Is CD248 involved in the resolution of inflammation during development of lung sarcoidosis?

and

The role of adipose tissue-derived stromal cells in the survival of lymphocytes

Presented by

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This project is submitted in partial fulfilment of the requirements for the award of the MRes

College of Medical and Dental Sciences

University of Birmingham

ABSTRACT

The antigenic trigger of sarcoidosis remains a scientific enigma, despite being the most common cause of interstitial lung disease. In 2010, a mouse model of pulmonary inflammation presented with granulomas, resembling sarcoidosis. Recent findings suggest the role of CD248 in inflammation and tissue remodelling/repair. Since these processes occur during inflammation and fibrogenesis, we explored the role of CD248 in a mouse model of sarcoidosis.

To investigate CD248 involvement in sarcoidosis, apolipoprotein knock-out ($ApoE^{-/-}$), CD248 knock-out ($CD248^{-/-}$) and double knock-out (DKO) mice were fed a high fat diet (HFD). Tissue histology was examined using haematoxylin and eosin and Van Gieson staining, and disease burden and fibrosis were quantified by Image J thresholding. CD248 distribution in wild type and $ApoE^{-/-}$ tissue sections was subsequently investigated by immunofluorescence.

ApoE deficiency combined with HFD induced sarcoid-like granulomas without supplementation with cholate, and our results suggest the involvement of CD248 in the resolution of inflammation during the development of sarcoidosis. CD248 deficiency influenced the severity of disease, and expression was increased in the sarcoid lung.

CD248's capacity to influence the resolution of inflammation in sarcoidosis is a promising finding. Investigating CD248 deficiency in other models of inflammation could further elucidate its role in immunology, and potential as a therapeutic target in the treatment of chronic inflammatory diseases.

ACKNOWLEGEMENTS

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1. INTRODUCTION

1.1 Immunopathology of sarcoidosis

Sarcoidosis is a multisystem inflammatory disease, which can affect many organs including the liver, lymphatics, spleen and heart, but most commonly presents in the lung [reviewed in Patterson *et al.* 2012], (Figure 1). It is the most common idiopathic interstitial lung disease in Europe, with an prevalence of 2 cases per 10,000 people in the UK [Muller-Quernheim 1998]. Familial correlation of sarcoidosis has been suggested, with some human leukocyte antigen (HLA) genes being associated with disease incidence implying a possible genetic predisposition. Associated genes include Class I HLA 1B8, Class II HLA CRB1, & Class II HLA DQB1 [reviewed in Iannuzzi *et al.* 2007]. The precise cause of sarcoidosis is currently unknown, but it is characterised by the presence of non-necrotising epitheliod granulomas in most cases, which persist around bronchovascular bundles and veins in the lungs during a cellular immune response [reviewed in Rosen 2007], (Figure 1b). Diagnosing sarcoidosis requires careful histological examination of biopsies from affected organs for the presence of sarcoid granulomas and the identification of any organisms which may cause granuloma formation, as the histological features of sarcoidosis are not unique to this condition [discussed in Mukhopadhyay & Gal 2010]. The majority of sarcoidosis patients do not require treatment as in most cases, it spontaneous resolves. However, in those patients where granulomatous disease persists and becomes chronic, several treatment options can be considered. The use of immunosuppressive corticosteroids such as prednisolone is the most common form of treatment and can have positive results, but for end-stage

patients, the only remaining treatment option is lung transplantation [Iannuzzi *et al.* 2007].

1.1.1 Aetiology of sarcoidosis

The causative agents of sarcoidosis are controversial, but it is the general consensus that a specific infectious or environmental trigger is involved in genetically susceptible individuals. Many antigens have been put forward as being associated with sarcoidosis, including bacteria and chemical compounds. Possible bacterial triggers include propionibacteria such as *P. acnes*, and mycobacteria including *M. tuberculosis* [reviewed in Ichikawa *et al.* 2008 & Du Bois *et al.* 2003]. Compounds such as silica and even nanoparticles have also been hypothesised as environmental triggers [discussed in Yeager *et al.* 2011 & Heffner 2007]. However, despite numerous efforts within the research community, whether a pathogenic relationship exists between such agents and sarcoidosis remains unclear.

1.1.2 Pathology of sarcoidosis

The sarcoid granuloma is a collection of inflammatory cells and occurs following an immune response mounted against an unknown antigen as illustrated in Figure 2a. Initially, antigen presenting cells (APCs) such as dendritic cells (DCs) or macrophages internalise, process and present antigen to other immune cells, for example T cells, in the context of major histocompatability complex (MHC) class II [reviewed in Rosen 2007, Patterson *et al.* 2012 & Baughman R. P *et al.* 2011]. The T cell recognises the antigen-MHC complex via its T cell receptor (TCR) and once activated, begins to produce interleukin-2 (IL-2). Expression of the interleukin-2 receptor (IL-2R) is upregulated at the lymphocyte plasma membrane. IL-2 binds to

its receptor and acts in an autocrine fashion, facilitating the proliferation and differentiation of T cells into a T helper 1 (T_H1) subset [Boyman & Sprent 2012]. These activated T cells produce a vast array of pro-inflammatory mediators including interferon-gamma (IFN-γ), interleukin-12 (IL-12), interleukin-18 (IL-18) [Shigehara *et al.* 2001], macrophage inflammatory protein (MIP) and monocyte chemotactic protein (MCP) [Gibejova *et al.* 2003], which are involved in the activation, recruitment, and polarisation of inflammatory cells. The recruitment of macrophages by T_H1 cells results in the local production of pro-inflammatory mediators such as the highly potent tumor necrosis factor-alpha (TNF-α) [Ferenback *et al.* 2003], but also IL-12 and interleukin-6 (IL-6) [discussed in Muller-Quernheim 1998]. This facilitates the orchestration of an immune response, involving the infiltration of T_H1 cells and macrophages to the site of inflammation. Here, they become organised into granulomas (Figure 1b), composed of a central aggregation of macrophages, which differentiate into multinucleated giant cells and epithelioid cells [Rosen 2007]. $CD4^+$ T Cells can be found in the centre, with small numbers of $CD8^+$ T cells, fibroblasts and B cells located at the edge [Muller-Quernheim 1998, Patterson *et al.* 2012]. To date however, the role of $CD8^+$ T cells and B cells in the pathology of sarcoidosis is unclear.

1.1.3 Pulmonary fibrosis in the sarcoid lung

Granuloma development in sarcoidosis may initially serve as a protective mechanism, encapsulating the offending inflammatory stimuli, e.g. bacteria, within the affected area, thus restricting further spread and resolving spontaneously in most cases. However inflammation can become chronic in up to 30% of individuals [reviewed in Lynch *et al.* 2007], and this protective mechanism becomes

dysregulated. Here, granulomas persist alongside pathological inflammation which causes gradual destruction of lung tissue due to the exacerbating actions of inflammatory cells and mediators released locally (Figure 2a). This can eventually lead to tissue destruction, loss of lung function and fibrosis. Pulmonary fibrosis is the development of excess fibrotic tissue due to fibroblast activation and proliferation. It is mediated by cytokines produced by inflammatory cells such as macrophages during chronic inflammation, including transforming growth factor-beta (TGF-β), interleukin-1 (IL-1), interleukin-8 (IL-8) and TNF-α (Figure 2b) [reviewed in Coker & Laurent 1998 & Agostini & Gurrieri 2006]. During sarcoidosis, fibrosis is found mainly around bronchovascular bundles [Patterson *et al.* 2012] similar to granuloma distribution. This causes permanent scarring, which severely decreases the capacity of the lungs to oxygenate blood via the diffusion of oxygen from alveolar spaces into pulmonary capillaries. Ultimately, the development of pulmonary fibrosis in sarcoidosis can lead to death in up to 5% of patients [reviewed in Lynch *et al.* 2007 & Rosen 2007].

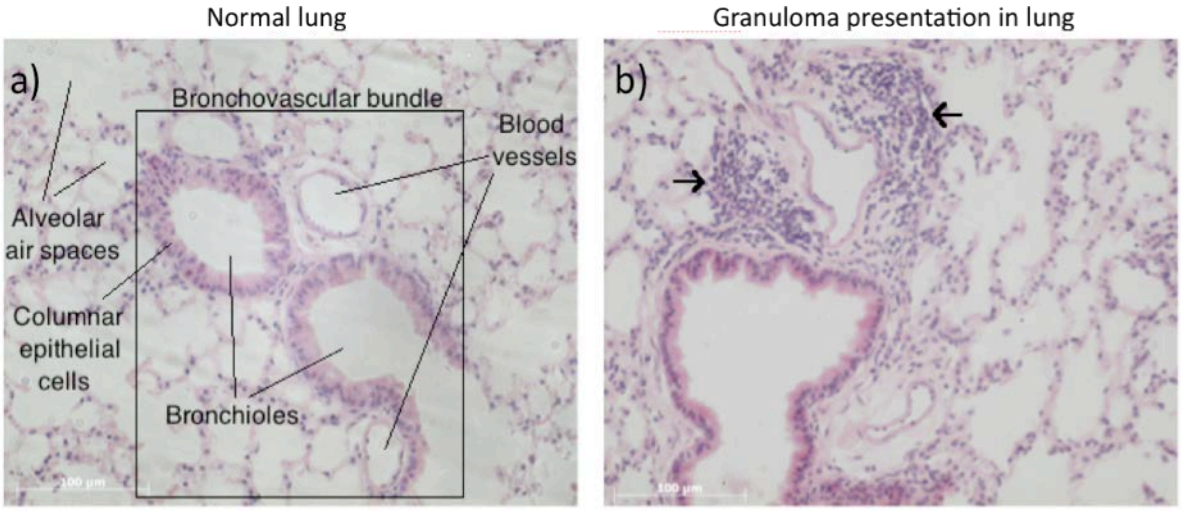


Figure 1. Representative images of normal and granulomatous lung. Formalin-fixed murine lung tissue sections were stained with haematoxylin and eosin (H&E) using standard protocols. a) Normal lung section. b) Section from ApoE$^{-/-}$ mouse on HFD. Arrows indicate granuloma formation. Original magnification x20. White bar represents 100μm. ***Images are the author's own.***

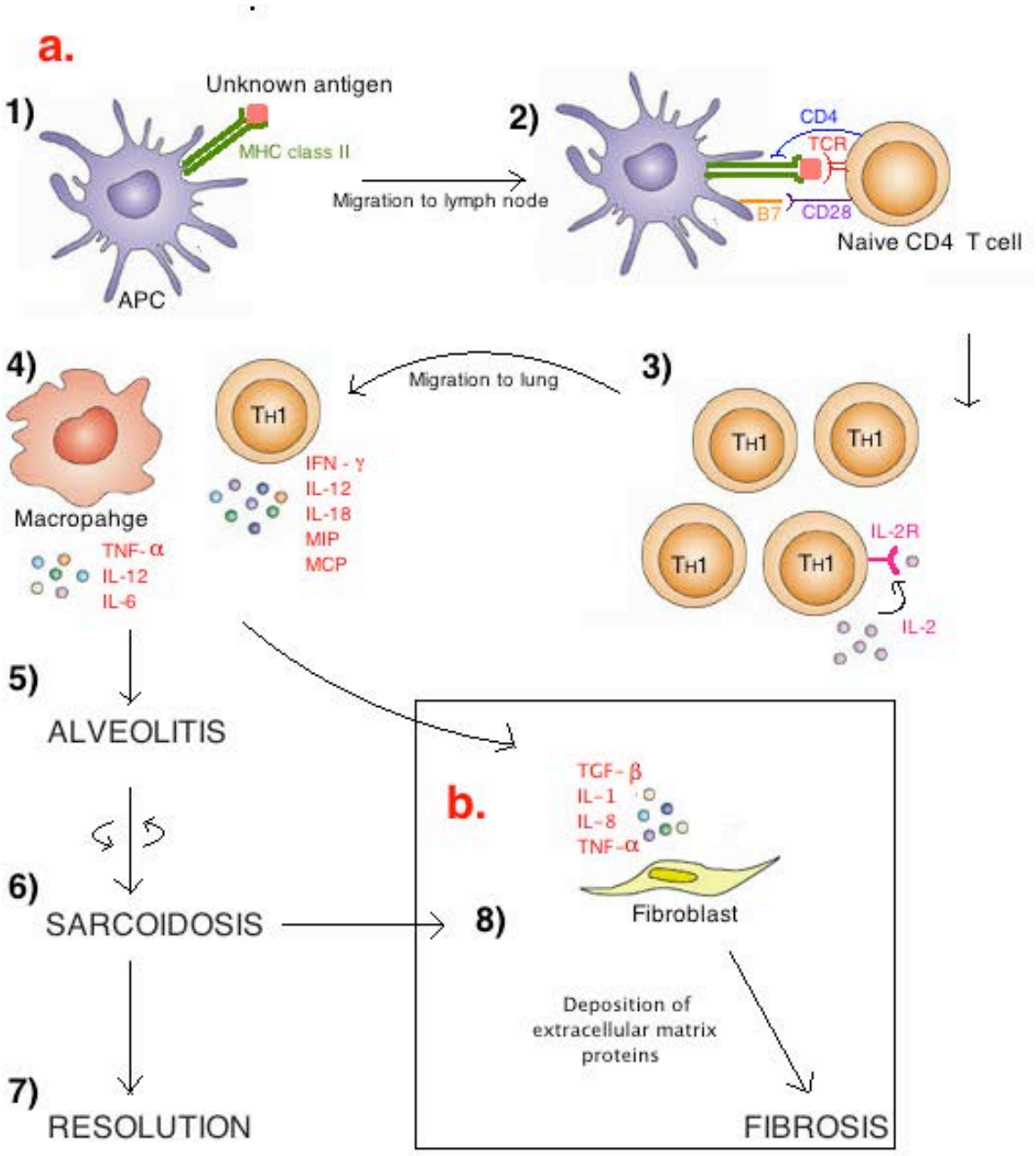


Figure 2. Central immunopathological mechanisms involved in the development of sarcoidosis (a) and pulmonary fibrosis (b). 1: Antigen recognition by APC and migration to the LN. 2: Antigen presentation to naïve T cells and interaction of MHC class II with TCR stabilised by co-receptor CD4 and confirmed by the interaction of co-stimulatory molecules B7 and CD28. 3: T cell activation differentiation into T_H1 subset, IL-2 production and increased IL-2R expression. 4: Migration of T_H1s to lung followed by secretion of pro-inflammatory cytokines and macrophage activation and polarisation of inflammatory response. 5: Macrophages produce cytokines which exacerbate inflammatory response and cause alveolitis through increased cell recruitment and proliferation. 6: Inflammatory cells organise into granuloma in sarcoidosis. 7: Spontaneous resolution. 8: Cytokines produced by local inflammatory cells activate fibroblasts. 9: Activated fibroblasts secrete extracellular matrix proteins and replace normal lung parenchyma with fibrotic tissue. ***Image created in Paintbrush.***

1.2 Current models of sarcoidosis

Although a lack of understanding of the complex and dynamic immunological processes occurring in granuloma formation currently prevents the advancement of sufficient and accurate *in vitro* models, several *in vivo* models of sarcoidosis have been developed in attempts to uncover key antigenic stimuli, and study the pathological processes of sarcoidosis.

i) The Kveim-Siltzbach model

Developed in the 1940s, the Kveim-Siltzbach model, reviewed in Altman & Boyton 2006, involves the inoculation of animals with sarcoid granuloma tissue extracts (Kveim extract). Granuloma formation in animals is representative of those seen in sarcoidosis, however due to the variability of conclusions drawn in laboratory reports, it is rare that the Kveim-Siltzbach model is used today.

ii) Pathogen driven models

Inoculation of experimental animals with granuloma-inducing pathogens may not accurately model sarcoidosis and assumes a pathogenic element is involved in aetiology, but allows the processes involved in the formation of sarcoid granulomas to be studied. Pathogens used in such models include *P. acnes*, which evokes cell-mediated granuloma formation [Altmann & Boyton 2006].

iii) Knock-out models

Selected gene knock-out (KO) models are commonly coupled with infection in order to induce granulomatous disease. For example, mice deficient for inflammatory

signalling protein MyD88 (myeloid differentiation primary response gene 88) infected with *Schistosoma mansoni* develop granuloma formation [Altmann & Boyton 2006, Layland *et al.* 2005]. However, an appealing model has recently emerged involving the dietary modulation and deficiency of Apolipoprotein E (ApoE), a protein involved in lipid metabolism, which drives granuloma formation in the lung without the requirement of infection [Samokhin *et al.* 2010], (Figure 2). ApoE-deficient mice on high fat diets (HFD) have been similarly used in the current investigation and thus the background to the model is described in more detail below.

1.3 Apolipoprotein E

Apolipoprotein E (ApoE) is a key plasma lipoprotein involved in the transport and metabolism of lipids such as cholesterol and triglyceride [reviewed in Mahley 1988]. It is synthesised primarily in the liver and is a component of liver-derived very low density lipoprotein (VLDL), but is also produced in the periphery, for example, by adipocytes [reviewed in Pendse *et al.* 2009], and in the brain and kidney [Mahley 1988]. ApoE serves as a ligand for low density lipoprotein (LDL) receptors [Mahley 1988], facilitating the distribution of lipids between cells for important biological processes including proliferation, energy storage and the maintenance of cell membrane integrity. Interestingly, ApoE plays a role in immunity. It has been shown to possess immunosuppressive effects, and has been implicated in inflammatory disorders such multiple sclerosis (MS) [reviewed in Zhang *et al.* 2010]. ApoE secreted by macrophages has been shown to modulate immune responses and its immunosuppressive properties include down-regulating T_H1 responses, suppressing lymphocyte proliferation and the activation of neutrophils, as well as inhibiting macrophage expression of pro-inflammatory cytokines [Zhang *et al.* 2010].

1.3.1 ApoE in immunity

ApoE is found localised to areas of tissue repair and its functions in repair and regeneration of tissues sheds light upon how it may function in immunological disorders. Injury of peripheral neurones leads to the local production of ApoE by macrophages following an inflammatory response [Mahley 1988]. This allows the distribution of lipids amongst cells as needed for biosynthesis to repair the injury and allow tissue regeneration to occur.

The detailed molecular mechanisms underlying the modulation of immunity by ApoE have not yet been elucidated, but initiation of signal transduction may occur via the binding of an 'immunosuppressive' receptor on the surface of T cells [Mahley 1988, Laskowitz *et al.* 2000]. It has also been hypothesised that ApoE may influence immune responses via nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) pathways [Zhang, *et al.* 2010], two pathways involved in the up-regulation of pro-inflammatory cytokines. As previously mentioned, ApoE has an inhibitory effect on the expression of pro-inflammatory signalling molecules by macrophages and in turn, the activation of macrophages leads to a down-regulation of ApoE expression [Zhang *et al.* 2010]. This reciprocal effect is suggestive of a regulatory feedback system within its signalling. These events may not be exclusive to peripheral nervous tissue, and may be generalised to other damaged tissues. Certainly ApoE-deficient mice show delayed olfactory healing responses [Nathan *et al.* 2010]. ApoE-null mice have been demonstrated to have altered lymphocyte-mediated immune responses, and possess macrophages with an increased capacity to express pro-inflammatory cytokines and MHC class II molecules [Zhang, *et al.* 2010, Laskowitz *et al.* 2000]. The established link between lipid metabolism and transport, and immune regulation is strengthened by new evidence generated using ApoE null mice.

1.3.2 The ApoE$^{-/-}$ model of sarcoidois

In early 2010, Samokhin and co-workers developed a mouse model of sarcoidosis mentioned briefly in section 1.1.2, by placing ApoE deficient mice on cholate-enriched HFD conditions [Samokhin *et al.* 2010]. These mice developed a high number of non-necrotising granulomas in the lungs representative of those seen in

human pulmonary sarcoidosis with similar immune cell compositions and induction of fibrosis. Interestingly this model does not incorporate a pathogen-derived initiating antigen as the mice are housed in sterile facilities.

HFD has been known to induce inflammation through increased production of inflammatory mediators, however ApoE$^{-/-}$ mice exhibit elevated lipid levels without the requirement of HFD [Naura *et al.* 2009]. Placing ApoE$^{-/-}$ mice on HFD has been shown to increase inflammation through the induction of pro-inflammatory mediators including TNF-α and IFN-γ [Naura *et al.* 2009]. Such a high-grade state of inflammation is likely to stimulate local generation of reactive oxygen species (ROS) by activated immune cells, for example macrophage production of superoxide, and can lead to further damage during the acute phase of lung inflammation [reviewed in Chabot *et al.* 1998], i.e. alveolitis. It is also possible that defects in immune cells, for example T cells, in ApoE$^{-/-}$ mice lead to a lack of immunosuppression or inhibition [Laskowitz *et al.* 2000], resulting in an impaired ability to prevent or repair injury. Such conditions could lead to chronic inflammatory states which promote granuloma development, and lead to pathological occurrences e.g. fibrogenesis. The mechanism which drives fibrogenesis in this model remains to be characterised but is hypothesised to involve a switch to T_H2-driven immune responses.

1.4 The role of CD248 in fibrosis and immune responses

CD248 is a transmembrane glycoprotein also known as endosialin or tumor endothelial marker-1 (TEM-1), with a controversial cellular expression pattern. It is widely considered as a stromal cell marker and its expression has been demonstrated on fibroblasts, pericytes, smooth muscle cells and mesenchymal stem cells (MSCs) *in vitro* [Christian *et al.* 2008]. The precise physiological role of CD248 is still unclear however research strongly suggests a central role in tissue remodelling and repair. Expression is high during embryogenesis; a period of intense remodelling, and decreases to virtually no expression in adults [Christian *et al.* 2008, Macfadyen *et al.* 2007]. However CD248-null mice develop normally with appropriate wound healing capacities and fertility [Nanda *et al.* 2006], suggesting the involvement of other compensatory factors in these circumstances. CD248 has been a prime candidate as a tumour endothelial marker (TEM) in recent research. It is up-regulated in many cancers including breast [Macfadyen *et al.* 2005] and colon [Christian *et al.* 2008] carcinomas. High expression within tumours is of significance since these represent areas of high levels of cell proliferation, remodelling, adhesion, and in the case of metastasis, migration. This supports the hypothesis that CD248 plays a key role in these processes.

1.4.1 CD248 and the extracellular matrix

CD248 has been shown to interact with extracellular matrix proteins such as collagen (types I and IV), and fibronectin [Tomkowicz *et al.* 2007]. The ability to bind extracellular matrix proteins is vital in cell adhesion and migration, required for tissue development and repair. Blocking CD248 has been shown to inhibit pericyte

migration [Bagley *et al.* 2008], suggesting that CD248 is also involved in angiogenesis due to the supportive function of pericytes to the vasculature, further demonstrating the importance of CD248 expression in tissue development and remodelling. In addition to this, fibroblast migration, which is important for efficient wound healing, is also reduced by removing CD248 expression [Christian *et al.* 2008]. Although important for physiological wound repair, there is also evidence that $CD248^{+}$ fibroblasts are linked to fibrogenesis in disease. For example, during chronic kidney disease, stromal fibroblasts show increased expression of CD248 which correlates with disease stage [Smith *et al.* 2011]. Similarly in lung fibrosis, extracellular matrix proteins are deposited to replace the normal lung parenchyma and CD248 has been demonstrated to bind to them. Thus CD248 may be important in the development of fibrosis in the chronic stage of sarcoidosis as well as having an involvement in the initiating immune response.

1.4.2 CD248 in inflammatory disease

The involvement of CD248 in the chronic inflammatory disease rheumatoid arthritis (RA) was highlighted by Maia and co-workers [Maia *et al.* 2010]. They demonstrated that CD248 expression increased within the human synovium during RA and that the severity of rheumatoid arthritis in murine models was reduced by deletion of the cytoplasmic domain of CD248. As intracellular domains are typically involved in signalling, this implies that the reduction in severity was due to effects downstream of CD248. Possible signalling roles of CD248 are supported by studies showing that CD248 regulates cell proliferation [Lax *et al.* 2010], via platelet-derived growth factor-receptor (PDGF-R)-dependent signalling [Tomkowicz *et al.* 2010]. Therefore CD248-dependent modulation of cellular responses may be key to the

pathogenesis of chronic inflammatory diseases such as RA and sarcoidosis.

1.5 Aim and objectives

Sarcoidosis is the most common cause of idiopathic lung disease in Europe [Muller-Quernheim 1998]. Despite the identification of immunological features in sarcoidosis, the molecular mechanisms underlying its pathology remain poorly understood. ApoE knockout ($ApoE^{-/-}$) mice fed HFD develop lung injury reflective of the clinical picture of sarcoidosis, and inactivation of CD248 has been shown to reduce the severity of rheumatoid arthritis, a chronic inflammatory disease. Therefore, we developed an ApoE-null CD248-null double knockout mouse (DKO) to uncover the role of CD248. Our major experimental aim was to explore the role of CD248 in a mouse model of sarcoidosis. To achieve this we had several experimental objectives:

- To investigate whether high fat diet alone (in the absence of cholate supplementation) is sufficient to cause sarcoid-like lesions in the mouse lung

- To examine whether absence of CD248 influences the severity of sarcoidosis in the $ApoE^{-/-}$ mouse

- To characterise the distribution of CD248 in wild type (WT) and $ApoE^{-/-}$ animals

2. METHODS

2.1 Animals and experimental protocol

ApoE-null (ApoE$^{-/-}$) CD248-null (CD248$^{-/-}$), and ApoE-null/CD248-null (DKO) C57BL/6 mice (bred in house under specific pathogen free (SPF) conditions: Biomedical Sciences Unit, University of Birmingham) were taken off a normal chow diet and placed on a high fat diet (HFD) containing 0.15% supplementary cholesterol (Western RD Diet, Special Diets Services, Essex, UK) after 10 weeks of birth. 13 weeks following this change, mice were sacrificed by terminal anaesthesia. Control groups remained on a normal chow diet. The number of mice per group differed but all consisted of at least 10 animals (Table 1). All tissue was collected under the appropriate Home Office licence.

Table 1. Numbers of mice in each experimental group. A total of 76 10-week old WT, CD248$^{-/-}$, ApoE$^{-/-}$ and DKO mice were placed on normal chow diet or HFD for 13 weeks. ApoE$^{-/-}$ and DKO mice on chow were unavailable for use in this investigation.

Group	Number of mice per group
WT on chow	11
WT on HFD	15
CD248$^{-/-}$ on chow	11
CD248$^{-/-}$ on HFD	15
ApoE$^{-/-}$ on HFD	10
DKO on HFD	14

2.2 Tissue preparation

Lungs were fixed in 10% formal saline for a minimum of 48h and paraffin embedded using standard procedures. Sections were cut at 4µm using a microtome and mounted on Xtra glass slides (Leica Biosystems, UK). On occasion spleens and livers were also collected from the animals for use as positive control samples.

2.3 Histology

Haematoxylin and eosin (H&E), and Van Gieson stains were performed on sections from all tissue samples within each group using standard protocols. Standard histological assessment of morphology was performed using a routine H&E staining protocol. Here, de-waxed sections were raised in dH_2O stained in haematoxylin for 4 minutes, in 0.3% acid alcohol for 30 seconds, tap water for 5 minutes and eosin for 2 minutes with intermittent 5-minute tap water rinses between each step. Finally Sections were then dehydrated rapidly in alcohols, cleared and mounted in DPX. Van Geison staining was used to quantify the +extent of fibrotic change. After de-waxing sections were stained with Celestin Blue for 5 minutes, rinsed in dH_2O and stained in Mayer's haematoxylin for 5 minutes. Sections were washed well in running tap water for 5 minutes and flooded with Van Gieson stain for 5 minutes. Sections were then dehydrated and mounted as before. All reagents were sourced from Leica Biosystems and slides were analysed using bright field microscopy. 9 images of each section were taken, with emphasis on bronchovascular bundles wherever possible. Images were subsequently analysed using Image J software (see section 2.5).

2.4 Immunofluorescence

Anti-CD248 antibody (Abcam) was used to determine CD248 expression in WT and ApoE$^{-/-}$ lung tissue sections. Formalin-fixed paraffin embedded tissue sections were de-waxed and re-hydrated. Antigen retrieval was performed using a microwave and EDTA buffer. Sections were blocked with 10% casein for 30 minutes followed by 10% goat serum for 30 minutes, and incubated with anti-CD248 antibody overnight at 4^{o}C. Sections were then washed for 5 minutes in Tris-buffered saline (TBS) 3 times before incubation with a FITC-conjugated secondary antibody (eBioscience) for 1h. Sections were washed again as before, stained with DAPI, and mounted in prolong gold (Invitrogen). All antibodies were used according to manufacturers instructions. For negative controls, the primary antibody was omitted and TBS used in its place. Images were captured by confocal microscopy using an inverted Zeiss LSM-510 UV confocal microscope at x10 magnification.

2.5 Data handling and statistical analysis

Nuclei in H&E stained sections and collagen in Van Gieson stained sections were quantified using Image J software via use of the colour threshold tool. This quantified the percentage of the total area stained for nuclei or collagen, indicating % disease burden or % fibrotic area. Statistical significance was determined using GraphPad Prism version 5.0 by performing a one-way analysis of variance (ANOVA). The mean ± the standard error of the mean (SEM) was calculated from data obtained and P=<0.05 was considered to be statistically significant.

3. RESULTS

3.1 High fat diets induce lung inflammation in WT and CD248$^{-/-}$ mice

The main histological feature of sarcoidosis is the formation of granulomas. H&E staining was used to examine the prevalence of inflammatory cells in tissue sections from the lungs of WT or KO mice on a normal chow-fed diet or HFD.

H&E staining revealed normal lung histology in chow-fed WT and CD248$^{-/-}$ mice (Figure 3a & 3c). Bronchovascular bundles and alveolar structures were normal in appearance and size in both mouse strains. They occurred at similar frequency in both WT and KO animals and no significant accumulations of inflammatory cells were present in either genotype on normal chow. In contrast, small aggregates of cells with similar morphology to granulomas were observed in a number of tissue sections from WT and CD248$^{-/-}$ mice on HFD (Figure 3b & 3d). These were discrete and localised in proximity to the vessels within the lungs. No other gross morphological features were associated with consumption of HFD.

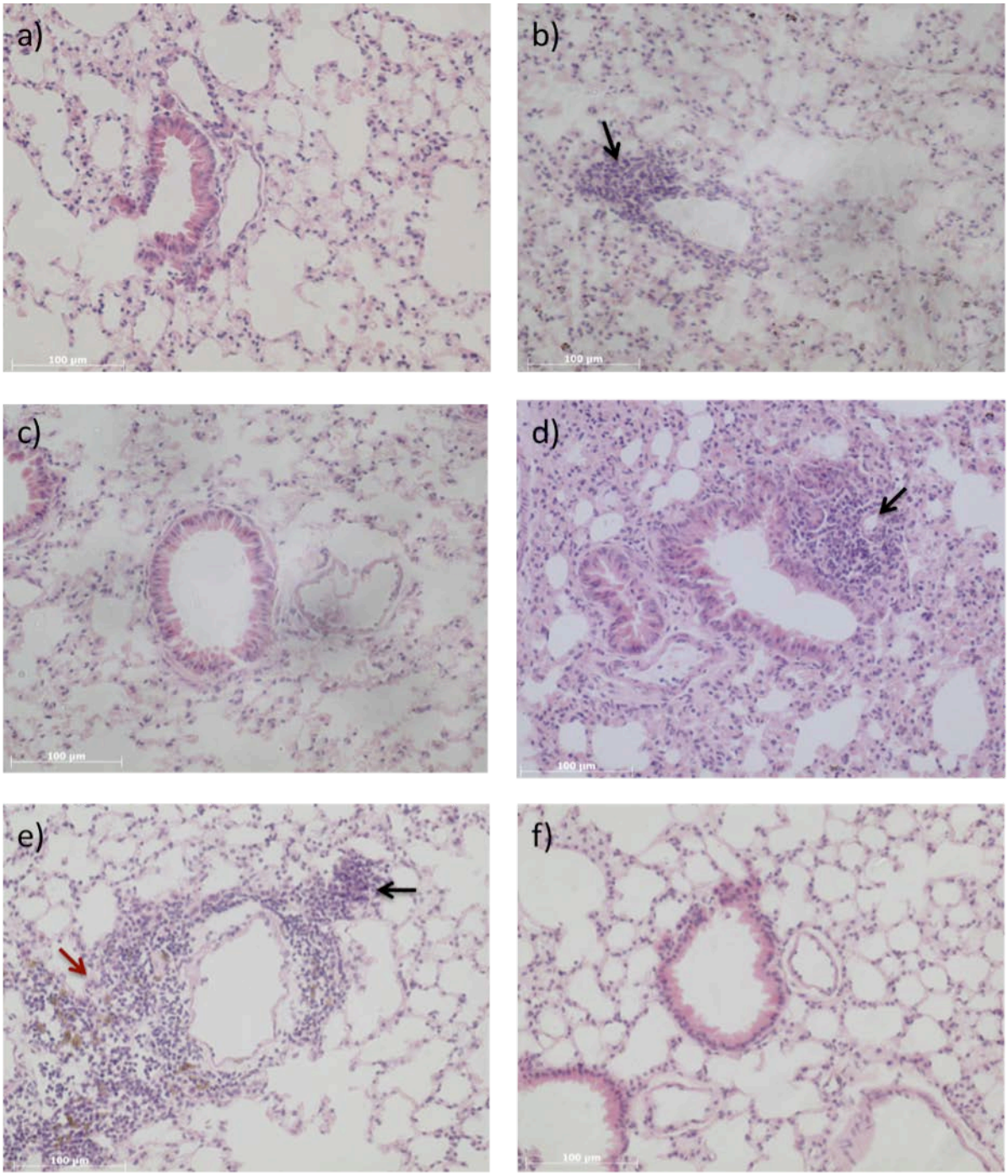


Figure 3. High fat diets induce inflammation in WT and CD248$^{-/-}$ mice. Representative images of formalin-fixed murine lung tissue stained with haematoxylin and eosin (H&E) using standard protocols. Sections from 10-15 animals per group were analysed (see Table 1). (a) WT on chow, (b) WT on HFD, (c) CD248$^{-/-}$ on chow, (d) CD248$^{-/-}$ on HFD, (e) ApoE$^{-/-}$ on HFD, and (f) DKO on HFD. Bright field microscopy was used at x20 original magnification and bar represents 100µm. Black arrows represent granuloma formation, while red arrow indicates infiltration by inflammatory cells.

3.2 Inflammatory lung injury is exacerbated in ApoE$^{-/-}$ mice, and CD248 deficiency reduces this response

On examination of tissue sections from Apo E$^{-/-}$ mice on HFD, large aggregates of cells with the morphology of inflammatory cells were observed reminiscent of sarcoid granulomas (Figure 3e). These aggregates appeared at a much higher frequency and density than those seen in the lungs of WT and CD248$^{-/-}$ mice on HFD. Additionally we noted occasional brown pigmentation in ApoE$^{-/-}$ mice on HFD, possibly representing hemosiderin uptake by macrophages. Interestingly, DKO mice exhibited very few, if any, such lesions and these lungs appeared histologically similar to chow-fed WT or CD248$^{-/-}$ mice (Figure 3f). It is notable that in all animals the majority of cell aggregates were located around bronchovascular bundles, as seen in figures 3b, 3d & 3e.

Images were then analysed using the threshold tool on Image J software to quantify the extent of granuloma formation (Figure 4). Where granulomas persist, an increase in haematoxylin-stained nuclei will be seen representing an increase in infiltrating inflammatory cells, therefore resulting in a higher % threshold. Although a small number of granulomas were observed in WT HFD mice, this was not statistically significant compared to WT mice on chow. No significant difference was seen between CD248$^{-/-}$ and WT mice on chow. CD248$^{-/-}$ mice on HFD had significantly increased numbers of lesions compared to WT mice on chow ($P=<0.005$). Disease burden was further increased in ApoE$^{-/-}$ mice on HFD compared to chow-fed WT ($P=<0.005$). Importantly the increase of disease burden in ApoE$^{-/-}$ mice on HFD was not apparent in DKO animals where disease burden

was not statistically significant from WT mice on chow.

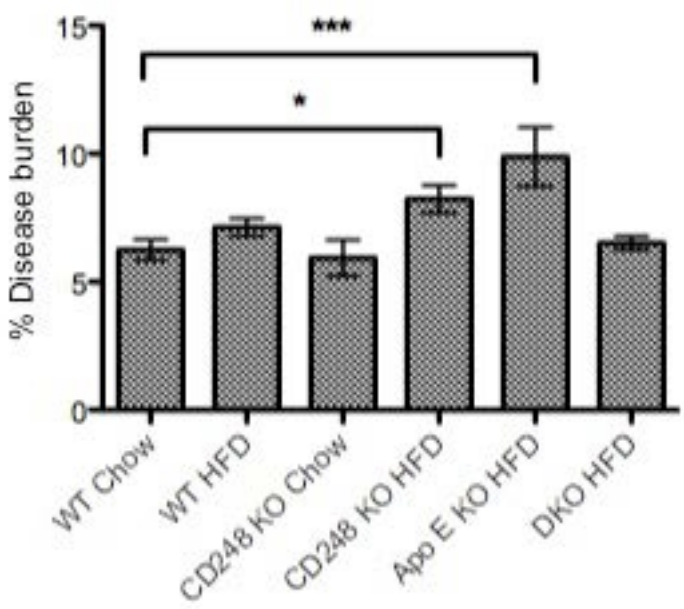


Figure 4. Percentage disease burden in histological images from mice on chow or HFD. Sections from 10-15 animals per group were imaged (See Table 1). WT, CD248$^{-/-}$ (CD248 KO), ApoE$^{-/-}$ (ApoE DKO) and double knock out (DKO) mice on chow-fed diets or HFD were stained with H&E, imaged using bright field microscopy and the percentage area of stained nuclei was analysed using Image J threshold analysis. Data represents mean ± SEM of 9 images from each mouse sample. ANOVA was used to compare all groups to WT chow. ***=P<0.0005, *=P<0.05

3.3 Assessment of lung fibrosis using Van Gieson staining suggests that CD248 is involved in lung fibrogenesis

In chronic inflammatory diseases such as sarcoidosis, fibrosis can occur involving the deposition of extracellular matrix proteins, including collagen, by activated fibroblasts. Here, Van Gieson staining was used to identify collagen in our lung sections. As expected, WT mice on chow-fed diets had no abnormal collagen staining, and appeared histologically similar to CD248$^{-/-}$ mice on chow (Figure 5a & 5c). In contrast large collagen deposits were noted in tissue samples from WT (Figure 5b) and ApoE$^{-/-}$ mice on HFD (Figure 5e). Interestingly CD248$^{-/-}$ mice on HFD presented with a lower degree of staining (Figure 5d). Unexpectedly, DKO mice on HFD exhibited the most intense collagen staining representing prominent fibrotic lesions (Figure 5f). In all mice fed HFD, fibrosis persisted mainly around bronchovascular bundles, similar to the granuloma distribution observed in H&E-stained tissue sections.

Again, Image J thresholding was used to quantify the extent of fibrosis in histological images (Figure 6). Where fibrotic lesions occur, intense pink staining will be seen demonstrating collagen. Therefore, the more pink-stained collagen, the higher the % threshold will be, representing the % fibrotic area. Collagen staining in tissue sections from WT and CD248$^{-/-}$ chow-fed mice was similarly low in both groups. Compared to WT mice on chow, the % fibrotic area was higher in WT (79.05% increase), CD248$^{-/-}$ (36.12% increase) and ApoE$^{-/-}$ mice on HFD (68.84% increase). We confirmed that levels of fibrosis were highest in DKO animals on HFD, and this change was statistically significant compared to animals on WT Chow

(P=<0.005). The fibrotic area in these animals increased by 176.45%, which corresponds well with the microscopic analysis.

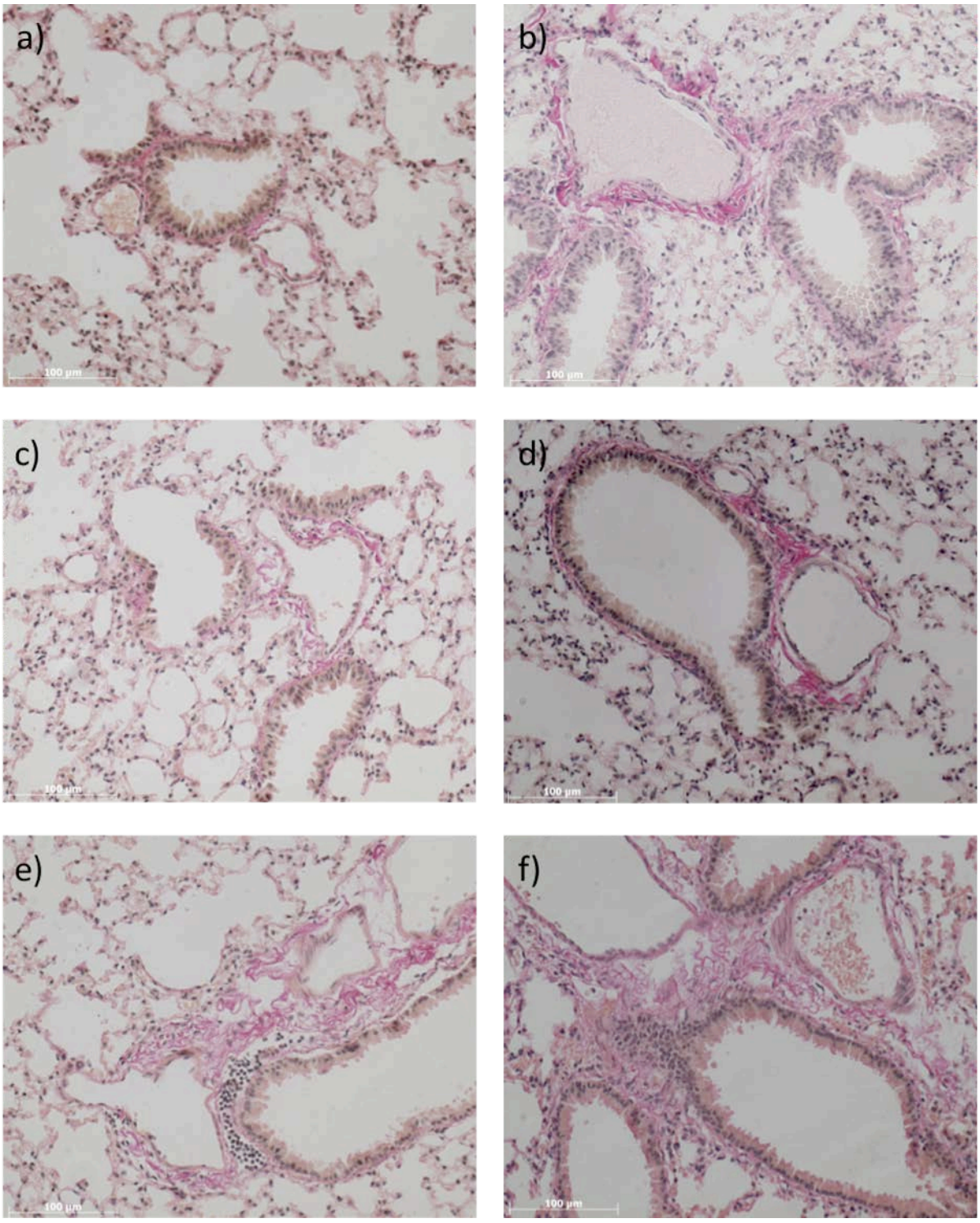


Figure 5. Assessment of lung fibrosis using Van Gieson staining suggests the involvement of CD248 in lung fibrogenesis. Representative images of formalin-fixed murine lung tissue sections stained with Van Gieson reagents using standard protocols. Sections from between 10 and 15 animals were anaylsed (See Table 1). (a) WT on chow, (b) WT on HFD, (c) $CD248^{-/-}$ on chow, (d) $CD248^{-/-}$ on HFD, (e) $ApoE^{-/-}$ on HFD, and (f) DKO on HFD. Pink staining localises to areas of collagen deposition. Images were photographed by bright field microscopy at x20 magnification and analysed by Image J threshold analysis.

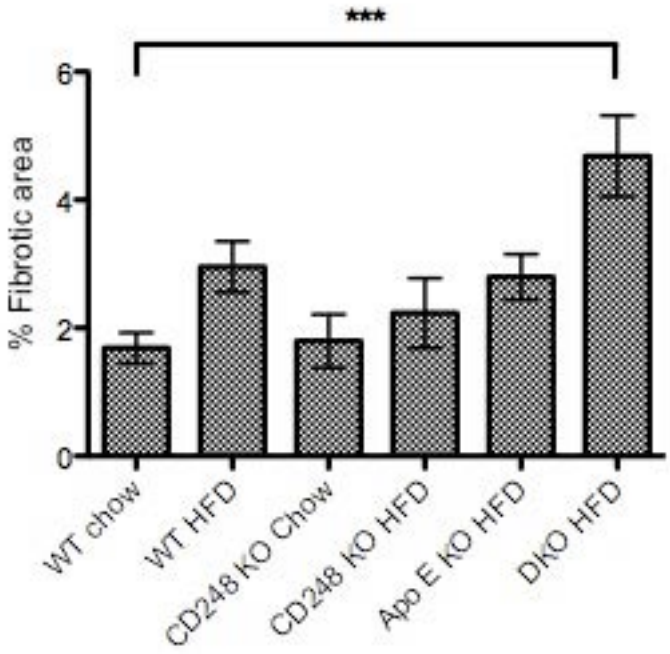
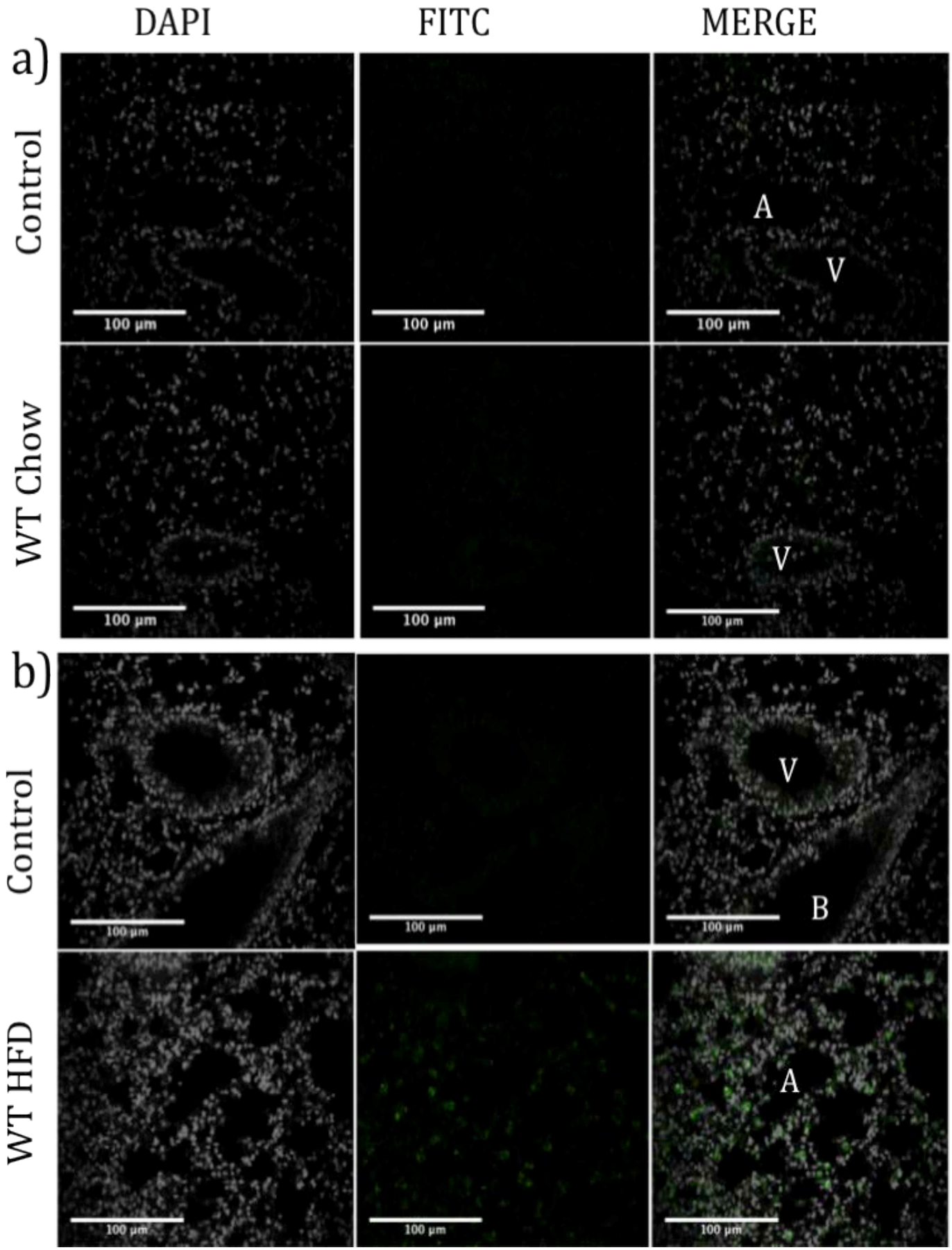


Figure 6. Percentage fibrotic area in histological images from mice on Chow or HFD. Sections from between 10 to 15 WT, CD248$^{-/-}$ (CD248 KO), Apo E$^{-/-}$ (Apo E KO) and double knock out (DKO) mice on chow-fed diet or HFD (see table 1) were Van Gieson-stained, imaged by bright field microscopy at x20 magnification, and the percentage area of stained collagen was analysed using Image J. Data represents mean ± SEM from 9 images per mouse sample. ANOVA performed on all groups compared to WT mice on chow revealed a significant difference between DKO mice on HFD and WT mice on chow (***=P<0.0005).

3.4 Assessment of pulmonary distribution of CD248 using immunofluoresent staining

Following the observation that fewer inflammatory infiltrates occurred in DKO mice on HFD, yet extensive fibrosis was evident in these animals, the distribution of CD248 was investigated in WT and ApoE$^{-/-}$ mice by immunofluorescence (Figure 7). Little immunofluorescent staining was visible in WT mice on chow, except for a minor quantity around a vessel (Figure 7a). More abundant staining was evident in sections from WT mice on HFD, and appeared to be evenly distributed throughout the tissue. Of note, some autofluorescence or non-specific staining was seen around vessels in controls (Figure 7b). Strong staining was observed in tissue sections from ApoE$^{-/-}$ mice on HFD, and followed a similar distribution to that seen in WT mice on HFD, however staining was much more prominent in sections from ApoE$^{-/-}$ mice on HFD (Figure 7c).


DAPI
FITC
MERGE
a)
Control
WT Chow
b)
Control
WT HFD
A
V
B
100 µm

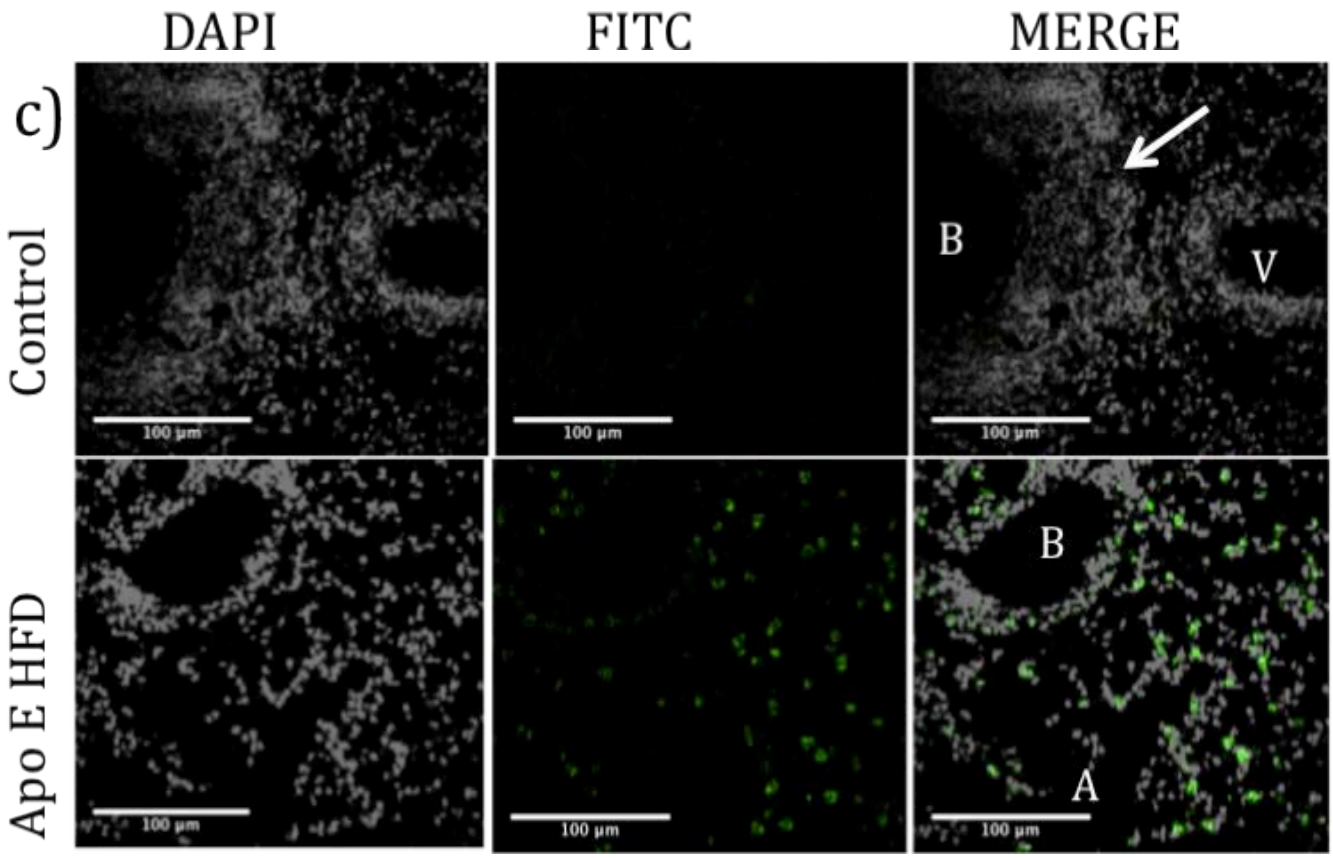


Figure 7. Assessment of pulmonary distribution of CD248 using immunofluorescent staining. Representative immunofluorescent images of formalin-fixed murine lung tissue sections. Samples from 5 WT Chow (a), WT HFD (b), and ApoE$^{-/-}$ HFD (c) mice were stained with anti-CD248 antibody followed by a FITC-conjugated secondary and analysed by confocal microscopy at x10 magnification. Grey staining represents nuclear counterstain (DAPI). Controls for each genotype were created by omitting the primary antibody, and the same acquisition settings were re-used when imaging all sections. Arrow indicates aggregate, V = vessels, B = bronchioles, A= alveolar space. White bar represents 100µm.

4. DISCUSSION

Granuloma formation occurs as a normal immunological mechanism to confine potential infectious agents identified by their immunostimulatory antigens. This protective process can become dysregulated in chronic immune responses, leading to pulmonary fibrosis, a key predisposing factor to death in sarcoidosis patients [reviewed in Baughman & Lower 2011]. Samohkin and co-workers recently described a mouse model of sarcoidosis [Samohkin *et. al* 2010], providing a novel means to study sarcoid granuloma formation and associated pathological features such as fibrosis. In this study, the role of CD248 in a similar model of sarcoidosis was investigated.

4.1 HFD is sufficient to induce sarcoid-like lung lesions in ApoE$^{-/-}$ but not DKO mice

In order to investigate the effects of CD248 in sarcoidosis, a mouse model similar to that previously developed by Samohkin and co-workers [Samohkin *et. al* 2010] was used. The model used in this investigation did not involve supplementation with cholate in the diet but we have demonstrated that HFD alone is sufficient to induce sarcoid-like granuloma formation in ApoE$^{-/-}$ mice.

The disease burden representing granuloma formation in CD248$^{-/-}$ mice on chow was not statistically different from WT mice on the same diet and lung histology was similar. This suggests that CD248 deficiency in mice on a standard diet does not appear to affect normal lung parenchyma, and may relate to our

observations that there is minimal expression of CD248 in WT mice on chow diet (Figure 7a). However, HFD was sufficient to induce the formation of granulomas in the lungs of WT, CD248$^{-/-}$ and ApoE$^{-/-}$ mice. This is of interest as Samohkin *et al.* 2010, did not see granuloma formation in WT mice on HFD without cholate supplementation. Cholate has been shown to induce inflammation in mice through the induction of pro-inflammatory genes [Quin *et al.* 2005]. This discrepancy could be due to the fact that the mice in our investigation remained on HFD for 13 weeks starting at 10 weeks of age, whereas the mice in the study by Samohkin and colleagues had HFD for 16 weeks but started the diet younger (6 weeks). Absence of requirement for cholate supplementation in our study to induce granuloma formation likely reflects genetic differences influencing the immune system between WT DBA1/J mice on HFD used in the investigation by Samohkin *et al.,* compared to the WT C57/BL6 background used in this study.

HFD induces inflammation in many organs including the colon and brain [Ding *et al.* 2010, Erdelyi *et al.* 2009, Pistell *et al.* 2010], and absence of granulomas in our chow-fed mice suggest it also contributes to pulmonary granuloma formation. Similarly our CD248$^{-/-}$ mice on HFD presented with more inflammatory aggregates than chow-fed WT mice, demonstrating that deficiency of this gene does not inhibit granuloma formation in the HFD mouse model.

ApoE$^{-/-}$ mice on HFD exhibited more extensive granuloma formation with occasional brown pigmentation near vessels potentially as a result of hemosiderin uptake by macrophages due to local haemorrhage, caused by severe inflammation (Figure 3e). ApoE$^{-/-}$ mice on HFD are used as a model to study inflammatory diseases, such as atherosclerosis [reviewed in Hofker *et al.* 1998], where by disease is accelerated by the inflammatory environment. Therefore the

exacerbating effects of ApoE$^{-/-}$ on granuloma formation are also possibly due to the inflammatory state seen with this phenotype on HFD. ApoE has been known to play many roles in immunity, including inhibition of lymphocyte proliferation [Laskowitz *et al.* 2000]. A lack of such immunosuppressive properties may contribute to granuloma formation, through the exaggeration of ordinary inflammatory responses, which under normal circumstances are protective and controlled mechanisms. Levels of circulating lipids are elevated in ApoE$^{-/-}$ mice [Moghadasian *et al.* 2001] which may make exacerbate inflammation itself, for example hyperlipidaemia is known to contribute to the inflammatory process which initiates and accelerates the chronic inflammatory disease atherosclerosis [reviewed in Steinberg 2002].

Unlike our study, in the study by Samohkin and co-workers, no granulomas presented in ApoE$^{-/-}$ mice after 16 weeks on HFD without cholate supplementation in the diet. This lack of granuloma formation may be due to the substantial difference in time on HFD compared to our study (10 week difference), with resolution of granulomas in ApoE$^{-/-}$ mice possibly occurring in the remaining 6 weeks of feeding HFD.

Surprisingly, DKO mice on HFD demonstrated reduced granuloma formation. The phenotype seen in ApoE$^{-/-}$ mice on HFD appeared alleviated in DKO mice and was phenotypical of WT Chow. This suggests that CD248 may contribute to the formation of granulomas in some way. Although the role of CD248 in immunity is poorly understood, it has been shown to promote inflammation [Maia *et. al* 2010] and there is a strong link between its expression and tissue remodelling/repair, possibly providing an explanation for why its deficiency appears to alleviate granuloma formation.

4.2 Absence of CD248 influences the severity of fibrosis in sarcoidosis.

Following the confirmation of granuloma formation in HFD animals, the extent of fibrosis in these animals was investigated. Fibrosis occurs as a result of chronic inflammation and can act as a means of resolution or can itself become chronic and dysregulated, driving further injury. Thus assessment of fibrosis may provide an indication of disease severity.

As expected, no abnormal Van Gieson staining was seen in WT and CD248$^{-/-}$ mice on chow fed diets and collagen deposition in CD248$^{-/-}$ mice was not statistically significant from WT mice, indicating the absence of fibrotic lesions. This correlates with the data seen in our H&E analysis, where few, if any granulomas were present. Low levels of collagen staining were evident in WT and CD248$^{-/-}$ mice on HFD, but were not statistically significant compared to chow-fed WT mice, and most likely corresponded to the granuloma formation seen in Figures 3 & 4. In contrast, large deposits of collagen were observed in ApoE$^{-/-}$ mice on HFD representing extensive fibrosis. This result was expected due to the size and complexity of inflammatory aggregates suggesting a state of chronic inflammation. Such chronic inflammatory events can commonly lead to the development of fibrosis due to cytokines in the environment released by immune cells, for example macrophage production of TGF-β, activating fibroblasts and stimulating collagen production.

Samohkin and co-workers did not find granulomas in ApoE$^{-/-}$ mice on HFD but did observe fibrosis. This was noted to be comparable to levels seen in ApoE$^{-/-}$ mice on cholate-supplemeted HFD after just 8 weeks [Samohkin *et al.* 2010]. It is

likely that the inflammatory properties of cholate previously described are exacerbating inflammation and thus, the formation of fibrosis. This data also supports our suggestion that granulomas may have occurred at an earlier time point, but all which remains is evidence of resolution and remodelling post-injury.

DKO mice on HFD exhibited the most prominent fibrosis, which was highly statistically significant compared to WT animals on chow. This was a very unexpected result, as almost no granulomas were observed on analysis of H&E-stained sections. Although the H&E analysis suggested that no disease was evident, analysis of Van Gieson-stained tissue sections suggested otherwise. Fibrosis occurs as a result of injury or inflammation, where a preceding inflammatory response is followed by the development of fibrotic lesions. Deletion of CD248 may have resulted in acceleration of this fibrotic process, or increased speed of resolution of granulomas, as opposed to prevention of disease. CD248 has been shown to interact with many extracellular matrix proteins [Tomkowicz *et al.* 2007], which may mediate interactions involved in normal inflammation between fibroblasts and immune cells. Our results suggest that the degree of fibrosis varies depending on granuloma severity in mice on HFD, but is affected by deficiency of CD248. Here, lack of CD248 may result in insufficient communication with immune cells, leading to immune cell dysfunction.

4.3 CD248 is not expressed in the lungs of chow-fed WT mice, but is up-regulated in sarcoidosis.

Granuloma formation was not observed in H&E data from DKO mice, however widespread fibrosis was evident following further investigation by Van Gieson-staining. The involvement of CD248 in the lung was therefore investigated by examining its distribution using anti-CD248 antibody.

Immunofluorescent staining for CD248 suggested that this surface protein is not normally expressed in the lungs of WT mice on chow-fed diets. Christian and co-workers describe little or no expression of CD248 in many organs in the adult [Christian *et. al* 2008], supporting this observation. CD248 expression was however observed in WT mice on HFD. Since HFD is pro-inflammatory and induced granuloma formation, this suggests increased CD248 expression due to inflammatory activation. This is supported by Lax and co-workers, who found increased CD248 expression in inflamed spleen [Lax *et al.* 2007]. Since the role of CD248 is concerned with fibroblast remodelling/repair of tissues, upregulation in the context of injury seems feasible.

A minor quantity of staining was seen around a vessel in Figure 7a. Some autofluorescence or non-specific staining for the secondary antibody was seen around a vessel in the control section for Figure 7b, suggesting that the small amount of staining seen in a) was most likely non-specific.

Our results implied that CD248 expression is increased by ApoE deficiency. CD248 staining appeared stronger in chronic inflammation in the $ApoE^{-/-}$ model of sarcoidosis, suggesting expression of even higher levels of CD248 by cells, most likely fibroblasts, than in WT mice on HFD. Further supporting this, the expression

of CD248 correlates with the level of disease burden (and therefore inflammation) seen in WT mice on chow, WT mice on HFD and ApoE$^{-/-}$ mice on HFD. Thus where very few, if any inflammatory aggregates were present in tissue sections, low levels of CD248 were observed, and where more severe inflammation was present, expression was increased. This correlates with reported expression patterns associated with severity of sarcomas [Bagley *et al.* 2008] and chronic kidney disease [Smith *et al.* 2011]. Interestingly the fact that the distribution of CD248 expression in inflamed (WT mice on HFD and ApoE$^{-/-}$ mice on HFD) tissues was similar, suggests that it is probably the same cells in both models which express CD248, and that either cell number or degree of expression per cell increases with disease severity.

4.4 Conclusion & Future implications

Granulomas form as a result of inflammation, and are often the result of treatable pathogens. The cause of sarcoidosis however remains an enigma. Our results and others [Samhokin *et al.* 2010], demonstrate that ApoE deficiency and HFD exacerbates inflammation, being sufficient to cause sarcoid-like lesions. However, we have shown that cholate in the diet is not required for this. We have also demonstrated that CD248 is involved in the pathology of sarcoidosis, influencing the severity of disease, as demonstrated in other chronic inflammatory diseases [Maia *et al.* 2010], although the cause of this effect is not clear. In our model, H&E analysis suggested that CD248 prevented granuloma formation, however the formation of fibrosis strongly suggests otherwise. Therefore, CD248 is most likely to be increasing the speed of disease progression, possibly through dysregulation of immune cells, promoting resolution. At this stage, it is not possible to determine the extent to which CD248 deletion increases the speed of granuloma formation, or the scope of fibrosis. In order to do this, different time courses would need to be analysed, ideally alongside lung function tests to confirm that granuloma formation and fibrosis correlate with *in vivo* observations of decreased lung function.

Despite achieving our aim to explore the role of CD248 in a mouse model of sarcoidosis, limitations of this study included a lack of control $ApoE^{-/-}$ and DKO mice on chow due to time restraints. This prevented the confirmation of observations from these genotypes. In addition to this, groups did not contain equal numbers of mice, possibly introducing statistical bias in smaller groups.

The role of CD248 in inflammation and fibrosis could be further investigated though CD248 deficiency in other mouse models of inflammation, and collection of

brochoalveolar lavarge fluid (BAL) from these animals to examine the present cytokine profile. The cellular makeup of granulomas could be identified through immunoflourescent staining to confirm their similarity to those found in sarcoidosis, and use of an isotype-matched control antibody could exclude non-specific staining of anti-CD248 antibody. Co-localisation of CD248 with specific markers of CD248-expressing cells such as fibroblasts, could uncover which cells were expressing CD248 in the lung. Subsequent real-time PCR could be used to quantify and compare CD248 expression in these cells in animals on normal chow diet or HFD.

The development of new models of CD248-deficient chronic inflammatory diseases will aid our comprehension of the pathological processes involved in granuloma formation and fibrosis. Such models may elaborate our understanding of the involvement of CD248 in disease and resolution, possibly leading to novel therapeutic targets/strategies in the future.

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PROJECT 2:

The Role of adipose tissue-derived stromal cells in the survival of lymphocytes

This project is submitted in partial fulfilment of the requirements for the award of the MRes

College of Medical and Dental Sciences

University of Birmingham

ABSTRACT

It is widely acknowledged that stromal cells play a key role in the homeostasis of lymphocytes in the lymph node (LN). Recent findings showed that LN stromal cells are derived from adipocyte precursor cells residing in the fat pad surrounding the LN. Interestingly, the adipose tissue is rich in lymphocytes but factors regulating their recruitment and retention are unknown. We thus assessed whether adipose tissue-derived stromal cells (ASCs) could play a role in the survival of lymphocytes in the adipose tissue.

To investigate the relationship between lymphocyte survival and ASCs, lymphocyte survival under different culture conditions with stromal cells was examined using flow cytometry, and expression of important lymphotrophic factors such as interleukin-7 (IL-7) and chemokine (C-C motif) ligand 19 (CCL19) was investigated with real-time PCR (RT-PCR).

Our findings suggest that pre-adipocytes in the stromal vascular fraction (SVF) of adipose tissue produce IL-7 and CCL19 contributing to T lymphocyte survival *in vitro*, however a cocktail of survival factors is likely to be involved *in vivo*.

The capacity of pre-adipocytes to modulate T cell survival is an important finding for adipose tissue biology. Although the reason for this physiological occurrence is unknown, a better understanding of how adipose tissue influences immune responses may lead to the development of novel anti-inflammatory therapies in cell-mediated diseases and obesity.

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1. INTRODUCTION

1.1 Structure and Function of Lymph Nodes

Secondary lymphoid organs (SLOs), such as lymph nodes (LNs), play a key role in the initiation of adaptive immunity. They are positioned along the lymphatic vessels at specific sites of the body. The lymphatic vasculature allows the drainage of small molecules, immune cells such as antigen-bearing dendritic cells (DCs), potential pathogens and antigens from surrounding tissues and organs via lymphatic vessels into the LNs. Recirculating lymphocytes enter the LN from the blood by extravasation at post-capillary venules known as high endothelial venules (HEVs) [reviewed in Willard Mack 2006]. The highly specialised structure of the LN enables antigen presentation, recognition, and the induction of appropriate immune responses. Communication between antigen presenting cells (APCs), T cells and B cells is crucial for the onset of an adaptive immune response.

The LNs consist of distinct regions within a surrounding capsule (Figure 1), with a highly specialised stromal architecture. The stroma is a supportive network of tissue comprised of stromal cells originally found to provide structural support to tissues and organs. However, these cells have since been shown to play additional roles in immunity. The importance of stromal cells in the development of the LN and other lymphoid organs has been recognised [Mebius 2003, Cupedo & Mebius 2004], along with their many other functions, including their ability to secrete homeostatic factors influencing the lymphocyte pool. So far, five stromal cell populations have been identified in LNs; $Gp38^{+}$ $CD31^{-}$ fibroblastic reticular cells (FRCs), $Gp38^{+}$ $CD35^{+}$ follicular dendritic cells (FDCs), $Gp38^{-}$ $CD31^{+}$ blood endothelial cells (BECs), $Gp38^{+}$ $CD31^{+}$ lymphatic endothelial cells (LECs) and

RANKL^{+} (Receptor activator of nuclear factor kappa-B ligand) marginal reticular cells (MRCs) [Link *et al.* 2007, Mueller & Germain 2009]. Lymphoid tissue stromal cells play an important part in the orchestration of immune responses, forming an intricate 3D meshwork and supporting maximal immune cell interactions through organisation of lymphocytes in the LN [reviewed in Mueller & Germain 2009, and Roozendaal & Mebius 2011], ensuring correct locations for interaction with their cognate antigen. The regions of the LN each perform individual functions, ultimately working together to provide an optimal microenvironment for lymphocytes to encounter and respond to antigens where necessary.

1.1.1 The Subcapsular Sinus

The subcapsular sinus (SCS), as it's name suggests, is located directly below the LN capsule and is the site at which lymph is delivered to the LN by afferent lymphatic vessels, and exits via efferent lymphatic vessels on the opposing side [reviewed in Mueller & Germain 2009]. Macrophages reside in the SCS, sampling antigen as it passes though in the lymph [Mueller & Germain 2009].

Also associated in this zone are a stromal cell population known as marginal reticular cells (MRCs), which form a layer beneath the SCS and express common stromal markers [Mueller & Germain 2009]. It is hypothesised that these cells represent a remaining population of lymphoid tissue organiser cells (LTo) from LN development [Katakai *et al.* 2008]. However, little is known about their true functionality.

1.1.2 The Cortex

Beneath the SCS is the cortex, which is composed mainly of B lymphocytes organised into primary follicles. These primary follicles become secondary follicles following antigen stimulation, and germinal centres are formed within, representing areas of high B cell proliferation [reviewed in Willard-Mack 2006, and Mueller & Germain 2009].

Within the cortex resides the stromal cell subset of follicular dendritic cells (FDCs) which interact with B cells and form a supportive network within B cell follicles in which B cells can locate their cognate antigen. To facilitate this, FDCs express C-X-C motif chemokine 13 (CXCL13), a B cell chemoattractant, and high levels of cell adhesion molecules (CAMs) such as vascular cell adhesion molecule-1 (VCAM-1) and mucosal addressin cell adhesion molecule 1 (MAdCAM1) [reviewed in Mueller & Germain 2009]**.**

1.1.3 The Paracortex

The paracortex is located between the cortex and medulla, and is populated predominantly by T cells and DCs [Mueller & Germain 2009]. It is here that T cells look for their cognate antigen presented by DCs and proliferate in response to it.

Fibroblastic reticular cells (FRCs) can be located in the paracortex, and it is these stromal cells which surround HEVs. They are the main source of interleukin-7 (IL-7) in the LN and also secrete the chemokine CCL19 (chemokine (C-C motif) ligand 19) [Link *et al.* 2007, Mueller & Ahmed 2008], two key mediators of naïve T lymphocyte survival and homeostasis of the T cell population within the paracortex.

1.1.4 The Medulla

Finally, the innermost region of the LN is the medulla, an area consisting mainly of plasma cells. Plasma cell precursors known as immunoblasts mature in the medulla, having migrated from germinal centres (GCs) in the cortex, and secrete antibodies into the lymph [Willard-Mack 2006].

Medullary fibroblasts constitute the stromal network alongside a scaffold of reticular fibres that support the structure of the LN [Mueller & Germain 2009].

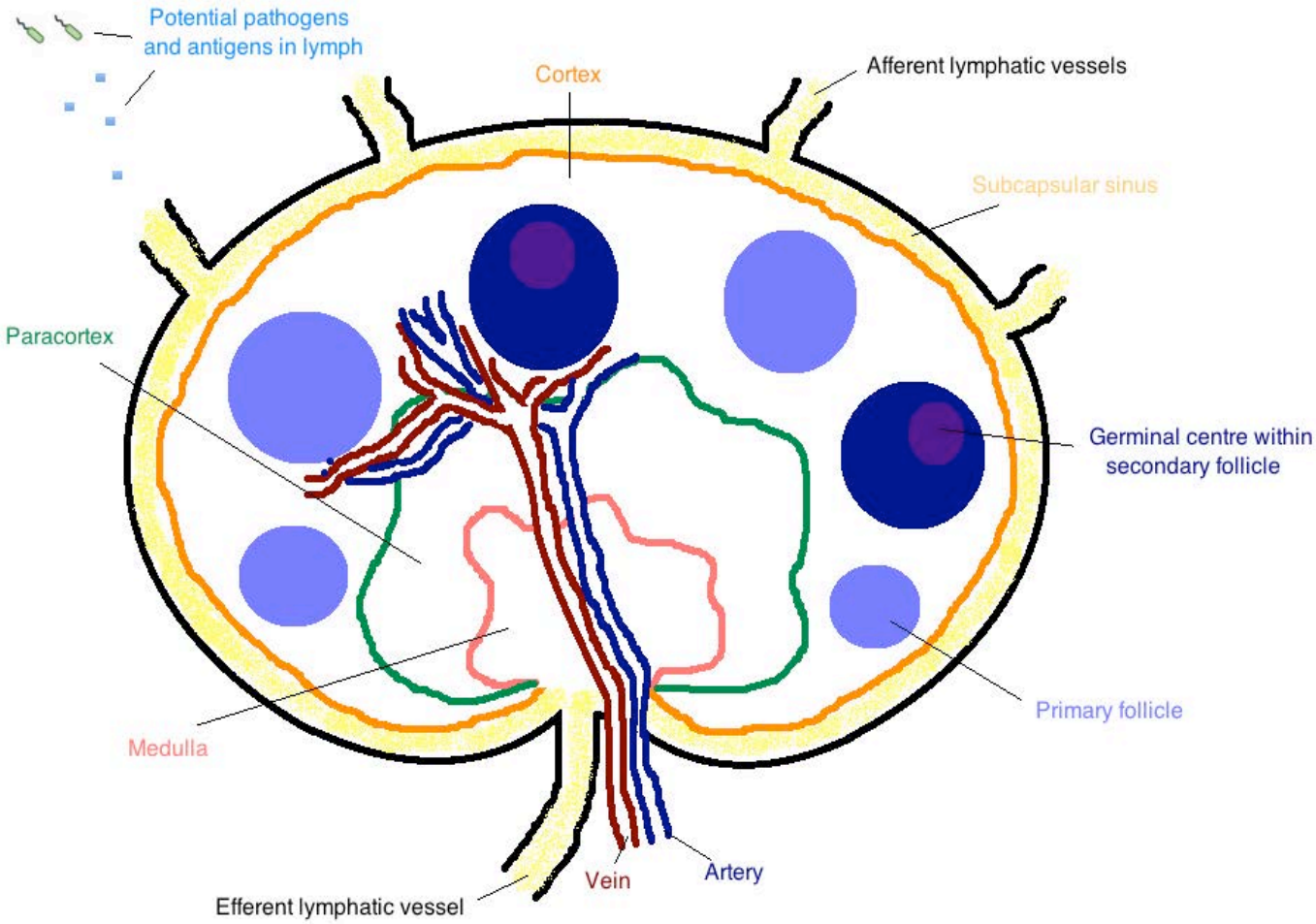

Figure 1. **The structure of lymph nodes.** Antigen enters LN via lymph in afferent lymphatics or migrating APCs in blood via HEVs. Antigen is processed and presented in the context of major histocompatibility complex (MHC) class II in cortex and paracortex. Communication between T cells, B cells and APCs leads to migration and proliferation of lymphocytes in the paracortex (T cells) or primary follicles in cortex to give rise to germinal centres (B cells). Activated B cells migrate to the medulla, where they mature into plasma cells and secrete antibodies into the lymph, which exits via the efferent lymphatic vessel to re-circulate the body. ***Image created in Paintbrush.***

1.2 Lymph Node Development

LN organogenesis occurs in the embryo and crosstalk interactions between lymphoid tissue inducer (LTi) cells and stromal organiser cells known as lymphoid tissue organisers (LTo) cells, is essential for the later stages of formation of these organs [White *et al.* 2007].

LTi cells express the TNF ligand family member Lymphotoxin (LT), and are found early on in LN organogenesis. Binding of LT to its Lymphotoxin Beta receptor (LTβR) expressed by stromal organiser cells activates the classical and alternative nuclear factor kappa-B (NF-κB) signalling pathways, resulting in the up-regulation of important CAMs, cytokines and chemokines [briefly reviewed in Cupedo & Mebius 2004]. It is these factors, which ultimately direct the formation of LNs, through the attraction, retainment, and organisation of haemopoietic cells within the developing LN anlagen.

LTβR signalling is essential for LN organogenesis as shown by the absence of these organs in mice deficient in this receptor or its downstream signalling molecules. A recent report has shown that stimulation through LTβR and the alternative (NF-κB2/RelB) pathway is required for full maturation of stromal cells to become “organizer” cells that express high levels of VCAM-1, intercellular adhesion molecule-1 (ICAM-1) and MAdCAM-1 and cytokines including IL-7 [Bénézech *et al.* 2010].

IL-7 can upregulate LT expression, and is involved in the regulation of the LTi cell pool, essential for LN formation, and the importance of this cytokine is demonstrated by the fact that IL-7 deficient mice have defective development of peripheral LNs [reviewed in Huang & Luther 2012]. Therefore, the expression of

genes induced though LTβR signalling leads to the recruitment of immune cells and the organisation of specific areas in the LN required for functional adaptive immunity.

1.3 The relationship between adipose tissue and lymph nodes

In past years, adipose tissue was originally regarded as a site of energy storage and release as triglycerides and fatty acids respectively, supplying cells with provisions for growth. Like stromal cells, subsequent research has uncovered the function of adipose tissue to be more elaborate than initially thought. Now, adipose tissue is widely considered as an endocrine organ with involvement in immunity [reviewed in Schaffler *et al.* 2007].

The cellular constituents of adipose tissue include adipocytes, pre-adipocytes, fibroblasts, endothelial cells, and immune cells such as macrophages and lymphocytes [Schaffler & Buchler 2007, Cawthorn *et al.* 2012]. The stromal cell compartment of adipose tissue can be found in the stromal vascular fraction (SVF) following collagenase digest [Schaffler & Buchler 2007].

1.3.1 Immune Functions of adipose tissue

The most obvious function of adipose tissue in immunity is the provision of energy to support inflammatory reactions and precursors of mediators such as cytokines and chemokines. Adipose tissue is slowly emerging as having greater and more complex involvements in immunity, including interactions with immune cells such as $CD4^+$ T cells and macrophages through the expression of cytokines, adipokines, and cell surface proteins.

Tumor necrosis factor alpha (TNF-α) is produced primarily by adipocytes in adipose tissue and is a potent pro-inflammatory mediator, activating macrophages and other immune cells. Interleukin (IL-6) is also produced by adipocytes, and contributes to the pro-inflammatory response through its involvement in the proliferation and differentiation of lymphocytes, but can also be anti-inflammatory as it activates IL-10 expression [reviewed in Rondinone C. M. 2006]. IL-10 is an anti-inflammatory cytokine that downregulates the expression of pro-inflammatory mediators and inhibits signalling though pro-inflammatory pathways such as NF-κB. Leptin and Adiponectin are adipokines produced by adipose tissue. Leptin is able to influence T cell proliferation, activation and apoptosis as well as modulating macrophage activities through directing the increased release of MCSF (macrophage colony-stimulating factor) in adipose tissues [Rondinone C. M. 2006]. Adiponectin modulates cytokine expression by exerting anti-inflammatory effects through decreasing the production of TNF-α and IL-6, whilst increasing IL-10 release [Rondinone C. M. 2006].

The capacity of adipose tissues to produce inflammatory mediators is further demonstrated in obesity and related pathologies, where an excess of adipose tissue results in a low grade state of chronic inflammation [reviewed in Lee & Pratley 2005], and the fact that most LNs are surrounded by adipose tissue (for example the inguinal (iLN), mesenteric (mLN), and popliteal LNs [Pond 2003]), supports the concept of its association with immunity.

1.3.2 Pre-adipocytes

Pre-adipocytes are fibroblastic adipose tissue-derived stromal cells of mesenchymal origin, representing adipose precursor cells. These cells have been previously

identified as Sca1^{+} CD31^{-} CD45^{-} [Rodeheffer *et. al* 2008]. Mesenchymal stem cells (MSCs) are a pluripotent cell population with the capacity to differentiate into several different cell lineages including osteoblasts, chondrocytes, myocytes and adipocytes [reviewed in Schaffler & Buchler 2007]**.** In this study, adipose tissue-derived stromal cells will be abbreviated to ASCs, which resemble adipose tissue-derived stem cells [reviewed in Cawthorn *et al.* 2012]. ASCs are multipotent, and like MSCs, can also differentiate into osteoblasts, chondrocytes, myocytes and adipocytes. It has been suggested that ASCs are equivalent to bone marrow MSCs, and share cell surface markers [Cawthorn *et al.* 2012].

As mentioned, pre-adipocytes are able to differentiate into adipocytes, through a process called adipogenesis. During adipogenesis, MSCs differentiate into adipose precursors (pre-adipocytes), which are then able to give rise to adipocytes. This process of differentiation involves the expression of transcription factors including CCAAT/enhancer binding proteins (C/EBPs), peroxisome proliferator-activated receptor gamma (PPARγ), and their target genes such as adiponectin [discussed in Berger J. P. 2005].

In addition to adipocytes, pre-adipocytes are also able to express cytokines and chemokines. This includes factors which are involved in lymphocyte homeostasis, such as IL-7 and CCL19 [Meijer *et al.* 2011]. Gp38^{+} CD31^{-} CD45-stromal cells (FRCs) are the main source of IL-7 in the LN [Link *et al.* 2007, Mueller & Ahmed 2008], and pre-adipocytes in the SVF of adipose tissue are able to produce IL-7. Rodeheffer and co-workers identified pre-adipocytes through lack of CD31 and CD45 expression [Rodheffer *et al.* 2008], and adipocyte precursors have been identified in the Gp38^{+} CD31^{-} fraction of adipocyte derived SVF [Bénézech *et al.* 2012]. Therefore, we hypothesised that pre-adipocytes in the SVF of adipose

tissue could be sorted on the basis of Gp38 expression and lack of CD31 and CD45 expression, and may also have supportive or regulatory functions towards SLOs, similar to that of LN FRCs. Increasing evidence for this comes from Bénézech and co-workers, who demonstrated that a common progenitor cell with pre-adipocyte characteristics can give rise to LN stroma, and confirmed that pre-adipocytes have the capacity to migrate to LN and contribute to the different stromal cell subsets *in vivo* [Bénézech *et al.* 2012].

1.4 IL-7 and LTβR signalling

The pattern of gene expression by cells determines their function and allows them to respond to their environment accordingly. Signal transduction is central to the control of gene expression in all cells, and governs the means by which physiological processes occur. In order to comprehend such behaviour, it is first important to understand the key molecular mechanisms underlying them.

1.4.1 IL-7 signalling pathway

IL-7 is a 25 kDa protein belonging to the haematopoietic family of cytokines [Huang & Luther 2012]. Its expression by non-haematopoietic cells such as LN stromal cells is a critical factor in the proliferation and survival of lymphocytes, in particular T cells.

IL-7 is the ligand for the interleukin-7 receptor (IL-7R), a heterodimer formed from an alpha chain (IL-7Rα) and a gamma chain (IL-7γ_c). Lymphocytes are one of the main cell types expressing both chains of the IL-7R, probably due to their requirement of IL-7 for survival [Huang & Luther 2012]. The IL-7R signals via the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway (Figure 2a). Following the ligation of the IL-7R by soluble IL-7, JAK 1 and JAK 3 bind to the intracellular domain of the α and γ_c chains respectively, causing them to dimerise. Subsequent autophosphorylation of the JAKs results in signal transduction. Target proteins include proteins involved in survival (e.g. Bcl-2) and proliferation (e.g. PI3K). The transcription factor STAT5 homo-dimerises on activation and translocates to the nucleus where it binds DNA and influences gene expression, such as CIS (cytokine-induced Src homology 2-containing protein), which is involved in the negative regulation of cytokines, and the chemotactic

cytokine MIP-1α (M protein 1 alpha) [Nelson *et al.* 2004], however many of STAT5's targets remain unknown.

1.4.2 LTβR signalling pathway

The Lymphotoxin Beta Receptor (LTβR) is a type 1 transmembrane glycoprotein belonging to the TNF-Receptor Superfamily of proteins [reviewed in Schneider *et al.* 2004]. LTβR is expressed on the surface of many cell types such as stromal cells, but not on lymphocytes [reviewed in Browning J. L. 2008]. Although LTβR has more than one ligand, LT will be focussed on due to its relevance to this investigation. LT is expressed on the surface of activated lymphocytes and is comprised of an α subunit and two β subunits, often being referred to as $LT\alpha_1\beta_2$ [Schneider *et al.* 2004].

The binding of LT to LTβR initiates the activation of the NF-κB family of transcription factors; the classical (canonical) NF-κB pathway or the alternative (non-canonical) NF-κB pathway (Figure 2b). In the classical NF-κB pathway, ligation of the LTβR leads to the interaction of TNF receptor associated factor (TRAF) adaptor proteins with the intracellular domain of the receptor, which leads to the mobilisation of NF-κB1 heterodimer (p50/RelA), enabling its translocation into the nucleus where it mediates the expression of genes involved in pro-inflammatory responses including MIP-1β (macrophage inflammatory protein-1B) and VCAM-1 [reviewed in Schneider *et al.* 2004 & Oeckinghaus *et al.* 2011].

In the alternative NF-κB pathway TRAFs are recruited to the LTβR like in the classical pathway however this leads to the activation of NIK (NF-κB-inducing kinase) instead of the IKK (inhibitor of NF-κB kinase) complex, resulting this time in

the translocation of the NF-κB2 (p52/RelB) heterodimers to the nucleus where they upregulate genes involved in lymphoid organ formation and organisation [Schneider *et al.* 2004], for example CCL19 and IL-7 [Bénézech *et al.* 2012].

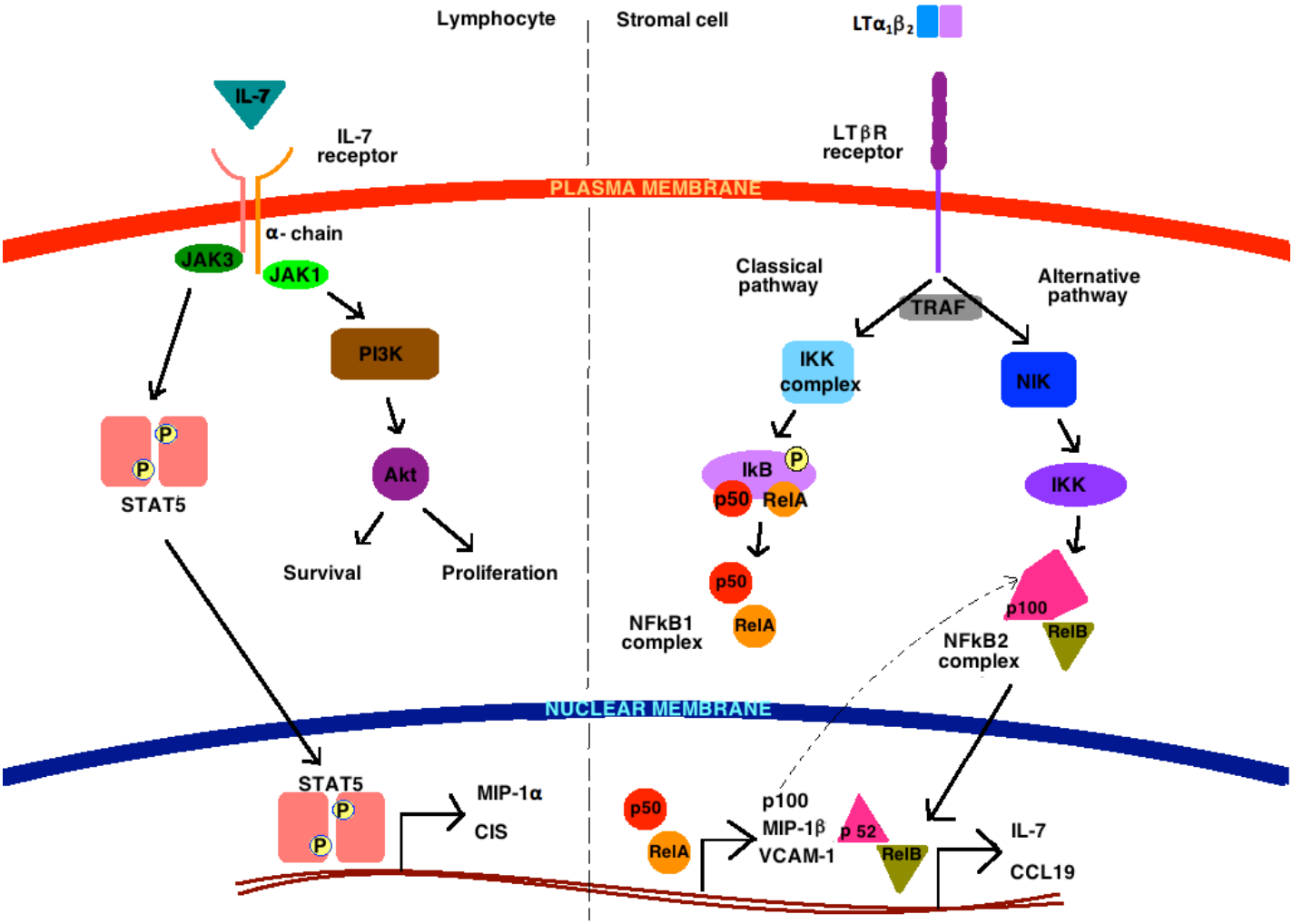

Figure 2. IL-7 and LTβR signalling. (a) IL-7/IL-7R signalling pathway. IL-7 binds to the IL-7R, causing the α chain and γ_c chain to recruit JAK1 and JAK3 respectively [Iwanami *et al.* 2011]. Autophosphorylation of the JAKs leads to the recruitment of STAT5 or activation of the PI3K pathway. On recruitment, STATs become phosphorylated and homodimerise, leading to their translocation to the nucleus where they bind DNA and alter gene expression. On stimulation of the PI3K pathway, AKT is activated, and leads to the regulation of proteins involved in survival and proliferation [Jiang *et al.* 2005].
(b) LT/LTβR signalling pathway. Ligation of LTβR by LT can lead to signalling via classical or alternative NF-κB pathways following the recruitment of TRAFs. In the classical NF-κB pathway, receptor activation results in the phosphorylation of IκB (inhibitor of κB) by the IKK complex (IκB kinase complex), releasing the NF-κB1 (p50/RelA) dimer and allowing its translocation into the nucleus, where genes involved in cell homeostasis and immunity are regulated [Schneider *et al.* 2004]. In the alternative pathway, NIK activates IKKα, leading to the phosphorylation of NF-κB1-induced p100, followed by its processing in the proteasome into p52. p52 then translocates to the nucleus with RelB as an NF-κB2 heterodimer [Oeckinghaus *et al.* 2011]. ***Image created in Paintbrush.***

1.5 Aim & Objectives

In recent years, the importance of ASCs has slowly emerged and we now understand them to play an important role in the development and regulation of immunity. However much more is yet to be known regarding the embryonic origin of ASCs, and their role in the regulation of lymphocyte survival in the adult. Understanding the contribution of adult pre-adipocytes in the modulation and survival of immune cells may hold potential applications in obesity or chronic inflammatory diseases involving adipose tissue. Investigating their properties will lead to an increased understanding about lymphocyte homeostasis by adipose tissue, and fat as an immunological organ.

Aim:

- To investigate the relationship between ASCs and lymphocyte survival

Objectives:

- Determine the role of ASCs in lymphocyte survival
- Examine the contribution of the LTβR pathway in lymphocyte survival
- Explore the involvement of IL-7 in lymphocyte survival
- Ascertain cell composition of adipose tissue SVF from WT and LTβR$^{-/-}$ mice.

2. METHODS

2.1 Mice

WT and LTβR$^{-/-}$ mice on a C57BL/6 background were bred in the University of Birmingham's Biomedical Services Unit (Birmingham, UK) under specific pathogen free (SPF) conditions, and in accordance with Home Office regulations. Mice were culled via CO_2 asphyxiation between 10 and 15 weeks of age.

2.2 Isolation of ASCs from adipose tissue and cell culture

Inguinal fat pads from age-matched mice were dissected and lymph nodes removed from WT tissues. Tissue was digested using RPMI with 2% FCS, 2.5mg/mL Collagenase D (Roche) and 100µg/mL DNase I (Sigma) for up to 20 minutes with constant shaking at 37^{o}C. Cells were passed through a cell strainer, and the resulting cell suspension was centrifuged yielding a pellet representing the SVF. Cells were cultured in RPMI 1640 medium containing 10mM HEPES buffer (Sigma), 50IU/mL penicillin, 50µg/mL streptomycin, (PAA) and 50µM b-mercaptoethanol with 10% FBS, (Sigma) and incubated at 37^{o}C.

For survival assays, WT or LTβR$^{-/-}$ SVF cells were seeded in triplicate at density of 1 million per well in 48-well plates and washed daily for 48h to remove non-adherent cells. After 5 days when SVF cells were approximately 30% confluent, fresh lymphocytes from disaggregated WT mLN were seeded at a density of 1 million per well. This was considered day 0. Lymphocyte survival was analysed by flow cytometry of non-adherent lymphocytes after 0, 1, 2, 4 and 7 days in co-culture, and calculated as a percentage of cells in the live gate.

2.3 Fluorescent antibody cell sorting (FACS) of pre-adipocytes for IL-7 investigation

WT SVF cells were stained with CD31, Gp38 and CD45 (eBioscience), disaggregated and sorted using MoFlo (Dako Cytomation) cell sorter (Figure 6a). $Gp38^{+}$ $CD31^{-}$ $CD45^{-}$ pre-adipocytes were seeded in triplicate at a density of 1 million per well in 48 well plates and washed after 24 and 48h to remove non-adherent cells. After 72h (Day 0), lymphocytes from disaggregated WT mLN were seeded at 1 million per well, accompanied by either 40µg/mL anti-IL-7Rα antibody (R&D systems) or 0.1µg/mL IL-7 (eBioscience)(Table 1). Cells were incubated at $37^{o}C$ and non-adherent lymphocytes analysed on day 2 by flow cytometry, following cell counting using Trypan blue dye exclusion to determine live cell numbers. Calculations were performed as a percentage of total live cells per well.

Table 1. Culture conditions for IL-7 investigation. Pre-adipocytes and/or mLN lymphocytes were seeded at 1 million per well in a 48-well plate, and treated with anti-IL-7Rα antibody, IL-7, or neither. After 2 days, lymphocyte survival was analysed by flow cytometry (Figure 6b).

	A	B	C	D	E
1	Pre-adipocytes + lymphocytes	Pre-adipocytes + lymphocytes + anti-IL-7Rα	Lymphocytes	Lymphocytes + anti-IL-7Rα	Lymphocytes + IL-7
2	Pre-adipocytes + lymphocytes	Pre-adipocytes + lymphocytes + anti-IL-7Rα	Lymphocytes	Lymphocytes + anti-IL-7Rα	Lymphocytes + IL-7
3	Pre-adipocytes + lymphocytes	Pre-adipocytes + lymphocytes + anti-IL-7Rα	Lymphocytes	Lymphocytes + anti-IL-7Rα	Lymphocytes + IL-7

2.4 Flow cytometry

Lymphocytes were stained with CD8a, CD19, CD62-L and CD4, and SVF cells stained with CD31, Gp38 and CD45. Flow cytometry was performed using a FACS Calibur (BD Biosciences), and analysed using FlowJo software (Tree Star). Non-viable cells were stained with fixable viability dye eFluor780 to be excluded from the live gate using forward scatter (FSC)/side scatter (SSC) gates. All antibodies/dyes purchased from eBioscience and used according to manufacturer's instructions.

2.5 Real-time PCR

cDNA was prepared from WT and $LTBR^{-/-}$ SVF cells and adipocytes using μMacs One-Step cDNA Synthesis Kit (Miltenyi Biotec) according to manufacturer instructions. Primers for IL-7 and CCL19 were used, alongside β-actin as a reference gene. Real-time PCR (RT-PCR) was performed using an Applied Biosystems 7900HT system and means from triplicate reactions ± SEM multiplied by 1000 were taken. Data represents a single experiment.

2.6 Statistical analysis

Statistical significance was calculated for all survival assays using GraphPad Prism 5. An unpaired t-test was performed and mean ± SEM was determined. P=<0.05 was considered statistically significant

3. RESULTS

3.1 Survival of lymphocytes in co-culture with WT ASCs

In order to establish the role of ASCs in the survival of lymphocytes, lymphocytes were co-cultured with stromal cells from inguinal fat pads of WT mice for 7 days. To determine lymphocyte survival, naïve $CD4^+$ $CD8^+$, and $CD19^+$ lymphocytes in the live gate were quantified using flow cytometry (Figure 3a).

By day 4, almost all B and T cells cultured alone had died (Figure 3a & 3b). For this reason, day 4 will be used as a key point of comparison in the analysis of these survival experiments. Survival was significantly higher on day 2 (P=<0.01) and day 4 (P=<0.05) for both T and B cells cultured with ASCs, when compared to being cultured alone (Figure 3b). Survival of $CD4^+$ T cells was also seen to be statistically significant on day 7 (P=<0.01) compared to single culture, suggesting that $CD4^+$ T cells survive better than $CD8^+$ T cells or B cells in co-culture with ASCs. A drop in survival of 28.5% and 39.3% for $CD4^+$ and $CD8^+$ T cells respectively supports this observation (Figure 3b). By day 7, $CD4^+$ T cell survival had decreased by 36%, and $CD8^+$ cells had decreased by 69.5%. B cell survival appeared to be least effected by co-culture with ASCs.

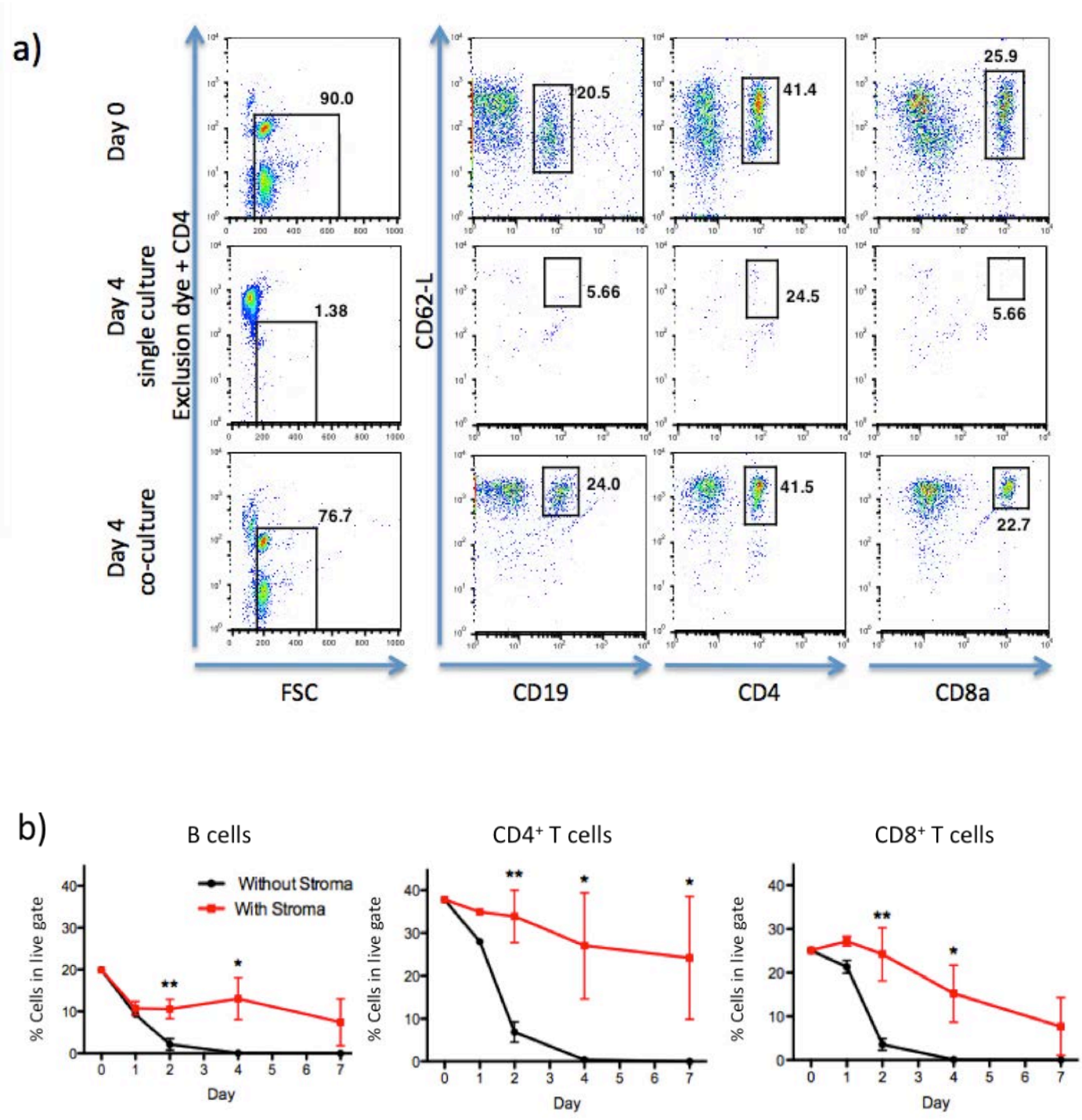

Figure 3. Lymphocyte survival in co-culture with ASCs. (a) Representative images of flow cytometric analysis performed on lymphocytes following antibody staining for lymphocyte markers. Live cells were gated on (first column) and then separated on the basis of naivety (CD62-L), and the expression of either CD19, CD4 or CD8a. (b) The survival of B cells, $CD4^+$ T cells and $CD8^+$ T cells in co-culture with ASCs derived from the SVF of inguinal fat pads of WT mice (red - with stroma) or alone (black – without stroma). Survival was determined as a percentage of cells in the live gate and expressed as the mean ± SEM of triplicates from one experiment. Statistical significance between single culture and co-culture was determined for days 2, 4 and 7. **= P<0.01, *=P<0.05.

3.2 Ability of LTβR$^{-/-}$ stromal cells to support lymphocyte survival

LTβR is a key factor governing the differentiation of stromal cells in the LN. We thus tested its role in ASCs in supporting T cell survival. Again, flow cytometry was used to quantify the survival of CD19^{+}, CD4^{+} and CD8^{+} lymphocytes in the live gate and stromal cells isolated from inguinal fat pads of LTβR $^{-/-}$ mice were used in co-culture (Figure 4a).

As shown above, lymphocyte survival decreased rapidly when cells were cultured alone and almost all cells were no longer viable by day 4 (Figure 4b). Lymphocytes co-cultured with WT ASCs presented with the highest levels of survival, and these results were statistically significant when compared to the survival of lymphocytes in the presence of LTβR$^{-/-}$ ASCs at day 4 and day 7 for B cells (Day 4: P=<0.01, Day 7: P=<0.05), CD4^{+} T cells (Day 4: P=<0.05, Day 7: P=<0.01) and CD8^{+} T cells (Day 4: P=0.05, Day 7: P =0.01). However, the survival of lymphocytes in co-culture with LTβR$^{-/-}$ ASCs was still higher than lymphocytes cultured alone, and was statistically significant on day 4 for B cells (P=<0.05), days 2, 4 and 7 for CD4^{+} T cells (P =<0.005, 0.05 & 0.01 respectively), and days 2 and 4 for CD8^{+} T cells (P=<0.01 & 0.05 respectively). T cells appeared to survive better on day 4 than B cells in all co-culture conditions, with the least decrease in survival from CD4^{+} T cells in co-culture with both WT (64.7%) and LTβR$^{-/-}$ (83.7%) ASCs, and the biggest decrease in B cells with WT (87.9%) and LTβR$^{-/-}$ (94.4%) ASC. CD4^{+} T cells survived the best in both WT and LTβR$^{-/-}$ co-cultures on day 7, continuing to be statistically significant in LTβR$^{-/-}$ co-cultures compared to single cultures (P=<0.01), and WT co-cultures compared to LTβR$^{-/-}$ co-cultures (P=<0.01).

Collectively, these results suggest a defect of LTβR$^{-/-}$ ASCs to sustain lymphocyte survival when compared to WT ASCs. However, LTβR$^{-/-}$ cells are not completely impaired as lymphocyte survival is still higher in co-culture assays than in the absence of ASCs. T cells, in particular CD4^{+} cells, appeared most influenced by ASCs in terms of survival, with B cell survival being influenced the least.

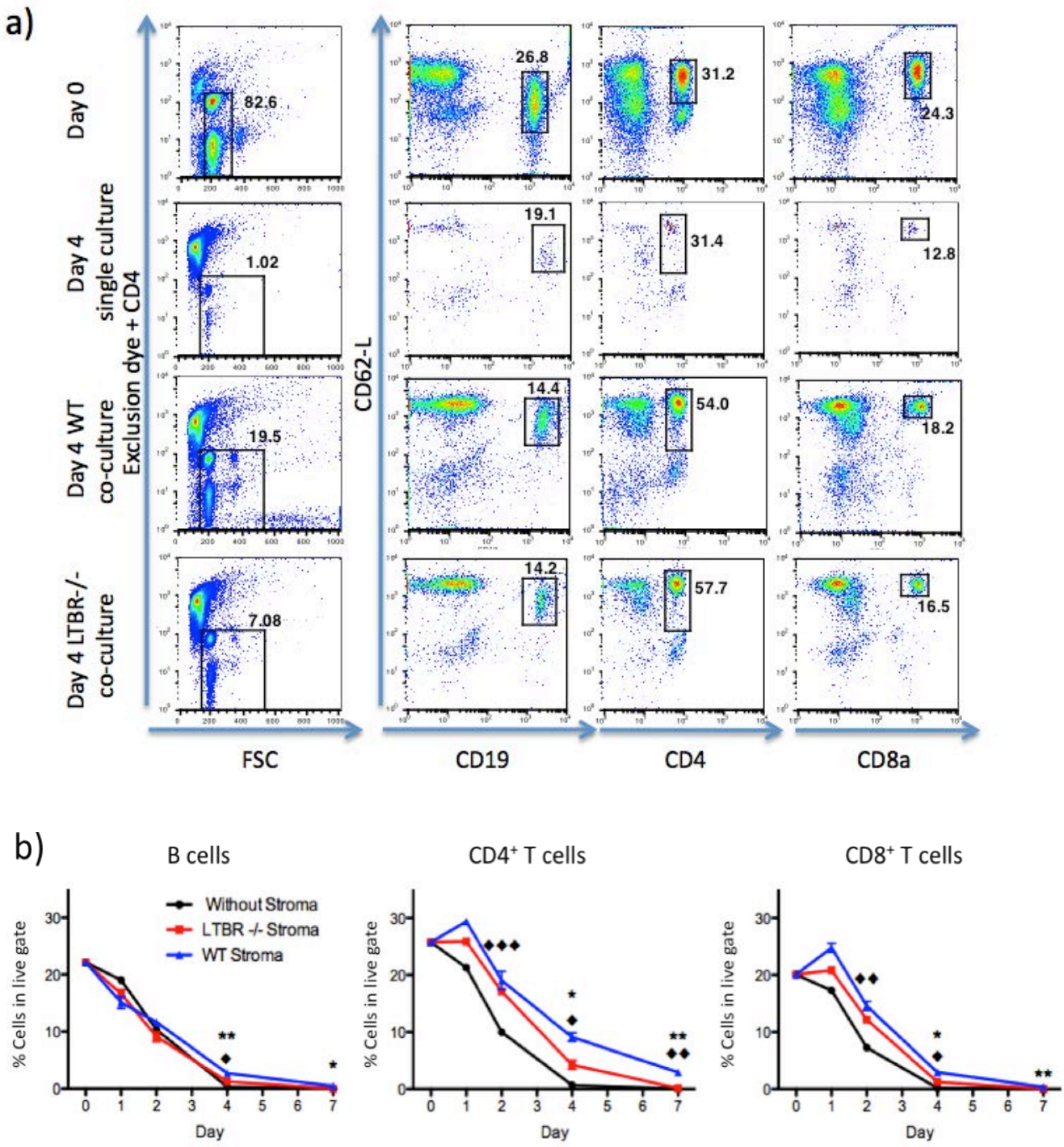

Figure 4. Lymphocyte survival in co-culture with WT and LTβR$^{-/-}$ ASCs. (a) Representative images of flow cytometric analysis of antibody-stained lymphocytes on day 0 and 4. (b) Survival of B and T lymphocytes in co-culture with ASCs (blue and red) or single culture (black). Survival was determined as a percentage of cells in the live gate (Figure (a), first column) and expressed as the mean ± SEM of triplicates from one experiment. Statistical significance between single culture and co-culture was determined for days 2, 4 and 7. Diamonds(♦) represent statistical comparison between LTβR$^{-/-}$ ASC co-culture vs. without stroma, and asterisks(*) represents statistical comparison between WT vs. LTβR$^{-/-}$ ASC co-culture. Statistical significance calculated for 2, 4 and 7 days. ♦♦♦=P<0.005, ♦♦/**= P<0.01, ♦/*=P<0.05.

3.3 Survival of T lymphocytes on adipose tissue-derived stromal cells is dependent on IL-7

Following the observation that ASCs express IL-7 (Figure 5a), the effect of this homeostatic cytokine on the survival of lymphocytes in co-culture with pre-adipocytes was next investigated using anti-IL-7Rα antibody, which binds to the α chain of the IL-7 receptor, inhibiting signalling. Initially, pre-adipocytes were sorted (Figure 6a). Then, WT lymphocytes were cultured alone or with sorted $Gp38^+$ $CD31^-$ $CD45^-$ pre-adipocytes and treated with IL-7 or anti IL-7Rα (Figure 6b). Day 2 was chosen as the point of analysis because the previous survival experiments demonstrated that cells in single culture were almost all dead by day 4 (Figures 3a, 3b, 4a & 4b) thereby making day 2 the best comparison point for analysis of any variations in survival.

T cell survival was 11.8% higher for $CD4^+$ T cells and 16.8% higher for $CD8^+$ T cells in co-cultures with sorted pre-adipocytes, compared to being cultured alone, and was statistically significant for $CD8^+$ T cells (P=<0.005), confirming that pre-adipocytes influence T lymphocyte survival, in particular $CD8^+$ T cells. B cell survival did not appear to be influenced by the presence of pre-adipocytes, IL-7 or anti-IL-7Rα antibody, and no statistical significance in survival was seen between experimental conditions and cells alone. As a positive control, IL-7 was added to lymphocytes cultured alone to demonstrate its effects on survival. Despite having no significant effect on B cells, IL-7 substantially increased T lymphocyte survival with statistical significance of P=<0.01 for $CD4^+$ cells, and P=<0.005 for $CD8^+$ cells compared to cells alone with anti-IL-7Ra, although these levels do not represent *in vivo* levels, as IL-7 was used at a higher concentration than physiologically normal.

Addition of anti-IL-7Rα antibody inhibited $CD4^+$ and $CD8^+$ T cell survival by 15.5% and 11.4% respectively compared with pre-adipocyte co-culture, and was statistically significant in both cases (P=<0.05). However, inhibiting IL-7 in cells alone did not completely abolish survival, suggesting the involvement of factors other than IL-7 in lymphocyte survival.

Next, the expression of IL-7 and CCL19 in the $Gp38^+$ fraction of SVF (pre-adipocytes) was measured by RT-PCR to determine whether adipose tissue-derived pre-adipocytes were the ASCs producing the survival factors as these cells have been shown to produce them in the LN. Both IL-7 and CCL19 were found to be expressed by the $Gp38^+$ $CD31^-$ $CD45^-$ fraction of cells isolated from inguinal fat pads of WT mice (Figure 6c). Levels of both IL-7 and CCL19 expression were much higher than seen in the LN, however this is most likely due to the fact that the only cells producing significant amounts of IL-7 in the LN are FRCs, and the $Gp38^+$ $CD31^-$ $CD45^-$ fraction was purified thereby yielding more cells in the $Gp38^+$ fraction, hence greater expression.

3.4 Expression of LTβR is not necessary for IL-7 expression

Since IL-7 and CCL19 are the main factors involved in lymphocyte survival expressed by LN stromal cells, the expression of these cytokines by cells in adipose tissue was investigated.

IL-7 and CCL19 were expressed in both the SVF of adipose tissue and adipocyte fractions of adipose tissue from WT and LTβR$^{-/-}$ mice (Figure 5). Expression of both of these survival factors did not differ greatly between SVF and adipocyte fractions. IL-7 expression in both fractions was comparable to that seen in the LN (Figure 5a), suggesting similar levels of lymphocyte homeostasis in both organs, whereas CCL19 expression was lower than in the LN (Figure 5b). Surprisingly, LTβR$^{-/-}$ SVF and adipocyte fractions expressed the same levels of IL-7 and CCL19.

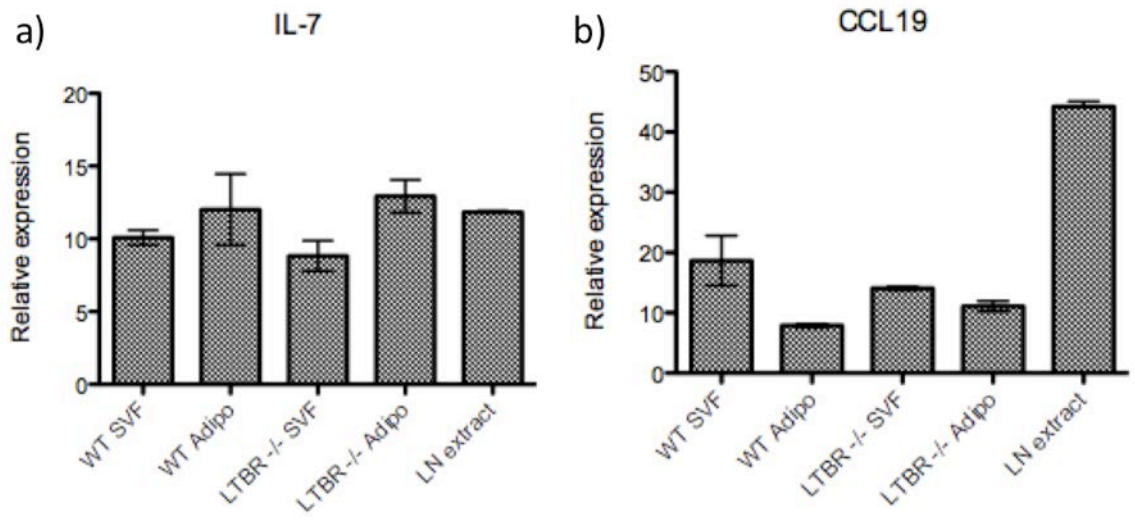

Figure 5. The expression of IL-7 and CCL19 in WT and LTβR$^{-/-}$ adipose tissue. IL-7 (a) and CCL19 (b) expression in SVF and adipocyte fraction of WT and LTβR$^{-/-}$ adipose tissue was determined via RT-PCR, using β-actin as reference gene. WT LN extract was analysed to provide a point of comparison of expression. Data is expressed as a mean of the relative expression from triplicate reactions in one experiment.

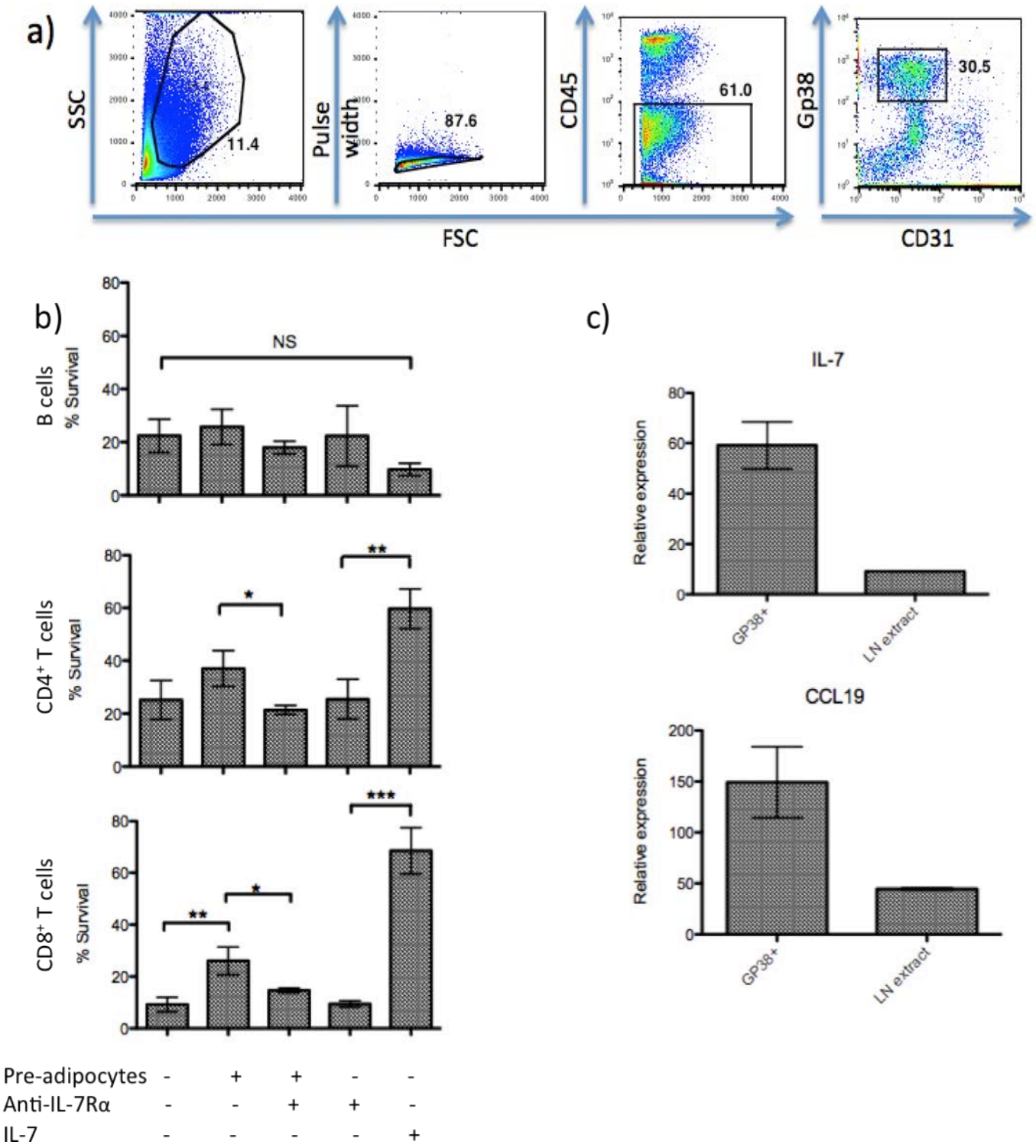

Figure 6. The effects of IL-7 on lymphocyte survival and its expression in pre-adipocytes. (a) WT SVF cells were stained and Gp38^{+} CD31^{-} CD45^{-} pre-adipocytes sorted. (b) Lymphocytes were treated with anti-IL-7Rα antibody on day 0 and analysed on day 2 by flow cytometry. The percentage of surviving lymphocytes was calculated from a viable cell count and data represents the mean ± SEM from one triplicate experiment. (c) RT-PCR was performed to determine the expression of IL-7 and CCL19 in Gp38^{+} SVF cells. LN extract was run alongside for comparison and β-actin was again used as a reference gene. This data is representative of triplicate reactions from a single experiment.***=P<0.005, **=P<0.01, *=P<0.05, NS=not statistically significant.

3.5 Stromal cell compositions in WT and LTβR$^{-/-}$ SVF derived from inguinal adipose tissue.

Finally, in order to investigate whether differences in stromal cell populations were responsible for the discrepancies seen in survival between WT and LTβR$^{-/-}$ SVFs, the cellular composition of the SVF from adipose tissue of WT and LTβR$^{-/-}$ mice was analysed. Flow cytometric analysis of SVF was conducted following staining for CD31, Gp38 and CD45, which have been previously described as markers of stromal cell populations [Link *et al.* 2007]. Similar percentages of cells were seen in all gates, except for the Gp38^{+} CD31^{-} gate, representing pre-adipocytes. In the LTβR$^{-/-}$ SVF, 57.1% of cells were observed in this gate (Figure 7b) compared to WT SVF (44.3%, Figure 7a), suggesting a slight difference in pre-adipocyte frequency.

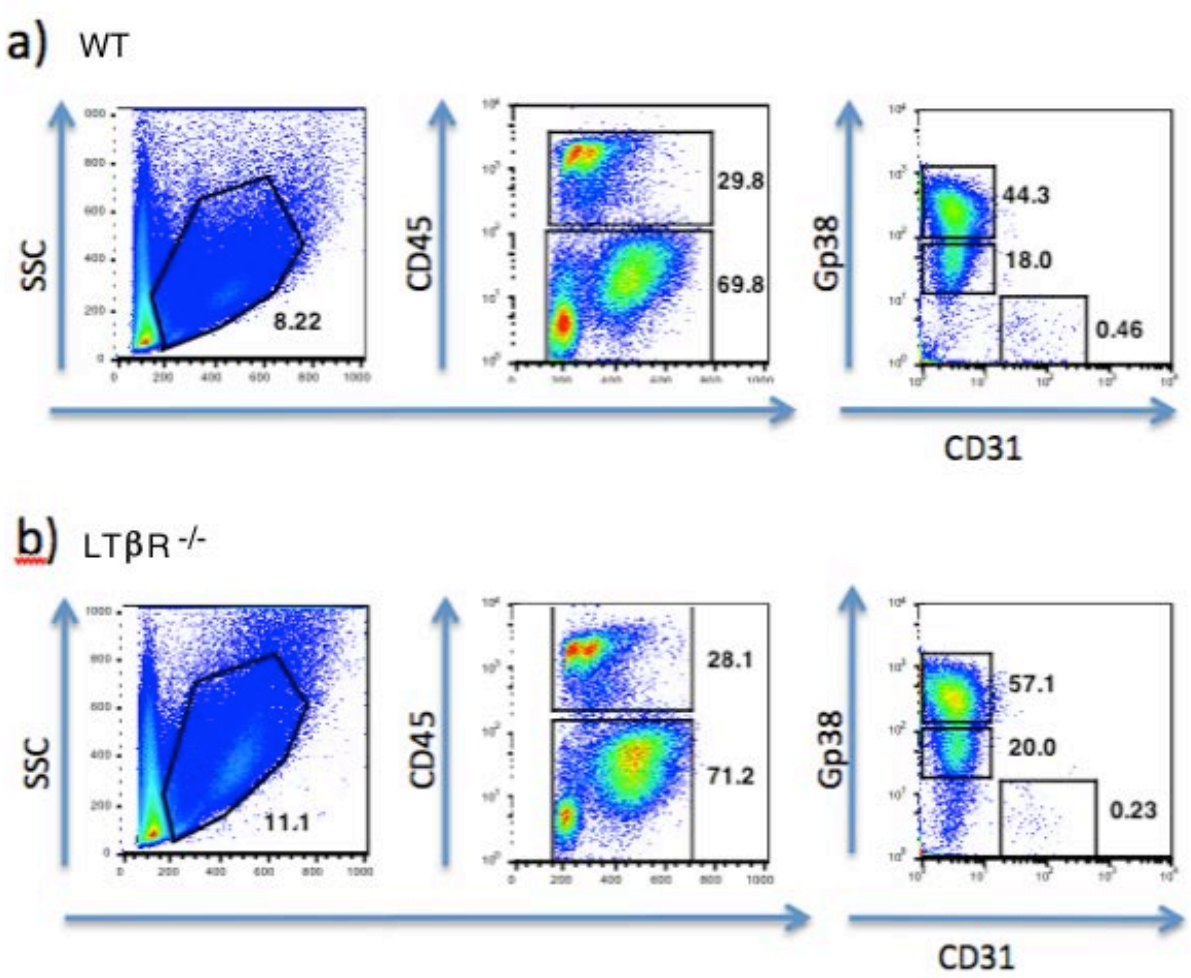

Figure 7. SVF cell composition. Representative images from flow cytometric analysis of 3 WT and 3 LTβR$^{-/-}$ SVFs. SVF cells derived from (a) WT and (b) LTβR$^{-/-}$ inguinal fat pads were stained for CD31, Gp38 and CD45 expression. Non-viable cells were excluded from the live cell gate using FSC/SSC gates. CD45^{-} cells were gated on, and three stromal cell populations were identified here, assuming the Gp38^{+} CD31^{-} population to represent pre-adipocytes.

4. DISCUSSION

Lymphocyte homeostasis is crucial in SLOs. The maintenance and organisation of a lymphocyte pool by stromal cells enables appropriate immune responses, and therefore a functional immune system. $Gp38^+$ $CD31^-$ $CD45^-$ FRC have been described to regulate the homeostasis of naive T cells in the LN. Adipocyte precursor cells which are able to migrate to the LN anlagen to become part of the lymphoid stroma share characteristics with the LN FRC [Link *et al.* 2007, Bénézech *et al.* 2012]. In this study, the ability of $Gp38^+$ ASCs to promote lymphocyte survival through involvement of the LTβR signalling pathway and expression of IL-7 was investigated *ex vivo*.

4.1 Adipose tissue-derived SVF cells promote lymphocyte survival

Lymphocyte survival was significantly enhanced by the presence of ASCs, although this effect was not as prominent in B cells. This observation is supported by Bénézech *et al.* 2012 and Link *et al.* 2007, who also found this effect to be specific to T cells, with B cell survival only minimally enhanced by the presence of ASCs. Link and co-workers found that IL-7 was produced mostly by $Gp38^+$ stromal cells in the LN [Link *et al.* 2007], and cells with this phenotype can also be found in the SVF of adipose tissues [Bénézech *et al.* 2012].

IL-7 can regulate both T and B cell survival [Taguchi *et al.* 2007, Tan *et al.* 2001], but has been shown to mainly support T cell survival [Link *et al.* 2007,

Bénézech *et al.* 2012]**.** The fact that IL-7 can regulate B cell survival to some extent provides a possible explanation regarding the smaller yet still evident influence of ASCs on B cell survival. In addition to this, the numbers of FDC-like cells producing supportive B cells factors such as CXCL13 in the SVF may have been sparse compared to FRC-like cells supporting T lymphocytes, unlike the LN which is massively populated by FRC and FDC subsets. More FRC-like cells than FDC-like cells in the adipose tissue SVF would result in a greater enhancement of T cell survival than B cell survival due to the production of more T cell than B cell survival factors, and this was observed in Figure 3b.

$CD4^+$ T cell survival was higher than $CD8^+$ T cells in co-cultures with WT ASCs. A large overlap in stromal cell derived factors modulating $CD4^+$ and $CD8^+$ lymphocyte survival exists, for example, interleukin 21 (IL-21) and IL-7 can regulate both $CD4^+$ and $CD8^+$ T cells [Ostiguy *et al.* 2007, Rochman *et al.* 2009]. One feasible explanation for the observed difference in lymphocyte survival may be that the net effect of the factors secreted by ASCs in the SVF influenced $CD4^+$ T cell survival to a greater extent than $CD8^+$ T cells. Despite reports that $CD8^+$ T cells have an innate ability to survive longer [Ferreira *et al.* 2000], it is unlikely that this is the cause of the descrepency in survival between T lymphocyte populations because survival decreased at approximately the same rate without ASCs, with $CD4^+$ and $CD8^+$ populations exhibiting similar decreases on day 2 of 81.6% and 85.6% respectively. The observation that $CD4^+$ T cells survived better than $CD8^+$ T cells was not found by Link *et al.* 2007, where no difference between these subsets is observed. This may be due to their use of sorted FRCs, not ASCs, in co-culture. Adult murine SVF cells were used in this study as the source of ASCs, and were not sorted in the experiment shown in Figure 3. Therefore, it is likely that the SVF

contained a larger variety of stromal cells, not just FRC-like cells, producing a different profile of survival factors.

4.2 LTβR$^{-/-}$ stromal cells exhibit decreased ability to support lymphocyte survival

LTβR$^{-/-}$ ASCs exhibited a decreased capacity to enhance the survival of lymphocytes in co-culture, but were still able to support survival to a greater extent than in the absence of any ASCs.

Similar to the findings of the prior assay (Figure 3b), only a minimal effect was seen on B cell survival in both co-culture with WT or LTβR$^{-/-}$ ASCs. This was most likely due to the same reasons as previously described in section 4.1; less B cell-supporting ASCs may have been present, and therefore fewer survival factors were available. B cell survival factors regulated by LβTR, such as BAFF (B cell activating factor) [Dejardin *et al.* 2002], would be reduced and therefore decrease B cell survival when co-cultured with LTβR$^{-/-}$ ASCs. Since B cells did not present with increased survival to the extent of T cells for both WT and LTβR$^{-/-}$ ASC co-cultures, it is possible that LTβR is not central to the regulation of B cell survival, and may not regulate the majority of B cell survival factors in adipose tissue. This could be confirmed by the investigation of WT and LTβR$^{-/-}$ expression of such factors in ASCs, and the presence of FDC-like CD35^{+} cells.

The involvement of LTβR in the differentiation of LN stromal cells has been demonstrated. LTβR engagement on pre-adipocytes inhibits adipocyte differentiation and promotes differentiation into LN stromal cells, and the expression of IL-7 [Bénézech *et al.* 2012]. A lack of signalling though LTβR can also result in the differentiation of ASCs into adipocytes, and therefore reduced IL-7 levels as decreased IL-7 production in adipocytes has been demonstrated by Maijer and co-workers [Maijer *et al.* 2011], possibly contributing to the decreased levels of survival

seen in Figure 5b. Another feasible explanation for the decrease in survival of lymphocytes in co-culture with LTβR$^{-/-}$ ASCs is that these stromal cells may have been dying at a higher rate than WT ASCs due to a lack of LTβR-mediated signalling, and were therefore producing less IL-7, resulting in the decreased survival of lymphocytes. Oshima and co-workers found that CD4^{+} T lymphocytes express significantly high levels of LT, which in turn supports stromal cell survival through LTβR signalling [Oshima *et al.* 1999]. In a recent investigation [Zeng *et al.* 2012], the depletion of CD4^{+} T cells resulted in significant loss of FRCs due to a lack of LTβR ligation by LT, further supporting the results seen in Figure 4b. The degree of which lymphocyte subsets affect ASC survival could be further investigated though co-culture of CD4^{+}, CD8^{+} and CD19^{+} lymphocytes individually, and monitoring of stromal cell survival. *In vivo* conditions would also allow the action of other factors and interactions promoting stromal cell survival, but this is an area of stromal cell research remaining to be explored.

A difference between CD4^{+} and CD8^{+} T cell survival was seen, with CD4^{+} T cells appearing to survive the best with or without WT or LTβR$^{-/-}$ ASCs. Once again, this could be attributed to the cocktail of survival factors produced by ASCs favouring CD4^{+} T cells.

Despite a lack of LTβR signalling, significant lymphocyte survival was still observed suggesting the involvement of other signalling pathways or activation of other receptors regulating genes which support lymphocyte survival or intrinsic regulation of stromal cell activities and fate, for example TNF-α [Meijer *et al.* 2011].

4.3 Pre-adipocyte-derived IL-7 supports T lymphocyte survival

Since pre-adipocytes share similar characteristics with FRCs, it was investigated whether it was the $Gp38^+$ adipocyte precursor cells which were responsible for the enhancement of lymphocyte survival in co-cultures.

No significant effects were observed under any culture conditions for B cells, suggesting that pre-adipocytes and IL-7 do not play an essential role in the survival of B lymphocytes. The remaining levels of B cell survival could reflect the minor support provided by pre-adipocyte derived IL-7, or the secretion of a different factor such as BAFF from $Gp38^+$ $CD31^-$ $CD45^-$ $CD35^+$ FDC-like cells [Hase *et al.* 2004, Link *et al.* 2007] which, if present, would not have been eliminated from the cell sort on the basis of CD35 expression.

However, T lymphocyte survival was enhanced by co-culture with sorted $Gp38^+$ pre-adipocytes, and was significantly increased in $CD8^+$ T cells, demonstrating the involvement of pre-adipocyte cells in lymphocyte survival. Inhibition of IL-7 using an antibody directed against the alpha chain of the IL-7R lead to a significant decrease in $CD4^+$ and $CD8^+$ T lymphocyte survival in the presence of pre-adipocytes. This strongly suggests that T cell survival is dependent on IL-7, and that it is most likely to be pre-adipocytes producing this factor. These observations are supported by the findings of Bénézech *et al.* 2012 who observed that $Gp38^+$ pre-adipocytes produce IL-7, and inhibition of this leads to decreased lymphocyte survival.

Further supporting the idea that pre-adipocytes are producing IL-7 which is enhancing T cell survival are our findings that IL-7 is expressed in the SVF, and sorting of $Gp38^+$ cells enriched IL-7 expression in this fraction. Similar levels of IL-7 expression were observed in the SVF and adipocyte fractions, however on

purification, IL-7 expression was higher despite removal of other cells in this fraction. This was also higher then IL-7 expression in the LN. Collectively these data suggest that $Gp38^{+}$ cells in adipose tissue produce high levels of IL-7, and is the reason for T lymphocyte survival, supporting the previous findings that WT ASCs are able to support lymphocyte survival in co-cultures.

Interestingly, complete inhibition of lymphocyte survival was not observed despite the addition of anti-IL-7Rα, suggesting that IL-7 is not independently supporting survival, but that other factors are also involved. This supports the $LT\beta R^{-/-}$ ASC survival assay as despite a lack of LTβR signalling, and likely decrease in IL-7 production, survival of lymphocytes still occurred. This survival could be attributed to the expression of the chemokine CCL19, which was expressed by ASCs (Figure 5b). In future, the synergistic effect of IL-7 and CCL19 on lymphocyte survival should be investigated to determine pre-adipocyte derived CCL19 involvement, and examine whether synergy results in even higher levels of lymphocyte survival in co-culture with pre-adipocytes, and whether inhibition of both factors can significantly knock this down.

IL-7 and CCL19 are two major factors contributing to lymphocyte survival in LN. The ability of purified $Gp38^{+}$ pre-adipocytes to express IL-7 and CCL19 was confirmed by RT-PCR, and was detectable at physiologically relevant levels in both WT and $LT\beta R^{-/-}$ SVF, suggesting that their expression is not dependent on LTβR. However, defective survival of T cells in co-culture with $LT\beta R^{-/-}$ ASCs suggests that other factors must be involved in their survival which do not involve LTβR signalling, again, supporting the idea that pre-adipocytes are capable of supporting T cell survival.

FRCs support T cell survival in LNs and present with a similar phenotype to

pre-adipocytes, producing CCL19 and IL-7. Therefore, pre-adipocytes may share similar functions in adipose tissue, hence enhanced T cell survival. This is feasible because FRCs do not support B cell survival to the extent of T cell survival, and this was seen in all survival assays.

4.4. Stromal cell populations are similar in WT and LTβR-/- SVF from adipose tissue

In order to confirm that defects in the stromal cell compartment of adipose tissue were not responsible for the diminished ability of LTβR$^{-/-}$ ASCs to support lymphocyte survival, three samples of adipose tissue from each group were individually digested and stained as described. No apparent differences in the percentage of cells in each gate from WT and LTβR$^{-/-}$ groups were observed, except in the Gp38^{+}CD31^{-} gate, where the WT fraction was 44.3% and the LTβR gate contained 57.1% cells (i.e. pre-adipocytes). However, due to the smallness of this difference, it is most likely to reflect individual variance between animals, and is not the cause of differential lymphocyte survival.

4.5 Conclusion & future implications

Stromal cells provide indispensible support to immune cells, providing them with optimal environments to respond to antigens. This investigation and others [Link *et. al* 2007, Bénézech *et al.* 2012] have demonstrated this supportive ability, which is particularly important to T cells.

T cell survival was shown to be dependent on ASC expression of IL-7, which was enriched following cell sorting of $Gp38^{+}$ $CD31^{-}$ $CD45^{-}$ pre-adipocytes. Adipose tissue-derived pre-adipocytes with phenotypic characteristics of FRCs were demonstrated to express lymphotrophic factors IL-7 and CCL19, however, the results strongly suggest that survival factors other than IL-7 must also be involved, as lymphocyte survival remained despite inhibition of IL-7R signalling.

Stromal cell LTβR was shown to be linked to lymphocyte survival as a lack of LTβR signalling correlated with a decrease of lymphocyte survival in co-cultures. However, some T cell survival remained, and $LT\beta R^{-/-}$ SVF cells were able to express IL-7 and CCL19, suggesting that other signalling is involved which is independent of LTβR.

Adipose tissue is known to be highly metabolic, and many lymphocytes are retained here, although the reason for this is unknown. Lymphocytes are able to communicate with adipose tissue through LT expression and adipose tissue LTβR, and can respond to adipose tissue-secreted factors such as adipokines [reviewed in Caspar-Bauguil *et al.* 2009]. Thus, it is possible that a dialogue exists between lymphocytes, adipose tissue and the cells contained in the SVF. Some understanding of the effects of adipose tissue on lymphocytes exists, but the effect of lymphocytes on adipose tissue is less well studied. Adipose tissue regulates

lymphocyte survival via IL-7, and lymphocytes can inhibit adipocyte differentiation thought LTβR signalling, suggesting a reciprocal relationship. Lymphocyte numbers differ greatly depending on obesity, metabolic state and inflammation [reviewed in Caspar-Bauguil *et al.* 2009], suggesting possible regulation of metabolic activities in adipocytes. In order to uncover the mechanisms by which lymphocytes regulate adipose tissue and the possible dialogue which exists between them, further investigation is required.

Although it is clear that ASCs are involved in the regulation of lymphocyte survival, the exact mechanisms are unknown. Future studies into the mechanisms underlying the support of lymphocyte survival by ASCs such as pre-adipocytes will lead to a better understanding of adipose tissue as an immunological organ. Investigation of the supportive capacity of other factors produced by pre-adipocyte may provide insight into the global signalling networks governing lymphocyte survival in adipose tissue. LT$\alpha^{-/-}$ mice could be used to determine whether similar effects in ASCs from these animals lacking the function ligand are seen in LTβR$^{-/-}$ animals, and further examine the involvement of LTβR in the survival of both ASCs and lymphocytes. In *ex-vivo* studies, pre-adipocytes are cultured in a closed system, out of context of their physiologically normal stromal environment *in vivo,* where many other influencing factors are present which are not represented in *vitro* experiments. Therefore, the development of *in vivo* models may facilitate the comprehension of the vast array of factors and interactions involved in stromal cell and lymphocyte survival in adipose tissues. In doing so, the pathological mechanisms of disorders involving lymphocyte dysregulation such as autoimmunity, or the low-grade state of chronic inflammation associated with obesity, can be better

recognised, possibly leading to the development of novel therapies involving stromal cells or their associated mediators.

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