DR3 expression ($p=0.021$), with higher Alum doses resulting in lower signal detection (Alum 50mg RF 8.6±1.1, Alum 1mg RF 20.1±2.9). As seen previously (Chapter 4), activated CD4$^+$ T cells were found to express more DR3 signal than CD44$^-$ naive CD4$^+$ T cells. In contrast to previous findings, DR3 expression did not differ between naive and activated CD8$^+$ T cells, although both exhibited less DR3 signal than CD4$^+$ T cell populations. (Figure 6.5) (Table 6.1). Alum was not required to induce DR3 expression, as comparable levels were seen on lymphocytes from OVA injected mice. Importantly, all DR3$^{wt}$ populations analysed displayed higher signals than control DR3$^{ko}$ cell types indicating the specificity of the reagents ($p<0.0001$) (Figure 6.5) (Table 6.1).
Figure 6.5. Ratio of DR3 MFI's on T cell populations in the spleens of DR3<sup>wt</sup> and DR3<sup>ko</sup> mice injected with Alum and OVA. Mice were injected on day 0 via the peritoneal cavity and sacrificed on day 7. Spleens were harvested and cells were stained with DR3 antibody. CD4<sup>+</sup> T cells (*Interaction=0.512, Genotype=***p<0.0001, Alum=0.112), CD8<sup>+</sup> T cells (*Interaction=0.393, Genotype=***p<0.0001, Alum=0.488), Activated CD4<sup>+</sup> T cells (*Interaction=0.075, Genotype=***p<0.0001, Alum=0.115), Activated CD8<sup>+</sup> T cells (*Interaction=0.220, Genotype=***p<0.0001, Alum=0.380) and NKT cells (*Interaction=**0.008, Genotype=***p<0.0001, Alum=*0.021). Ratio DR3 was calculated by the MFI fold increase compared to control IgG antibody. Results determined using 2 way ANOVA; *p<0.05, **p<0.01, ***p<0.001. Bars represent mean ± SEM (n=3).
Table 6.1. DR3 expression on T cell populations in the spleen following injection with OVA and varying concentrations of Alum

<table>
<thead>
<tr>
<th>Alum concentration*</th>
<th>DR3wt</th>
<th></th>
<th></th>
<th></th>
<th>DR3ko</th>
<th></th>
<th></th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>CD4+ T cells</td>
<td>Act.CD4+ T cells</td>
<td>CD8+ T cells</td>
<td>Act.CD8+ T cells</td>
<td>NKT cells</td>
<td>CD4+ T cells</td>
<td>Act.CD4+ T cells</td>
<td>CD8+ T cells</td>
<td>Act.CD8+ T cells</td>
</tr>
<tr>
<td>OVA</td>
<td>8.1±0.4</td>
<td>10.5±0.5</td>
<td>4.7±0.1</td>
<td>4.3±0.1</td>
<td>13.0±0.8</td>
<td>2.0±0.1</td>
<td>1.9±0.1</td>
<td>2.3±0.1</td>
<td>2.2±0.1</td>
</tr>
<tr>
<td>0.1mg</td>
<td>10.3±1.2</td>
<td>13.8±2.0</td>
<td>5.3±0.3</td>
<td>5.3±0.3</td>
<td>19.5±2.3</td>
<td>2.1±0.1</td>
<td>2.0±0.1</td>
<td>2.2±0.1</td>
<td>2.3±0.2</td>
</tr>
<tr>
<td>0.4mg</td>
<td>9.5±0.6</td>
<td>10.6±0.7</td>
<td>4.9±0.4</td>
<td>4.2±0.3</td>
<td>13.0±1.3</td>
<td>2.0±0.1</td>
<td>2.0±0.2</td>
<td>2.1±0.2</td>
<td>2.3±0.2</td>
</tr>
<tr>
<td>1mg</td>
<td>11.1±0.3</td>
<td>1501±0.4</td>
<td>5.9±0.1</td>
<td>5.7±0.1</td>
<td>20.1±2.9</td>
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<td>2mg</td>
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<td>5.0±0.2</td>
<td>16.3±1.5</td>
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<tr>
<td>10mg</td>
<td>8.2±1.4</td>
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<td>4.8±0.9</td>
<td>4.7±0.4</td>
<td>13.7±2.7</td>
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<td>2.4±0.2</td>
<td>2.5±0.1</td>
<td>2.3±0.1</td>
</tr>
<tr>
<td>50mg</td>
<td>7.0±1.8</td>
<td>9.9±1.7</td>
<td>4.3±0.5</td>
<td>4.6±0.7</td>
<td>8.6±1.1</td>
<td>1.8±0.4</td>
<td>2.2±0.2</td>
<td>2.5±0.2</td>
<td>2.4±0.3</td>
</tr>
</tbody>
</table>

* Corresponds to the concentration of Alum injected into mice (mg/ml)

\(^{\dagger}\) Significance of genotype measured by 2 way ANOVA.

Median fluorescence intensity (MFI) ratio calculated by dividing the test median by the isotype following gating on the specified population ± SEM following flow cytometry.
6.3.3 Antigen specific responses following adjuvant and OVA injection

6.3.3.1 Antigen specific antibody production

Alum was found to significantly affect OVA specific IgG production ($p<0.0001$). Mice treated with higher concentrations of the adjuvant showed higher levels of OVA specific IgG in their serum, with a dose response between 0.1mg Alum ($\text{DR3}^{\text{wt}} 0.1\pm0.02$, $\text{DR3}^{\text{ko}} 0.1\pm0.04$) and 50mg (Alum 50mg $\text{DR3}^{\text{wt}} 0.5\pm0.1$, $\text{DR3}^{\text{ko}} 0.6\pm0.03$). Very little OVA specific IgG was produced in mice injected solely with OVA ($\text{DR3}^{\text{wt}} 0.05\pm0.02$, $\text{DR3}^{\text{ko}} 0.1\pm0.1$) (Figure 6.6) (Table 6.2). Importantly, no significant differences were observed in antibody production between $\text{DR3}^{\text{wt}}$ and $\text{DR3}^{\text{ko}}$ mice, suggesting DR3 does not regulate antibody responses quantitatively, despite the differences noted in B cell number, T cell activation and DC numbers.
Figure 6.6. Antigen specific antibody response of DR3<sup>wt</sup> and DR3<sup>ko</sup> mice injected with Alum and OVA. Mice were injected on day 0 via the peritoneal cavity and sacrificed on day 7. Serum was isolated from blood obtained from cardiac puncture and tested for concentrations of OVA specific IgG (Interaction=0.415, Genotype=0.511, Alum=***p<0.0001). Significance determined using 2 way ANOVA and Bonferroni post test; *p<0.05, **p<0.01, ***p<0.001. Values represent mean ± SEM (n=3). Data shown is representative of 2 experiments.
Table 6.2. Serum levels of OVA specific IgG following injection with OVA and varying concentrations of Alum

<table>
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<tr>
<th>Alum Concentration*</th>
<th>DR3&lt;sup&gt;wt&lt;/sup&gt;</th>
<th>DR3&lt;sup&gt;ko&lt;/sup&gt;</th>
<th>Significance&lt;sup&gt;§&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>OVA</td>
<td>0.05±0.02</td>
<td>0.1±0.1</td>
<td>N.S.D</td>
</tr>
<tr>
<td>0.1mg</td>
<td>0.1±0.02</td>
<td>0.1±0.05</td>
<td>N.S.D</td>
</tr>
<tr>
<td>0.4mg</td>
<td>0.3±0.1</td>
<td>0.2±0.1</td>
<td>N.S.D</td>
</tr>
<tr>
<td>1mg</td>
<td>0.4±0.1</td>
<td>0.3±0.5</td>
<td>N.S.D</td>
</tr>
<tr>
<td>2mg</td>
<td>0.5±0.03</td>
<td>0.5±0.01</td>
<td>N.S.D</td>
</tr>
<tr>
<td>10mg</td>
<td>0.5±0.1</td>
<td>0.6±0.01</td>
<td>N.S.D</td>
</tr>
<tr>
<td>50mg</td>
<td>0.5±0.1</td>
<td>0.6±0.03</td>
<td>N.S.D</td>
</tr>
</tbody>
</table>

* Corresponds to the concentration of Alum injected into mice

§ Significance between DR3<sup>wt</sup> and DR3<sup>ko</sup> mice measured by 2 way ANOVA. N.S.D= no significant difference.

Values represent the mean±SEM of the optical density reading from the OVA specific IgG ELISA using serum from DR3<sup>wt</sup> and DR3<sup>ko</sup> mice from 2 different experiments (n=6).
6.3.3.2 DR3\textsuperscript{wt} and DR3\textsuperscript{ko} splenocytes displayed comparable proliferative responses

Whole splenocyte populations from DR3\textsuperscript{wt} and DR3\textsuperscript{ko} mice were stimulated \textit{in vitro} with 2 different concentrations of OVA, 10µg/ml and 50µg/ml. Cells were then pulsed with \textsuperscript{3}H-thymidine to assess the proliferative response. Results confirmed that DR3\textsuperscript{wt} and DR3\textsuperscript{ko} mice had comparable proliferative responses when challenged with OVA, suggesting that the defects noted were not due to a lack of antigen-specific T cell expansion. At no concentration of Alum were significant differences seen between the 2 genotypes, although \textsuperscript{3}H-thymidine incorporation did increase in response to higher doses of OVA. Cells treated with 50µg/ml of OVA proliferated more than those treated with 10µg/ml, with this difference being significant in all experimental dosage groups (Figure 6.7) (Table 6.3). Furthermore, proliferation was greater in cells from mice treated with 50mg Alum than the lower concentrations of Alum, particularly 0.1mg.
Figure 6.7. Proliferation of DR3\textsuperscript{wt} and DR3\textsuperscript{ko} splenocytes in response to OVA following injection with Alum and OVA. Mice were injected on day 0 via the peritoneal cavity and sacrificed on day 7. Splenocytes were extracted via mashing of the spleen and stimulated \textit{in vitro} with 2 concentrations of OVA; 10\(\mu\)g/ml, 50\(\mu\)g/ml. Alum 50mg (Interaction=0.947, Genotype=0.592, Stimulation=***0.0004), Alum 10mg (Interaction=0.211, Genotype=0.067, Stimulation=***p<0.0001), Alum 2mg (Interaction=0.807, Genotype=0.259, Stimulation=***p<0.0001), Alum 1mg n.s.d, Alum 0.4mg (Interaction=0.591, Genotype=0.08, Stimulation=**0.007), Alum 0.1mg (Interaction=0.603, Genotype=0.135, Stimulation=*0.040), OVA (Interaction=0.335, Genotype=0.904, Stimulation=***0.0002). Significance determined using 2 way ANOVA; *p<0.05, **p<0.01, ***p<0.001. Values represent mean ± SEM (n=3). Data shown is representative of 2 experiments.
Table 6.3. Proliferative response of DR3<sup>wt</sup> and DR3<sup>ko</sup> mice injected with OVA and varying concentrations of Alum

<table>
<thead>
<tr>
<th>Alum Concentration*</th>
<th>DR3&lt;sup&gt;wt&lt;/sup&gt; OVA 10ug/ml&lt;sup&gt;^&lt;/sup&gt;</th>
<th>DR3&lt;sup&gt;ko&lt;/sup&gt; OVA 50ug/ml&lt;sup&gt;€&lt;/sup&gt;</th>
<th>DR3&lt;sup&gt;wt&lt;/sup&gt; OVA 10ug/ml&lt;sup&gt;^&lt;/sup&gt;</th>
<th>DR3&lt;sup&gt;ko&lt;/sup&gt; OVA 50ug/ml&lt;sup&gt;€&lt;/sup&gt;</th>
<th>Significance&lt;sup&gt;$&lt;/sup&gt;</th>
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<td>OVA 0.1mg</td>
<td>750±112</td>
<td>1051±106</td>
<td>634±165</td>
<td>1285±210</td>
<td>N.S.D</td>
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<td>OVA 0.4mg</td>
<td>537±286</td>
<td>905±297</td>
<td>258±113</td>
<td>491±127</td>
<td>N.S.D</td>
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<td>OVA 1mg</td>
<td>1612±452</td>
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<td>752±439</td>
<td>1341±541</td>
<td>N.S.D</td>
</tr>
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<td>2104±818</td>
<td>751±147</td>
<td>1129±362</td>
<td>N.S.D</td>
</tr>
<tr>
<td>OVA 10mg</td>
<td>1337±79</td>
<td>2570±162</td>
<td>1029±147</td>
<td>2049±298</td>
<td>N.S.D</td>
</tr>
<tr>
<td>OVA 50mg</td>
<td>1869±592</td>
<td>2972±1018</td>
<td>2156±215</td>
<td>3332±346</td>
<td>N.S.D</td>
</tr>
</tbody>
</table>

* Corresponds to the concentration of Alum injected into mice

^ Corresponds to cells stimulated with 10ug/ml of OVA

€ Corresponds to cells stimulated with 50ug/ml of OVA

$ Significance between DR3<sup>wt</sup> and DR3<sup>ko</sup> mice measured by 2 way ANOVA. N.S.D= no significant difference.

Values represent mean±SEM of the cpm readings from 3h-thymidine pulsed DR3<sup>wt</sup> and DR3<sup>ko</sup> splenocytes from 2 different experiments (n=6).
6.3.4 Investigating the mechanisms for DR3\textsuperscript{wt} cellular accumulation in the spleen

6.3.4.1 Spleens from DR3\textsuperscript{wt} mice contained higher levels of CXCL10 than DR3\textsuperscript{ko} mice

Reduced cellular accumulation in the spleens of DR3\textsuperscript{ko} mice was not found to be dependent on antigen-specific proliferation, therefore chemokine levels were measured from spleen lysates to determine whether a defect in recruitment was potentially contributing to reduced cellular accumulation. Results showed that DR3\textsuperscript{ko} mice had lower levels of CXCL10, as 2-way ANOVA analysis showed genotype to be a significant factor ($p=0.001$). This difference was particularly apparent in mice injected with 50mg of Alum (DR3\textsuperscript{wt} 2127±264 pg/ml, DR3\textsuperscript{ko} 1438±120 pg/ml) ($p<0.05$). No other chemoattractants measured were found to significantly differ between DR3\textsuperscript{wt} and DR3\textsuperscript{ko} mice, although Alum concentration did affect the levels of many. CCL3, CXCL2 and CXCL1 concentrations were found to be higher in mice treated with 50mg of Alum, which correlated with an increase in neutrophils, the known responder of CXCL1 ($p=0.004$). In contrast CCL5 levels decreased with the higher doses of Alum ($p=0.013$). No significant differences were seen in CXCL13 release, a typical B cell chemoattractant, although levels did correlate with B cell number (Figure 6.8) (Table 6.4).
Figure 6.8. Chemokine levels in the spleens of DR3wt and DR3ko mice injected with Alum and OVA. Mice were injected on day 0 via the peritoneal cavity and sacrificed on day 7. Spleens were harvested to form a lysate, from which chemokine levels were measured using ELISA. CCL3 (Interaction=0.743, Genotype=0.571, Alum=0.019), CCL4 n.s.d, CCL5 (Interaction=0.257, Genotype=0.948, Alum=0.013), CXCL1 (Interaction=0.802, Genotype=0.553, Alum=0.004), CXCL2 n.s.d, CXCL10 (Interaction=0.596, Genotype=0.002, Alum=0.146)and CXCL13 n.s.d. Significance determined using 2 way ANOVA and Bonferroni post test; *p<0.05, **p<0.01, ***p<0.001. Symbols represent mean ± SEM (n=3).
### Table 6.4. Chemokine levels in the spleens of DR3\textsuperscript{wt} and DR3\textsuperscript{ko} mice challenged with varying concentrations of Alum with OVA

<table>
<thead>
<tr>
<th>Chemokine (ng/ml)</th>
<th>DR3\textsuperscript{wt}</th>
<th>DR3\textsuperscript{ko}</th>
<th>Significance$^\S$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Alum conc.*</td>
<td>50mg</td>
<td>10mg</td>
</tr>
<tr>
<td>CCL3</td>
<td>0.4±0.2</td>
<td>0.2±0.05</td>
<td>0.2±0.02</td>
</tr>
<tr>
<td>CCL4</td>
<td>0.4±0.1</td>
<td>0.3±0.1</td>
<td>0.3±0.01</td>
</tr>
<tr>
<td>CCL5</td>
<td>15±11.1</td>
<td>30.1±7.4</td>
<td>25.6±4.5</td>
</tr>
<tr>
<td>CXCL1</td>
<td>0.7±0.2</td>
<td>0.2±0.02</td>
<td>0.4±0.1</td>
</tr>
<tr>
<td>CXCL2</td>
<td>0.3±0.06</td>
<td>0.2±0.01</td>
<td>0.2±0.03</td>
</tr>
<tr>
<td>CXCL10</td>
<td>2.1±0.3</td>
<td>1.8±0.1</td>
<td>2.0±0.2</td>
</tr>
<tr>
<td>CXCL13</td>
<td>85.6±15.9</td>
<td>93.2±34.4</td>
<td>85.7±19.8</td>
</tr>
</tbody>
</table>

*Corresponds to the concentration of Alum injected into mice (mg/ml)

$^\S$Significance of genotype measured by 2 way ANOVA. N.S.D= no significant difference, *p<0.05, **p<0.01.
6.4 Discussion

To examine the splenic response observed in Chapter’s 4 and 5, whereby a reduction in splenocyte accumulation was noted in DR3\(^{ko}\) mice exposed to multiple OVA/Alum challenges during acute and chronic inflammatory models of allergic lung inflammation, a more basic model was utilised to explore this phenotype. Mice were injected once in the peritoneal cavity with an OVA/Alum mix followed by a detailed analysis of splenic myeloid and lymphocyte populations at day 7. Results showed that 1 sensitisation injection was sufficient to initiate alterations in the cellular content of DR3\(^{wt}\) spleens, and this was significantly different in DR3\(^{ko}\) mice.

This novel phenotype was made even more so by the defect in multiple myeloid cell subset numbers. Little research exists regarding DR3 and the regulation of myeloid cell numbers, despite numerous reports citing DR3 as a co-stimulator of T cell expansion (Meylan, Davidson et al. 2008; Jones, Stumhofer et al. 2011; Slebioda, Rowley et al. 2011; Buchan, Taraban et al. 2012; Twohig, Marsden et al. 2012). In the model of antigen induced arthritis (AIA), mice were sub-cutaneously immunised twice 1 week apart, with methylated BSA (mBSA) and complete freunds adjuvant (CFA). AIA was then induced in the joint via an intra-articular injection of mBSA 21 days after the initial immunisation. No impairment was found in F4/80\(^{+}\) (a marker for macrophages) cell number between DR3\(^{wt}\) and DR3\(^{ko}\) mice at either 3 or 21 days post arthritis induction, suggesting DR3 did not control myeloid cell recruitment to the joint (Bull, Williams et al. 2008). F4/80\(^{+}\) macrophages act as the precursor for the bone resorbing cells, osteoclasts, identified using TRAP staining. Within the same model, DR3 was found to affect osteoclast generation at sites of bone pathology, particularly the pannus area, as
reduced numbers were noted in DR3KO mice. As well as this, *in vitro*, it was found that TL1A could promote osteoclast differentiation from bone marrow derived macrophages in the presence of RANK-L and M-CSF, as well as human peripheral blood monocytes (Bull, Williams et al. 2008).

In contrast, in this thesis DR3 was found to affect the numbers of multiple myeloid cell subsets in the spleen following immunisation, including F4/80+ macrophages at higher adjuvant concentrations. This increase occurred after only 1 peritoneal sensitisation injection, as opposed to the 2 priming events and inflammatory hit used in the AIA model. This may be due to the use of different adjuvants, as Alum was used in this study compared to CFA, as well as the use of the i.p route of sensitisation in this Chapter as opposed to subcutaneous and intra-articular injections utilised by Bull et al (Bull, Williams et al. 2008).

DR3 expression has itself been reported on the human macrophage cell line THP-1 in the basal state (Kang, Kim et al. 2005; Su, Chang et al. 2006), as well as foam cells in carotid endarterectomy tissue (Kim, Lee et al. 2001; Kang, Kim et al. 2005; Kim, Kang et al. 2008). Foam cells are essential in the development of atherosclerotic plaques via the ingestion of low-density lipoprotein (LDL) (Lusis, Mar et al. 2004). DR3 has been reported to directly stimulate human macrophage foam cell formation (McLaren, Calder et al. 2010). THP-1 cells and human monocyte derived macrophages stimulated with TL1A for 24hrs, demonstrated a significantly increased uptake of acetylated and oxidised LDL compared to untreated controls. This uptake was ablated upon administration of a soluble DR3-Fc fusion protein. As well as LDL uptake, caused by
TL1A driven up-regulation of scavenger receptors, cholesterol efflux was also reduced, via a decrease in the levels of apoE and ABCG-1 gene expression. To ensure that TL1A was acting though DR3, DR3 expression was reduced using a recombinant adenovirus expressing DR3 shRNA. These DR3 knock down macrophages failed to uptake LDL or effect cholesterol efflux in response to TL1A, confirming increased foam cell formation was DR3 dependent. The results were mirrored using murine bone marrow derived macrophages where DR3<sup>ko</sup> cells displayed impaired LDL uptake, correlating with a reduction in scavenger receptor mRNA (McLaren, Calder et al. 2010). This role for DR3 in myeloid cell function and differentiation implies that, like in this Chapter, DR3 is important beyond T cells in the immune response.

As well as macrophages, DR3<sup>ko</sup> mice also displayed reduced numbers of myeloid DCs following OVA/Alum injection. Some studies report Alum to act directly on myeloid DCs, increasing antigen uptake and internalisation and enhancing the expression of co-stimulatory molecules (Sun, Pollock et al. 2003; Morefield, Sokolovska et al. 2005). More recently however, Flach et al used atomic force microscopy to hypothesise that as opposed to internalisation, Alum actually binds to DCs via lipid moieties, particularly sphingomyelin and cholesterol, delivering antigen via endocytosis. Binding promoted lipid sorting in the DC plasma membrane, including clustering of immunoreceptor tyrosine based activation motif (ITAM) containing receptors, which in turn activated the spleen tyrosine kinase (Syk) and PI3K pathways through a phosphorylation cascade. This led to the initiation of the immune response via an up-regulation of the co-stimulatory molecules CD86, CD80 and ICAM-1, promoting tight interactions with CD4<sup>+</sup> T cells (Flach, Ng et al. 2011). As DR3 has been shown to regulate cholesterol levels upon macrophages (McLaren, Calder et al. 2010), it may be acting in a similar
manner on myeloid DCs. It is tempting to speculate that the Alum-triggered reduction of myeloid DC numbers in DR3<sup>ko</sup> spleen is related to DR3’s capacity to regulate cholesterol uptake, but further studies would be required to confirm this.

Myeloid DC differentiation and proliferation is driven by, amongst other proteins, GM-CSF. GM-CSF has been shown to enhance conventional DC expansion both <i>in vitro</i> (Inaba, Steinman et al. 1992), and <i>in vivo</i> when over-expressed (Vremec, Lieschke et al. 1997). Proliferation relies on the PI3K pathway, and more particularly, the downstream effector mammalian target of rapamycin (mTOR), inhibition of which prevents GM-CSF human monocyte derived DC development due to reduced proliferation and survival (Kim, Kang et al. 2010). GM-CSF levels increase during inflammation, with some reporting the cytokine promotes monocyte conversion to murine spleen myeloid DCs (Naik, Metcalf et al. 2006), as well as DC generation in disease models, including acute inflammatory arthritis and antigen induced peritonitis (Campbell, van Nieuwenhuijze et al. 2011). TL1A has previously been found to augment GM-CSF release from the splenocytes of a model of acute graft versus host response <i>ex vivo</i>, as well as suboptimally stimulated peripheral blood T cells (Migone, Zhang et al. 2002), suggesting TL1A is able to influence GM-CSF production and so potentially myeloid DC proliferation.

Splenic CD<sub>11c</sub><sup>+</sup> DCs have been shown to rapidly release TL1A in response to multiple stimuli, including STAg, via the TLR pathway (Scanga, Aliberti et al. 2002) and cross linked IgG (Meylan, Davidson et al. 2008), although no expression was seen following <i>Salmonella enterica</i> Typhimurium infection (Buchan, Taraban et al. 2012). In a mouse
model of chronic ileitis, TL1A and CD11c⁺ cells were found to co-localise using lamina propria mononuclear cells from inflamed SAMP1/YitFc and TNFΔARE mice, with flow cytometry results suggesting cells were DCs due to their CD11c⁺ MHCII⁺ phenotype (Bamias, Mishina et al. 2006). Despite this, little has been published specifically relating to DR3 function in plasmacytoid DCs.

Plasmacytoid DCs are characterised by their ability to release high levels of type I interferons (Cella, Jarrossay et al. 1999), hence they are predominantly known for their functions in anti-viral immunity. Plasmacytoid DC development is thought to be dependent on Flt3-L, as Flt3-L deficient mice were found to have fewer plasmacytoid DCs compared to control animals (Brawand, Fitzpatrick et al. 2002), while GM-CSF has also been shown to support the survival of plasmacytoid DC precursors (Ghirelli, Zollinger et al. 2010). Flt3-L treatment was also shown to greatly enhance the number of plasmacytoid DCs in the lungs of allergen challenged mice, leading to reduced Th2 associated eosinophilia. They were also shown to suppress the ongoing immune response, as their depletion, using the antibody 120G8, abrogated inflammation (Kool, van Nimwegen et al. 2009). Similar results were presented by De Heer et al, as depletion of plasmacytoid DCs led to IgE sensitisation, airway eosinophilia, goblet cell hyperplasia and Th2 cytokine release, in response to normally inert antigen, suggesting plasmacytoid DCs could be tolerogenic as opposed to immunogenicity exhibited by myeloid DCs. They were also found to suppress the generation of effector T cells (de Heer, Hammad et al. 2004). In the spleen, antigen pulsed plasmacytoid DCs induced minimal proliferation and cytokine polarisation in antigen-specific T cell receptor-transgenic T cells, even in the presence of IL-2. They instead gained a regulatory phenotype and suppressed antigen-specific T cell proliferation (Martin, Del Hoyo et al. 2004).
However, others have suggested plasmacytoid DCs hold T cell stimulatory and expansion abilities once activated, although to a lesser extent than conventional myeloid DCs (Salio, Palmowski et al. 2004; Schlecht, Garcia et al. 2004; Villadangos and Young 2008). When virus activated they have been shown to elicit Th1 responses (Liu, Kanzler et al. 2001), although Ito et al observed plasmacytoid DCs to prime Th2 cells when activated by IL-3 and CD40-L in an OX40-L dependent manner (Ito, Amakawa et al. 2004).

DR3\textsuperscript{ko} spleens contained significantly fewer plasmacytoid DCs than DR3\textsuperscript{wt} mice following immunisation with Alum and OVA or OVA alone. Unlike myeloid DCs, the results of this Chapter show that plasmacytoid DC accumulation is enhanced by, but not dependent upon adjuvant, but rather just antigenic stimulation, suggesting DR3\textsuperscript{ko} plasmacytoid DC deficiencies are due to a lack of expansion. It would therefore be interesting to examine the effects of TL1A on Flt3-L levels, to determine if the DR3/TL1A pathway regulates production and consequently plasmacytoid DC proliferation and survival. Alternatively, TL1A, like OX40-L, may induce plasmacytoid DCs to prime Th2 cells, or increase the levels of IFN\(\gamma\), thereby enhancing the immune response. Whilst, no cytokine measurements were made in this study, it is clear that the role of DR3 in plasmacytoid DC accumulation and function requires further investigation.

Both myeloid and plasmacytoid DCs arise from a common DC precursor (Naik, Sathe et al. 2007), expanding through stimulation of the previously mentioned tyrosine kinase, Flt3 and cytokine GM-CSF, as well as M-CSF and IL-3 amongst others (Karsunky,
Merad et al. 2003; Liu, Victora et al. 2009). However, unlike DR3 effects on osteoclastogenesis (Bull, Williams et al. 2008), the Receptor is unlikely to have a role in myeloid or plasmacytoid DC differentiation and homeostasis as comparable numbers were recorded between DR3\textsuperscript{wt} and DR3\textsuperscript{ko} mice in the basal state (Chapter 3). Furthermore, Tian et al found TL1A to have no effect on murine bone marrow derived DC maturation, compared to its shorter variant VEGI, which was found to induce early DC activation. This included up-regulation of the maturation markers CD83, CCR7, CD40, CD80 and CD86 as well as early activation of the maturation signalling molecules NF-κB, STAT3, p38, JNK, leading to cytoskeleton reorganisation and premature dendrite formation (Tian, Grimaldo et al. 2007). TNF\textalpha too, has been shown to aid umbilical cord derived CD34\textsuperscript{+} haematopoietic cells to develop into DCs when cultured with stem cell factors and GM-CSF. DCs were apparent by day 14, except when treated with an anti-TNFRI antibody, so verifying that TNF\textalpha was acting through TNFRI (Morrison Iii, Cruse et al. 2003). Similarly in the mouse, using bone marrow precursors from TNF\textalpha\textsuperscript{ko} C57BL/6 mice, DCs displayed an inability to mature, instead retaining the phenotype of immature DCs (Ritter, Meissner et al. 2003). It would therefore be interesting to measure the levels of DC co-stimulatory molecules, as although DR3 does not appear to be important in DC differentiation, like TNF\textalpha, it may affect myeloid and plasmacytoid DC activation and maturation upon antigenic challenge.

DCs are essential for T cell priming and sensitisation, leading to the clonal expansion of antigen-specific T cells, for which Alum assists, acting as an additional co-stimulatory signal. Whilst it has been suggested that Alum does not activate DCs \textit{in vitro} (Sun, Pollock et al. 2003), \textit{in vivo}, Alum has been shown to initiate DC induced adaptive
immunity via NLRP10. NLRP10\(^{-/-}\) mice were unable to migrate to the draining lymph nodes, despite antigen capture, resulting in absolute loss of CD4\(^+\) T cell priming (Eisenbarth, Williams et al. 2012). The results obtained within this Chapter however, suggest T cell priming is unaffected by the loss of DR3, as despite reduced T cell accumulation in the spleens of DR3\(^{ko}\) mice, proliferation in response to OVA was comparable between the 2 genotypes.

The DR3/TL1A pathway has previously been shown to have a role in the proliferation of murine T cells including, Th17 cells in the presence of low anti-CD3 stimulation (Pappu, Borodovsky et al. 2008; Jones, Stumhofer et al. 2011), CD4\(^+\) memory cells when stimulated with IL-12 and TL1A (Bamias, Mishina et al. 2006) and whole CD4\(^+\) T cell populations when stimulated with high concentrations of TL1A in the absence of CD28 co-stimulation (Meylan, Davidson et al. 2008). Furthermore, reduced peptide-specific proliferation of DR3\(^{ko}\) splenocytes was observed in a model of EAU (Calder and Wang 2012). Splenocytes were removed 21 days after subcutaneous injection of interphotoreceptor retinoid binding protein (IRBP) emulsified with CFA and *Mycobacterium tuberculosis*, stimulated with IRBP and pulsed with \(^{3}\)H-thymidine. Whilst the assay revealed no proliferative differences at 10 days post injection, at day 21, DR3\(^{ko}\) splenocytes displayed a significant reduction in proliferation compared to DR3\(^{wt}\) cells, suggesting DR3 was required for sustained splenic T cell responses rather than priming (Calder and Wang 2012). DR3 altogether failed to enhance T\(_{reg}\) proliferation *in vitro* under suboptimal signalling conditions (Schreiber, Wolf et al. 2010). Adding to this, DR3 was reported to inhibit the expansion of human B cells, though survival was unaffected (Cavallini, Lovato et al. 2013). The experiments performed within this Chapter imply DR3 has a redundant role in the generation of
antigen-specific T cells, as comparable \(^3\)H-thymidine uptake was recorded from both DR3\(^{wt}\) and DR3\(^{ko}\) in vitro cell stimulations. Having said that, only 1 timepoint was measured and no attempt was made to assess proliferation in vivo, using for example, a Ki67 stain. In contrast to the methods employed in this Chapter, the majority of the above studies investigated the expansion of T cells that had been activated via their TCR and co-stimulated with TL1A. Like Calder et al (Calder and Wang 2012), the data presented here implies DR3 has no significant effect on T cell priming, despite the difference in DC numbers.

Although T cell priming was not impaired in DR3\(^{ko}\) mice, the numbers of activated T cells were, as a lack of expansion was observed following antigenic stimulation. Memory T cells have previously been reported to demonstrate a higher rate of proliferation in response to TL1A than naive lymphocytes (Bamias, Mishina et al. 2006), whilst Meylan et al showed TL1A to have a role in expanding effector T cells at the site of inflammation in vivo. The same group also observed DR3 to be dispensable for the differentiation of naive T cells (Meylan, Davidson et al. 2008), suggesting the Receptor is required for expansion rather than activation. Furthermore, DR3/TL1A interactions were found to be required for activated T cell function against viral proteins, experiments of which were not performed in this Chapter, despite reduced numbers.

Recruitment was also examined as a possible means by which DR3\(^{wt}\) splenocytes increased in comparison to DR3\(^{ko}\) mice. The spleen lysates from mice injected with OVA and Alum were analysed for a range of chemokines, with results showing that
DR3<sup>wt</sup> and DR3<sup>ko</sup> mice had significant differences in the levels of CXCL10, also known as IP-10. CXCL10 is thought to act as a chemoattractant for a range of cells, including CD4<sup>+</sup> and CD8<sup>+</sup> T cells, NKT cells, NK cells, monocytes (Campanella, Tager et al. 2008) and plasmacytoid DCs (Sozzani, Vermi et al. 2010) via the receptor CXCR3. It is also secreted by various immune subsets, including DCs, as well as structural cells, such as endothelia (Luster and Ravetch 1987), in response to IFN<sub>γ</sub> stimulation (Dufour, Dziejman et al. 2002).

TL1A has previously been reported to synergise with the IL-12/IL-18 pathway to induce IFN<sub>γ</sub> expression from human NK cells (Papadakis, Prehn et al. 2004) as well as CD4<sup>+</sup> T cells (Prehn, Mehdizadeh et al. 2004; Cassatella, da Silva et al. 2007). Shih et al further treated CD4<sup>+</sup> T cells with bacteria treated DCs (Shih, Kwan et al. 2009), whilst Migone et al stimulated T cells with anti-CD3 and anti-CD28 (Migone, Zhang et al. 2002). Biener et al took a different approach, using HEK-293 cells expressing membrane bound TL1A and co-culturing them with IL-12/IL-18 primed CD4<sup>+</sup> T cells (Biener-Ramanujan, Gonsky et al. 2010). However, the results were consistent; TL1A was able to stimulate IFN<sub>γ</sub> release. Similar reports have been published using murine cells, as IL-12/IL-18 primed CD4<sup>+</sup> T cells up-regulated IFN<sub>γ</sub> in response to TL1A (Kamada, Hisamatsu et al. 2010). Bamias et al also stimulated murine CD4<sup>+</sup> lymphocytes with anti-CD3 and anti-CD28 along with recombinant mouse TL1A, which was shown to induce IFN<sub>γ</sub> release in a dose dependent manner. Suboptimal anti-CD3 concentrations revealed an additive effect of TL1A, enhancing IFN<sub>γ</sub> release (Bamias, Mishina et al. 2006). Using DR3<sup>ko</sup> cells from a model of EAU, Calder et al recorded reduced IFN<sub>γ</sub> production from splenocytes stimulated <em>ex vivo</em> with IRBP compared to DR3<sup>wt</sup> mice (Calder and Wang 2012). A TL1A-driven induction of IFN<sub>γ</sub>
could therefore explain the significant increases noted in CXCL10 levels in DR3\textsuperscript{wt} mice, that further impacts on the recruitment of cell types thought to produce IFN\textgamma, including plasmacytoid DCs and CD4\textsuperscript{+} T cells (Vremec, O'Keeffe et al. 2007). However, Alum is typically thought to induce a Th2 response. Further studies would be needed to determine the polarisation of the T cell response following OVA and Alum challenge in the absence of DR3.

CXCL10 has an already established role during inflammation and has been found to be elevated in several disease models, including EAE, whereby anti-CXCL10 treatment blocked the recruitment of antigen-specific effector T cells into the CNS (Fife, Kennedy et al. 2001) and CIA, where treatment with anti-CXCL10 significantly inhibited the infiltration of CD4\textsuperscript{+} T cells and F4/80\textsuperscript{+} cells into the synovium and attenuated bone destruction, as well as inhibiting RANK-L and TNF\textalpha levels (Kwak, Ha et al. 2008). CXCL10\textsuperscript{ko} mice have also been shown to have impaired T cell responses, including proliferation to allogeneic and antigenic stimulation and IFN\textgamma secretion in response to antigen challenge (Dufour, Dziejman et al. 2002). Unlike the data presented by Dufour et al, proliferation was found to be intact in DR3\textsuperscript{ko} mice, despite CXCL10 levels being decreased. This suggests CXCL10 works downstream of DR3, perhaps affecting cellular accumulation due to a lack of TL1A signalling, which may directly stimulate chemokine release, or function by inducing IFN\textgamma, creating a positive feedback loop regulated by the DR3/TL1A pathway. In order to test this theory, immunisations could be performed using CXCL10\textsuperscript{ko} mice. Alternatively, CXCL10 levels could be silenced in DR3\textsuperscript{wt} mice using antibody inhibition prior to Alum/OVA injection. Spleens could then be examined to see whether they present a similar phenotype to DR3\textsuperscript{ko} mice. However,
due to the pleiotrophic nature of chemokine, as well as ensuring absolute depletion, experiments such as these are difficult to execute.

DR3 has previously been shown to influence the release of other chemokines, albeit using human cells. Following the IFNγ priming of THP-1 cells, TL1A was found to induce IL-8, the murine ortholog being CXCL1 (Kang, Kim et al. 2005). IL-8 was also shown to be released from monocyte derived macrophages in a dose dependent manner upon TL1A stimulation, as well as on HEK-293 cells via DR3 over expression (Su, Chang et al. 2006). As well as IL-8, DR3 has previously been shown to induce CCL2 release from THP-1 cells. Levels were further increased after LPS stimulation (Kang, Kim et al. 2005). In this Chapter, CXCL1 levels did not differ between DR3\(^{wt}\) and DR3\(^{ko}\) mice, whilst CCL2 measurements were not taken.

Despite the analysis of multiple chemokines, significant differences were only observed between DR3\(^{wt}\) and DR3\(^{ko}\) mice in CXCL10 levels. No differences were noted in typical myeloid cell chemoattractants including CCL3, CCL4 or CCL5, despite differences in the numbers of some of these subsets. However, data within the laboratory has shown DR3 to elicit an extremely early effect in an un-sensitised model of peritoneal inflammation. Mice were peritoneally injected with an inactivated bacterial suspension (Hurst, Wilkinson et al. 2001), followed by assessments of leukocyte subsets in the peritoneal cavity. Chemokine analysis showed DR3\(^{ko}\) mice had multiple defects, including CXCL1, CCL3 and CCL4 (Perks, 2013 PhD). This suggests that DR3 acts up-stream of several chemoattractants, including those directed at myeloid cell recruitment, exerting its effects as early as 6hrs post injection. Therefore, it
may be that the timepoint chosen to assess chemokine levels in this model were too late for some of those tested. Considering the results recently obtained within the laboratory, it would be interesting to examine chemokine levels over a time-course following OVA/Alum injection, to determine how early DR3 acts in this model regarding chemokine regulation.

Alum itself is thought to affect the cellular recruitment of innate immune cells to the injection site (Goto and Akama 1982; Goto, Kato et al. 1997), via the regulation of chemokines. Mosca et al used genome wide microarray to demonstrate the ability of Alum to modulate chemokine expression following intra-muscular injection (Mosca, Tritto et al. 2008), whilst intra-peritoneal injection of Alum has been shown to induce the local production of the chemoattractants CCL2 and CXCL1. This led to neutrophil, eosinophil and monocyte recruitment, the latter of which were shown to migrate to the draining lymph node and differentiate into inflammatory DCs (Kool, Soullie et al. 2008). Moreover, Alum has also been shown to induce IL-8 and CCL2 release from macrophages and monocytes (Seubert, Monaci et al. 2008), as well as recruit CD11b+ myeloid cells following intra-muscular injection. These recruited cell types were found to take up adjuvant and antigen and transport to the draining lymph nodes, with the authors proposing Alum injection leads to chemokine driven immune amplification, increasing the numbers of antigen presenting cells contributing to the onset of immunity (Calabro, Tortoli et al. 2011). Adding to this, innate cell depletion post Alum injection had little effect in the generation of an adaptive immune response (McKee, Munks et al. 2009). As multiple DR3KO innate cell subsets are depleted between 2mg and 50mg, including macrophages, eosinophils, monocytes, NK cells, NKT cells and γδ T cells, as well as the chemokine CXCL10, it is possible Alum is influencing cellular recruitment.
via DR3. However, the data suggests a certain threshold for this effect, as the biggest
differences are observed at the higher adjuvant concentrations.

Splenic differences have also been published between DR3<sup>wt</sup> and DR3<sup>ko</sup> mice in a
model of MCMV infection (Twohig, Marsden et al. 2012). The spleen is a site of robust
viral specific T cell and NK cell responses during MCMV infection. Whilst NK cell
expansion was found not to differ, a significant reduction was noted in DR3<sup>ko</sup> CD4<sup>+</sup> T
cell numbers at day 6 post-infection, and CD8<sup>+</sup> T cells throughout the studied time-
course. These T cell decreases were also observed using recombinant Vaccinia virus.
Priming was found to be intact, indicated by similar levels of cytokine-producing and
degranulating T cells, assessed using a CD107 assay. However, DR3<sup>ko</sup> mice were found
to have fewer virus-specific IFNγ expressing T cells in the early response, but not the
late response, suggesting the memory response was unaffected. This impairment in T
cell expansion was shown to be DR3 specific, using adoptive transfer of congeneric
DR3<sup>wt</sup> and DR3<sup>ko</sup> T cells differing at the Thy1 locus, demonstrated that only DR3<sup>wt</sup> but
not DR3<sup>ko</sup> CD4<sup>+</sup> and CD8<sup>+</sup> T cells expanded. This expansion was found to be due to
increased proliferation rather than reduced cell death.

Though similar results were obtained in this Chapter, in that T cell splenic differences
were observed by day 7, the means by which this was achieved were quite different. An
MCMV viral model is a chronic model of pathogen infection, as once mice have been
infected they are constantly exposed to the virus and therefore suffer constant immune
activation. The model employed in this Chapter involved only 1 injection of non-
infectious OVA/Alum. Furthermore, Twohig <i>et al</i> considered a lack of proliferation to
be responsible for reduced $\text{DR3}^{\text{ko}}$ T cell numbers (Twohig, Marsden et al. 2012), whilst
the results of this Chapter suggest antigen-specific proliferation is unaffected by the
absence of DR3. Therefore, the mechanism by which T cell numbers increase in the
spleen is likely to differ between the MCMV model and that described in this study,
with the results presented here implying a contribution from defects in recruitment.

Despite significant differences in B cell number between $\text{DR3}^{\text{wt}}$ and $\text{DR3}^{\text{ko}}$ mice, no
differences were found in OVA specific IgG production, again signifying priming was
unimpaired. However, little antibody was produced in the absence of Alum. Alum has
been shown to directly modulate B cells to produce IgG1 (Jin, Kim et al. 2013), by
inducing uric acid and activating inflammatory DCs (Kool, Soullie et al. 2008). DCs
were found to influence the humoral response as DC depleted mice injected with
OVA/Alum, displayed significant reductions in the levels of OVA-specific IgG1 10
days after injection (Kool, Soullie et al. 2008). Alum, even without antigen, was found
to influence the in vivo priming activity of splenic B cell responses via an increase in
calcium following B cell surface MHCII aggregation, so leading to antigen specific B
cell expansion. This was reported to be mediated by a population of Gr1$^+$/CD11b$^+$ IL-4
producing cells (Jordan, Mills et al. 2004), which were later identified as eosinophils
(Wang and Weller 2008). NKT cells have also been reported to be involved in Alum
stimulated antibody enhancement (Shah, Devera et al. 2012).

Limited research has been published regarding the role of DR3 on B cells. Naive murine
splenic and human peripheral blood derived B cells have been reported as DR3 negative
(Fang, Adkins et al. 2008; Cavallini, Lovato et al. 2013), however, activation through
the B cell receptor (BCR) induced its expression *in vitro* and it has been detected *in vivo* in tonsil germinal centres (Cavallini, Lovato et al. 2013). Cavallini et al also stated that DR3 reduced B cell proliferation, but had no effects on survival. However, no functional experiments were performed to decipher the role of DR3 in somatic hypermutation or affinity maturation.

Affinity maturation occurs in germinal centres and precedes B cell differentiation into either memory B cells or plasma cells. It is the process by which rare mutants expressing BCRs with high affinity for antigen are expanded through cycles of mutation and cell death. The TNFSF have an established role in the B cell response, such as CD40/CD40-L, which is required for a sustained immune response and isotype switching (Grewal and Flavell 1998), BAFF which can provide the necessary signals in the absence of CD40-L (Mackay and Browning 2002) and TACI, which is thought to maintain B cell homeostasis (Yan, Wang et al. 2001). B cells are able to assess the affinity of antigen using BCR-intrinsic mechanisms during the early phase of the B cell response, leading to activation. High affinity BCRs form oligomers which in turn grow into microclusters. This leads to enhanced recruitment of Syk kinases and calcium fluxes, so triggering signalling cascades and activation (Liu, Meckel et al. 2010). Affinity discrimination is key in the initiation of BCR responses. Those with low affinity undergo apoptosis, whilst those with high affinity receive survival signals rather than a proliferative advantage (Anderson, Khalil et al. 2009). Therefore, although it appears class switching is not altered by the absence of DR3, shown by the comparable antigen specific IgG levels, no studies have examined the effects of DR3 on B cells with high affinity for antigen.
6.5 Summary

The results of this Chapter show accumulation of multiple populations of splenocytes to be reduced in DR3\(^{\text{ko}}\) mice after one intra-peritoneal injection of non-pathogenic antigen and the adjuvant Alum. As well as confirming its previously described role in influencing T cell numbers, this thesis also shows DR3 to regulate the numbers of B cells, DC subsets, macrophages and eosinophils. The functional consequences of this remain unknown as antigen-specific T cell proliferation and antibody production were unchanged in this model. Results suggest that recruitment may be an important factor, as one chemokine, CXCL10, was significantly reduced in DR3\(^{\text{ko}}\) compared to DR3\(^{\text{wt}}\) spleens, although further experiments need to be conducted to determine whether other chemokines may also be involved at earlier timepoints. Furthermore, while most splenocyte subsets demonstrated an impaired capacity to increase numbers in DR3\(^{\text{ko}}\) mice, myeloid DCs showed a decrease relative to baseline levels, suggesting involvement of other mechanisms separate to recruitment. These are novel descriptions of a previously undiscovered immune phenotype in DR3\(^{\text{ko}}\) mice and indicate there may be more basic impairments in their immunity than initially reported.
Chapter 7:

General Discussion
7.1 Summary

The results of this thesis indicate that whilst DR3 is not required for the homeostatic maintenance of leukocytes in the lung, spleen and peripheral blood upon the induction of allergic lung inflammation, it induces a multitude of effects. Following acute allergic lung inflammation, DR3\(^{ko}\) mice displayed reduced cellular inflammation into the lung characterised by decreased 7/4\(^{-}\) monocytes, NK cells and NKT cells, as well as into the BAL fluid, whereby numbers of 7/4\(^{-}\) monocytes, myeloid DCs, CD4\(^{+}\) T cells, CD8\(^{+}\) T cells and NKT cells were reduced. In a more clinically relevant model of chronic allergic lung inflammation, DR3\(^{ko}\) mice exhibited lower mucin levels and inflammation in the lung parenchyma compared to DR3\(^{wt}\) mice. Interestingly, both acute and chronic models induced DR3 dependent changes in the spleen, as DR3\(^{ko}\) mice displayed reduced accumulation of multiple myeloid and lymphocyte subsets compared to DR3\(^{wt}\) mice. This phenotype was further explored, with results showing that one intra-peritoneal immunisation was sufficient to induce these altered splenocyte numbers, although as comparable antigen-specific T and B cell responses were observed, the functional biological consequences of this remain unclear.

7.2 DR3 is not required for leukocyte homeostasis

Despite the presence of DR3 on naive T cell populations, lung, spleen and peripheral blood leukocyte numbers did not differ between DR3\(^{wt}\) and DR3\(^{ko}\) mice, suggesting DR3 is not involved in the immunological maintenance of these organs. However, DR3’s constant presence and the lack of an inflammatory response suggest that either soluble DR3 or TL1A may be regulated to prevent constant bouts of inflammation and an unintended effector response.
7.3 DR3 in allergic lung inflammation

Data within this thesis implicates the DR3/TL1A pathway during specific phases of allergic lung inflammation. Reduced cellular accumulation was observed in the lungs and BALF of DR3\(^{3\text{ko}}\) mice following acute allergic lung inflammation, raising the possibility that DR3 could be a potential therapeutic target during acute exacerbations of the disease. As previously reported, reduced effector T cell numbers correlated with reduced mucin levels in mice lacking functional DR3. Meylan et al suggested DR3 is required for effector T cell expansion at the site of inflammation, as well as the production of the pathogenic cytokines IL-5 and IL-13 after re-challenge with antigen (Meylan, Davidson et al. 2008). DN DR3 transgenic mice exhibited similar resistance to OVA induced acute allergic lung inflammation, whilst blockade with an anti-TL1A antibody as late as 1 day before challenge also inhibited disease. Adding to this, Fang et al showed DR3\(^{+}\) NKT cells to be central in disease development. J\(\alpha\)18-deficient mice, which lack invariant NKT cells and are resistant to OVA induced airway inflammation, were transferred with DN DR3 NKT cells, which unlike WT NKT cells, failed to restore airway inflammation. The authors therefore proposed that NKT cells secrete IL-13 in response to TL1A, which acts on mucus producing cells and promotes the asthma phenotype (Fang, Adkins et al. 2008). Both of these hypotheses are supported by the data of this thesis, where reduced CD4\(^{+}\) T cell and NKT cell numbers were recorded in the DR3\(^{3\text{ko}}\) BALF, which corresponded to reduced mucin levels. Together this data implies that in acute inflammation, DR3 is required for disease progression as its absence hinders the development of both cellular infiltration and a resulting remodelling effect.
During chronic disease, DR3 appears to be essential for architectural changes in the lung, as DR3\textsuperscript{ko} mice exhibited reduced peribronchial and perivascular cuffing as well as lower mucin levels, a causative of mucus plugging and bronchoconstriction. Though the mechanisms behind these structural changes were not examined, data in this thesis implicates DR3 as a driver of mucin production during lung inflammation. Mucus overproduction is commonly associated with Th2 derived cytokines, although the data of Chapter 5 shows T cell numbers to be comparable between DR3\textsuperscript{wt} and DR3\textsuperscript{ko} mice. There are a number of plausible explanations for this mucin hyperplasia, despite the absence of T cell increases.

Firstly, cellular accumulation in the chronic treated DR3\textsuperscript{wt} lung may have been transient. T cells may have been present in increased numbers before the point of harvest, releasing the necessary cytokines to induce airway remodelling before migrating from the lung. However, as neither cytokine levels nor multiple timepoints were examined in this thesis, this hypothesis requires further experiments to confirm.

A second potential explanation for the observed mucin increases is the involvement of alternative cells types besides CD4\textsuperscript{+} T cells. Asthma, and consequently allergic lung inflammation is increasingly being considered a heterogeneous disease involving more than just Th2 cells. While multiple T cell and myeloid cell subsets were examined, no account was taken of mast cells during this study, irrespective of their central role in allergic inflammation and more specifically airway alterations. Increased mast cell numbers have been reported in the airway smooth muscle of asthmatic patients, corresponding with disease severity (Brightling, Bradding et al. 2002). In vitro, human
mast cells have been shown to adhere to airway smooth muscle through smooth muscle membrane bound SCF, soluble IL-6 and cell adhesion molecule 1 (CADM1), which is also required for mast cell adhesion to fibroblasts (Moiseeva, Roach et al. 2013). This smooth muscle and mast cell interaction was shown to support mast cell survival, proliferation, as well as de-granulation in co-culture (Hollins, Kaur et al. 2008). Through the release of inflammatory mediators, including, β-tryptase (Berger, Girodet et al. 2003), chymase (Lazaar, Plotnick et al. 2002), leukotriene D4 (Espinosa, Bosse et al. 2003), histamine (Panettieri, Yadish et al. 1990) and TGFβ (Woodman, Siddiqui et al. 2008), mast cells have been reported to influence smooth muscle proliferation, although this has been disputed by some (Kaur, Hollins et al. 2010). Mast cell importance in allergic lung inflammation has been further confirmed using mice lacking FcRγ chain, a signalling adaptor used by FcεRI. FcRγ deficiency results in the inhibition of mast cell activation by antigen and IgE dependent FcεRI aggregation, and thereby resistance to airway remodelling (Yu, Tsai et al. 2006). Human mast cells have also been shown to release amphiregulin, an epidermal growth factor ligand, causing an up-regulation in mucin gene expression in epithelial cells, leading to goblet cell differentiation (Okayama, Okumura et al. 2009). The same study reported intracellularly stored TSLP release by mast cells in asthmatic subjects following FcεRI aggregation and IL-4 stimulation (Okayama, Okumura et al. 2009), leading to smooth muscle actin and collagen up-regulation in a STAT3 dependent manner (Wu, Liu et al. 2013). IL-9, produced by a newly described subset known as Th9 cells (Veldhoen, Uyttenhove et al. 2008), as well as mast cells themselves (Hültner, Kölsch et al. 2000), has been shown to enhance mast cell growth, differentiation and protease expression. In both OVA and house dust mite induced chronic models of allergic lung inflammation, IL-9 neutralisation was reported to attenuate structural changes including mucus secretion,
sub-epithelial collagen deposition and release of pro-fibrotic factors (Kearley, Erjefalt et al. 2011). This overwhelming evidence of mast cell involvement in airway remodelling combined with the structural changes observed in this study in the absence of T cell differences, suggests mast cells as a good candidate for inducing DR3-dependent mucin release.

γδ T cells have also been reported to enhance Th2 cytokine production following allergen challenge (Krug, Erpenbeck et al. 2001), with some observing an increase in intracellular IL-4 in γδ T cells isolated from the airways of asthmatic patients (Pawankar, Okuda et al. 1996; Spinozzi, Agea et al. 1996), suggesting γδ T cells have a pro-inflammatory role in allergic asthma. The absence of γδ T cells in mice was reported to prevent the development of airway hyper-reactivity and eosinophil and T cell infiltration in an OVA induced model of acute allergic lung inflammation, although timing of depletion was found to be important (Lahn, Kanehiro et al. 1999). Depletion just before challenge resulted in airway hyper-responsiveness, whilst before sensitisation, hypo-responsiveness was observed in response to Methacholine (Hahn, Taube et al. 2004). Mice lacking γδ T cells also displayed reduced IgE and IgG release following acute allergic lung inflammation (Zuany-Amorim, Ruffie et al. 1998), similar to an OVA chronic model of allergic lung inflammation whereby mice demonstrated reduced airway hyper-reactivity, inflammation and allergen specific IgE (Tamura-Yamashita, Endo et al. 2008). Jaffar et al observed an increase in intra-epithelial associated IL-17 producing γδ T cells following allergic lung inflammation, up-regulated through prostaglandin I2 and IL-6 (Jaffar, Ferrini et al. 2011). IL-17 has previously been shown to have a role in allergic lung inflammation (Nakae, Komiyama et al. 2002; Song, Luo et al. 2008) and is increased in the airways and sputum of
asthmatic patients, correlating with airway hyper-responsiveness (Molet, Hamid et al. 2001; Barczyk, Pierzchala et al. 2003). Furthermore, IL-17 was also shown to stimulate the mucin genes MUC5B and MUC5AC from both human and mouse tracheo-bronchial epithelial (TBE) cells in combination with IL-6 (Chen, Thai et al. 2003), suggesting γδ T cell derived IL-17 could possibly be initiating mucus hyperplasia. In contrast, Murdoch et al proposed these IL-17 γδ T cells, which were more prevalent than IL-17 αβ T cells, to promote resolution in an acute OVA model of allergic lung inflammation (Murdoch and Lloyd 2010). γδ T cells were transferred intra-tracheally at day 13, the peak of acute allergic lung inflammation, ameliorating asthmatic features as opposed to γδ T cell blockade, which led to disease exacerbation. Transfer of Th17 cells was found to have no effect on disease outcome, whilst IL-17 deficient γδ T cells were unable to promote resolution, suggesting γδ T cell derived IL-17 as important in controlling allergic lung inflammation (Murdoch and Lloyd 2010) Others also suggest γδ T cells to have a regulatory role, inhibiting airway responses through the release of IFN-γ (Isogai, Athiviraham et al. 2007). Though conflicting results exist regarding the role of γδ T cells in asthma pathogenesis, this evidence of cytokine release suggests they could be a contributing factor to the mucus increase observed in repeatedly challenged DR3wt mice. It would therefore be interesting to analyse DR3 expression and compare numbers between DR3wt and DR3ko mice to determine any differences and thereby potential contribution to the mucin over-production noted in the chronic allergic lung inflammation model described here.

Alternatively, TL1A may be acting directly on goblet cells, bypassing the requirement for Th2 derived cytokines and therefore effector T cell increases in DR3wt mice. DR3wt mice did display a significant increase in myeloid DCs which have previously been
shown to release TL1A in inflammatory circumstances, suggesting they could be a direct source of TL1A and therefore goblet cell induced mucus release. TSLP, released by mast cells (Okayama, Okumura et al. 2009), has previously been shown to initiate DC maturation via the release of the TNFSF member OX40-L (Ito, Wang et al. 2005). Due to the DR3wt myeloid DC increases observed in the lung during the chronic model, it is tempting to speculate that TSLP may be stimulating DC activation via TL1A. TSLP storage by mast cells suggests early release in the innate response, whereby it may directly activate DCs. This in turn may initiate TL1A release and thereby potentially activate goblet cells to release mucin in the absence of T cells. In support of this theory, DR3 expression also appeared to correlate with goblet cell location, though experimental attempts to prove this association proved unsuccessful. Definitive conclusions regarding this theory are difficult to draw as no account was taken of either mast cells or TSLP. Adding to this, due to a lack of murine reagents at the time of experimentation, TL1A levels were not examined in this model. Despite these drawbacks, this is the first report of DR3 in chronic allergic lung inflammation and its potential as a therapeutic target for antagonism of goblet cell hyperplasia and associated mucus over-production as a result of allergic disease.

Mast cells, as mentioned previously, are known to produce inflammatory mediators during inflammation. However, whilst the main mediator in humans is histamine, it is serotonin release which dominates in rodents (Canning 2003). Serotonin is a chemo-transmitter which is predominantly sourced from platelets in humans, though it is also found in neuroendocrine cells of the respiratory tract (Mao, Morimoto et al. 1996). It’s role in human asthma remains unclear, although Lechin et al reported a positive correlation between serotonin concentration and asthma symptoms (Lechin, van der
Dijs et al. 1996). Treatment with tianeptine, which acts to reduce free plasma serotonin, also decreased clinical asthma features (Lechin, van der Dijs et al. 1996; Lechin, van der Dijs et al. 1998; Lechin, van der Dijs et al. 1998), whilst corticosteroids, the mainstream asthma treatment, have also been reported to reduce serotonin levels (Pretorius 2004). Menard et al. found serotonin to inhibit the production of the Th1 cytokines TNF, IL-12 and IL-10 whilst enhancing the production of nitrous oxide and prostaglandin E2 from alveolar macrophages (Menard, Turmel et al. 2007). In rodents, serotonin is thought to work by potentiating acetylcholine release, inducing contraction of bronchial muscle via activation of 5-HT2 and 5-HT3 receptors (Cazzola, Matera et al. 1995; Takahashi, Ward et al. 1995; Lechin, van der Dijs et al. 2004). This contraction requires an intact parasympathetic system, as ablation of the sensory neurons compromised the cholinergic pathways, thereby inhibiting bronchoconstriction after antigen challenge in a mouse model of asthma (Cyphert, Kovarova et al. 2009). In immunised BP2 mice, which produce high titres of antibody, serotonin was shown to provoke bronchoconstriction upon challenge via acetylcholine release (Eum, Norel et al. 1999), whilst the chemo-transmitter has also been shown to induce microvascular leakage in rodents (Michel and Kendall 1997). This emphasises the importance of serotonin in rodent allergic disease in initiating bronchoconstriction, for which mast cells have frequently been implicated (Margulis, Nocka et al. 2009). As serotonin is known to be released by mast cells, it may explain their proximity to the airway nerves (Myers, Undem et al. 1991).

Interestingly, aging DR3KO mice were observed to have abnormal neurochemistry, in that dopamine levels increased by 60% in the striatum of the brain whilst serotonin levels decreased 4 fold. This differential neurotransmitter expression was accompanied
by progressive behavioural defects in the absence of inflammation and neuropathology (Twohig, Roberts et al. 2010). The consequences of this unexpected finding were not examined beyond the brain by Twohig et al, though it is tempting to speculate that DR3<sup>ko</sup> resistance to allergic lung inflammation may stem from a defect in serotonin production. This in turn may lead to reduced bronchoconstriction and inflammation if DR3<sup>ko</sup> mast cells are impaired in their ability to produce serotonin and thus lack acetylcholine-induced constriction. It may then have been worthwhile to examine plasma serotonin levels to determine if a link exists between DR3 neurological function and immunity.

Though modelled on the protocols employed by Fernandez Rodriguez et al, the results of this thesis differ somewhat from those noted by the former. These discrepancies may be a result of using C57BL/6 mice as opposed to the BALB/c strain. Variation is known to occur in the development of allergic lung inflammation between different mouse strains. Shinagawa et al found A/J mice to exhibit the features of allergic lung inflammation to a greater degree than BALB/c and C57BL/6 following repeated intranasal challenge (Shinagawa and Kojima 2003), agreeing with Zu et al who also reported decreased eosinophils, IgE, IL-5 and IL-13 in the BALF of C57BL/6 mice compared to FVB/NJ and BALB/c mice (Zhu and Gilmour 2009). Using an inhalation model of allergic lung inflammation, Brewer et al reported marked variability amongst strains in the development of pulmonary responsiveness, eosinophilia and IgE levels, suggesting, like Whitehead et al, disease progression is genetically determined (Brewer, Kesselgof et al. 1999; Whitehead, Walker et al. 2003). Interestingly, Kumar et al could not elicit any airway lesions or airway hyper-reactivity in sensitised C57BL/6 mice chronically exposed to aerosolised antigen (Kumar and Foster 2002). Adding to this,
conflicting data has been published regarding the importance of eosinophils in disease progression, as ablation on a C57BL/6 background showed eosinophils to be integral to airway inflammation and hyper-reactivity (Lee, Dimina et al. 2004; Walsh, Sahu et al. 2008), whilst on a BALB/c background, mice were not protected from AHR, but rather airway remodelling (Humbles, Lloyd et al. 2004).

BALB/c mice are generally considered better Th2 responders than C57BL/6 mice, judged by their increased pulmonary eosinophilia and cytokine production (Zhu and Gilmour 2009; De Vooght, Vanoirbeek et al. 2010). Though many of the expected asthma-like features materialised in the acute model, chronically challenged mice failed to display increased accumulation of some subsets within the lung and the BAL. Potential reasons for this have previously been discussed (Chapter 5, section 5.4.1), though another contributing factor may have been the use of C57BL/6 mice. An alternative would have been to generate DR3\(^{ko}\) mice on a BALB/c background, though this was beyond the scope of this thesis in terms of time and resources. The above examples illustrate the need to standardise murine models as well as assess multiple endpoints to decipher associations between the immune response and allergic lung disease.

### 7.4 DR3 and splenic accumulation

In addition to the lung, a novel phenotype was also noted in the spleen following acute and chronic allergic lung inflammation. Further investigation found DR3\(^{ko}\) mice to exhibit a global reduction in splenocytes after just one i.p immunisation with non-
pathogenic substances, associated with a reduction in the chemokine CXCL10. However, this deficit appeared to have little effect on spleen function or immunity, as neither antigen-specific T cell proliferation nor antibody production were impaired. This is in contrast to previous studies following infection with either viral (Twohig, Marsden et al. 2012) or bacterial (Buchan, Taraban et al. 2012) organisms, as reductions were noted in the expansion of T cell populations. Buchan et al reported reduced numbers of activated/memory CD4$^+$ T cells in the DR3$^{ko}$ spleen following Salmonella infection (Buchan, Taraban et al. 2012). Twohig et al too, observed reduced numbers of splenic CD4$^+$ and CD8$^+$ T cells in response to MCMV infection using Ki67. This reduction was shown to be due to decreased DR3$^{ko}$ proliferation. Peptide specific CD8$^+$ T cells were also Ki67$^+$, suggesting that impaired anti-viral T cell generation in DR3$^{ko}$ mice was caused by intrinsic defects in the capacity of CD4$^+$ and CD8$^+$ T cells to expand in response to antigen (Twohig, Marsden et al. 2012). In a model of HSV-1 infection, an anti-DR3 antibody was found to expand T$_{reg}$S in the spleen, peripheral blood and draining lymph node of C57BL/6 mice when administered either 2 days pre infection or day 0 (J Reddy, Schreiber et al. 2012). This led to decreased numbers of neutrophils as well as IFN$\gamma$ producing CD4$^+$ T cells, leading to reduced lesions. However, in order to function as a therapeutic as well as prophylactic treatment, the anti-DR3 antibody required galectin-9. Anti-DR3 enhanced the proliferation of T$_{reg}$S, whilst galectin-9 increased effector T cell apoptosis, resulting in reduced mRNA levels of IFN$\gamma$, IL-6 and CXCL1, and increased levels of IL-10 and TGF$\beta$ (J Reddy, Schreiber et al. 2012). The former mentioned viral and bacterial models involved the use of infectious organisms, with both alluding to faulty pathogen clearance in DR3$^{ko}$ mice, resulting in constant immune stimulation and a sustained immune response due to constant pathogen presence. Meanwhile, Reddy et al demonstrated the therapeutic potential of an agonist
DR3 antibody, ablating the development of disease in a pathogenic viral model of infection. The results described in this thesis suggest the DR3/TL1A pathway as a booster of the immune response, including both myeloid and lymphocyte populations. This could be particularly beneficial in chronic conditions, such as those presented by MCMV and HSV-1, whereby DR3 is required to clear infection and protect from pathogen induced disease.

Whilst it is clear that DR3\textsuperscript{ko} mice suffer defective leukocyte accumulation in the spleen following intra-peritoneal sensitisation, the biological and functional impact of this is unclear. Readouts tested, including antigen-specific proliferation and antibody release, were unaffected by the loss of DR3 suggesting further research is required into the functional consequences of this decreased cellular accumulation, such as affinity maturation and splenic organisation. Attempts were made to examine T and B cell interactions in the spleen following OVA/Alum injection using IHC to examine whether broad splenic structure was DR3 dependent following challenge, as well as measure germinal centre size using peanut agglutinin lectin (PNA) (Appendix figure 1.3). However, problems arose concerning staining and due to time constraints, meant this line of investigation could not be concluded.

Other TNFSF members have been shown to influence splenic architecture, including TNF, lymphotoxin (LT)\textalpha and LT\textbeta. TNF was found to be essential for the formation of primary B cell follicles, follicular DC networks and germinal centres (Pasparakis, Alexopoulou et al. 1996; Pasparakis, Alexopoulou et al. 1997), whilst LT\textalpha\textsuperscript{ko} mice display severe disorganisation of the splenic architecture, including lack of marginal
zones, follicular DC networks and germinal centres as well as un-segregated B and T cell areas (De Togni, Goellner et al. 1994; Matsumoto, Fu et al. 1997). However, TNF$^{\text{ko}}$ mice maintain the capacity to Ig class switch (Pasparakis, Alexopoulou et al. 1996), whilst splenic T cells of LT$\alpha^{\text{ko}}$ mice exhibit normal lytic function (De Togni, Goellner et al. 1994), thus implying that despite defective organisation and development, particular functions remained unaltered. With this in mind, it would be interesting to optimise splenic T and B cell staining to determine whether structure and organisation were compromised in DR3$^{\text{ko}}$ mice following challenge.

As well as reduced leukocyte numbers, spleen lysates of DR3$^{\text{ko}}$ mice also displayed decreased levels of CXCL10, which could have aided a potential loss of architecture. Leukocytes are positioned in distinct locations by chemoattractants in the spleen, allowing interactions between cells as well as antigen and thereby an effective immune response. The observed deficiencies in CXCL10, could have altered splenocyte positioning and as a result splenic architecture, altering DR3$^{\text{ko}}$ response to antigen. In a model of MCMV infection, the virus was found to preferentially infect the endothelial cells of the stroma, suppressing the release of CCL21. This chemokine loss resulted in the failure of T cells to locate to the T cell zone in the spleen, which was partially restored via the activation of the LT$\beta$ signalling pathway (Benedict, De Trez et al. 2006). Although using a virus, Benedict et al showed splenic remodelling to impede efficient contact between cells by altering the migration of T cells. A similar situation could be occurring in DR3$^{\text{ko}}$ mice, in that CXCL10 decrease may impair leukocyte accumulation into the spleen as well as arrangement without affecting select functional responses.
Results of this thesis show DR3 to have differential roles in different phases of allergic lung inflammation. DR3\textsuperscript{wt} mice displayed increased numbers of leukocytes in the lung and BAL compared to DR3\textsuperscript{ko} mice in acute disease, whilst chronically challenged mice exhibited goblet cell hyperplasia. This may be due to increased IL-13 levels or direct TL1A action on goblet cells. In the spleen, DR3\textsuperscript{wt} mice showed increased levels of CXCL10, promoting splenocyte accumulation, which may lead to further release of TL1A and IFN\(\gamma\), thereby acting as a positive feedback loop.
7.5 Future Directions

Although, a broad assessment of the DR3/TL1A pathway in allergic lung inflammation was examined in this thesis, further experiments could be performed to strengthen data and improve analysis:

- A lack of reagents to identify both TL1A and DR3 have hindered profiling of their expression during this project. Upon continuation of these experiments, specific TL1A primers will be used to measure levels of the cytokine using qPCR. It would also be beneficial to develop a soluble murine DR3 ELISA to quantify membrane bound isoforms in comparison to soluble DR3, as well as produce antibodies capable of differentiating between the different isoforms. RT-PCR could also be used to examine these, though post-translational DR3 gene expression could not be accounted for. Using these techniques, the expression of DR3 and TL1A can be further elucidated allowing a comprehensive assessment of their levels in both naive and inflammatory situations.

- Mast cells and γδ T cells could also be investigated. Mast cells can be identified using flow cytometry and the markers CD117 and FceRI (Chen, Grimbaldeston et al. 2005), whilst DR3 expression on γδ T cells can be studied to explore whether these cells may respond to TL1A signalling.

- Key cytokines, such as IL-5, IL-13 and TGFβ, could also be measured, due to their known importance in mucin production and structural remodelling. These
cytokines could be measured in the BALF and the spleens. Adding to this, it would be interesting to examine the serum concentration of serotonin in DR3\textsuperscript{ko} and DR3\textsuperscript{wt} mice, to determine whether levels are reduced in DR3\textsuperscript{ko} mice beyond the brain, as well as in an inflammatory setting.

- As impaired myeloid cell numbers were observed in the spleens of DR3\textsuperscript{ko} mice following immunisation, myeloid cell analysis could be extended using IHC to identify rare cell types. CD35 would allow the identification of follicular DCs whilst SIGN-R1 could be used to examine marginal zone macrophages. SIGLEC-1 and MOMA-1 are also known markers of metallophilic macrophages (Mebius and Kraal 2005).

- Alternative routes of antigen administration (intra-nasal, subcutaneous) could also be studied, to determine if the observed splenic phenotype is specific to intra-peritoneal immunisation. I.P injections are commonly used in murine models as a means of antigen sensitisation and enhancement, including DR3\textsuperscript{ko} models of allergic lung inflammation (Meylan, Davidson et al. 2008) and EAU (Calder and Wang 2012). However, the results of this thesis suggest that DR3\textsuperscript{ko} splenic accumulation is reduced following I.P injection compared to DR3\textsuperscript{wt} mice. Though the biological impact of this is unknown, it cannot be said for certain that splenic defects did not affect previously published data using DR3\textsuperscript{ko} mice.
• Conditional knockouts could be used to confirm the role of DR3 in the generation of the splenic immune response and define Receptor function in individual cell subsets. This could be achieved by using Cre-lox recombination driven by tissue-specific promoters to remove the DR3 gene, which in order to study DC function, could be CD11c.

• Murine TL1A injections could be utilised in addition to OVA, to examine if effects are TL1A dose dependent or even independent of allergen once priming has occurred. Injections of anti-TL1A antibody in DR3\textsuperscript{wt} mice following sensitisation in both allergic lung inflammation and the splenic model described in this thesis, could be interesting, to observe whether effects were ameliorated and align with the phenotype of DR3\textsuperscript{ko} mice.

• Human BAL samples from healthy controls, mild and severe asthma patients could be tested for TL1A, DcR3 and potentially, soluble DR3 levels to determine just how translatable and clinically relevant the data within this project is. This would also allow an arbitrary assessment of how TL1A levels fluctuate with disease severity and for what stages of asthma development it may be important.

• To examine the effects of TL1A on DC proliferation, DCs could be isolated from the spleens of DR3\textsuperscript{wt} and DR3\textsuperscript{ko} mice following injection using the marker CD11c and FACS. Cells could be stimulated \textit{in vitro} with TL1A, and proliferation measured using BrdU. The supernatants from cell cultures could
also be used to study the effects of TL1A on GM-CSF and Flt3-L levels in comparison to un-stimulated cells.

- The data of Chapter 6 suggests Alum reduces splenic DR3<sup>ko</sup> myeloid DC numbers, whilst reduced macrophage and eosinophil numbers are only observed upon injection of higher Alum concentrations. It would therefore be interesting to use alternative adjuvants, such as LPS or CpG, to determine whether the noted effects are Alum specific. Adding to this, in contrast to Alum, both LPS and CpG are known to be dependent on the TLR pathway.

- As reduced numbers of activated T cells were observed in the DR3<sup>ko</sup> spleen, it would be interesting to examine whether the function of these cells is also impaired. Cytokine release in response to OVA could be examined, including the Th1 cytokine IFNγ, as well as the Th2 cytokine IL-4. Additionally, the proportion of T cells releasing TNFα or de-granulating could be measured using a CD107 assay.

- To assess whether affinity maturation is affected by the loss of DR3, the BCR repertoires of DR3<sup>wt</sup> and DR3<sup>ko</sup> mice could be analysed using high throughout sequencing following immunisation. The BCR could be amplified using specific primers and analysed using a sequencer to identify clones. Bioinformatics could be used to identify gene segments and correct for sequencing errors. Any differences between the 2 genotypes could then be analysed and used as an indicator to assess whether affinity maturation is impaired in DR3<sup>ko</sup> mice.
7.6 Final conclusions

The research described in this thesis has, for the first time, shown an important role for the DR3/TL1A axis in chronic allergic lung inflammation. Blockade of this pathway through gene knockout reduced cellular accumulation in acute inflammation and severity of histopathological changes in chronic models of allergic lung inflammation, in particular mucin production. These experiments in turn led to the discovery of a novel splenic phenotype, as DR3$^{ko}$ mice displayed impaired T cell and DC accumulation after a single peritoneal injection of non-pathogenic substances. These results suggest that DR3 exerts effects beyond just effector T cell expansion and myeloid differentiation, around which publications in the field have previously been orientated. Though further investigation is required to fully elucidate the effects of DR3 absence on spleen function, this data suggests chemokine levels and recruitment to be affected.
Chapter 8:

References


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Appendix
## Appendix Table 1.1. Chemoattractant levels in BAL fluid at 24 and 72hrs post inhalation following chronic allergic lung inflammation

<table>
<thead>
<tr>
<th>Chemokine</th>
<th>DR3&lt;sup&gt;wt&lt;/sup&gt;</th>
<th>DR3&lt;sup&gt;ko&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PBS&lt;sup&gt;*&lt;/sup&gt;</td>
<td>OVA&lt;sup&gt;^&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>24hrs&lt;sup&gt;ε&lt;/sup&gt;</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCL3</td>
<td>36±10</td>
<td>31±5</td>
</tr>
<tr>
<td>CCL4</td>
<td>N/A†</td>
<td>N/A†</td>
</tr>
<tr>
<td>CCL5</td>
<td>145±40</td>
<td>125±22</td>
</tr>
<tr>
<td>CXCL1</td>
<td>74±16</td>
<td>45±6</td>
</tr>
<tr>
<td>CXCL2</td>
<td>254±54</td>
<td>216±20</td>
</tr>
<tr>
<td>CXCL10</td>
<td>799±144</td>
<td>955±246</td>
</tr>
<tr>
<td>CXCL13</td>
<td>551±99</td>
<td>556±46</td>
</tr>
<tr>
<td><strong>72hrs&lt;sup&gt;¥&lt;/sup&gt;</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCL3</td>
<td>28±3</td>
<td>30±4</td>
</tr>
<tr>
<td>CCL4</td>
<td>N/A†</td>
<td>N/A†</td>
</tr>
<tr>
<td>CCL5</td>
<td>152±15</td>
<td>154±18</td>
</tr>
<tr>
<td>CXCL1</td>
<td>72±7</td>
<td>76±16</td>
</tr>
<tr>
<td>CXCL2</td>
<td>237±16</td>
<td>228±48</td>
</tr>
<tr>
<td>CXCL10</td>
<td>943±59</td>
<td>940±178</td>
</tr>
<tr>
<td>CXCL13</td>
<td>1110±64</td>
<td>1197±178</td>
</tr>
</tbody>
</table>

* PBS corresponds to mice challenged via inhalation with PBS

^ OVA corresponds to mice challenged via inhalation with OVA

ε 24hrs corresponds to mice harvested 24hrs after the final inhalation challenge

¥ 72hrs corresponds to mice harvested 72hrs after the final inhalation challenge

† N/A corresponds to chemokines either not being present or too low to be tested

$ Significance between groups using 1 way ANOVA, N.S.D = no significant difference
Appendix figure 1.1. Chemokine levels within the BAL of DR3<sup>wt</sup> and DR3<sup>ko</sup> mice following chronic allergic lung inflammation (24hrs). Mice were sacrificed 24hr post final inhalation challenge with either OVA or PBS (n=5). BAL fluid was isolated and the supernatant used to determine chemokine levels using ELISA. CCL3 n.s.d, CCL5 n.s.d, CXCL1, n.s.d, CXCL2 n.s.d, CXCL10 n.s.d and CXCL13 n.s.d. No significant differences seen. Significance determined using 1 way ANOVA and Bonferroni post test Bars represent mean ± SEM.
Appendix figure 1.2. Chemokine levels within the BAL of DR3<sup>wt</sup> and DR3<sup>ko</sup> mice following chronic allergic lung inflammation (72hrs). Mice were sacrificed 72hr post final inhalation challenge with either OVA or PBS (n=7). BAL fluid was isolated and the supernatant used to determine chemokine levels using ELISA. CCL3 n.s.d, CCL5 (*p=0.0266), CXCL1 (**p=,<0.0001), CXCL2 (*p=0.0256), CXCL10 (*p=0.0354) and CXCL13 (*p=0.0223). Significance determined using 1 way ANOVA and Bonferroni post test. Bars represent mean ± SEM.
Appendix figure 1.3. Examples of T cell (blue) and B cell (brown) splenic interactions in DR3<sup>ko</sup> mice following OVA/Alum injection. IHC was used to examine T and B cell interactions in frozen spleen sections using CD3 and B220 staining. (A) is an example of a relatively successful stain compared to (B) where the section is lifting from the slide resulting in ‘patchy’ staining. Scale bar=100μM.